Supercritical fluid chromatography and two-dimensional liquid chromatography

From new applications to technical innovations

Sun, Mingzhe

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PhD Route Map

Supercritical fluid chromatography and two-dimensional liquid chromatography

From new applications to technical innovations

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Abstract

Lignin depolymerisation can be utilized to produce value-added aromatic compounds. One of the major challenges in lignin depolymerisation study is the development of effective analytical tools to unravel the huge complexity of the depolymerisation product mixture. Traditional gas chromatography (GC) and high- performance liquid chromatography (HPLC) methods applied for this purpose are often in lack of a high resolving power to separate the various components, which exist in a large number in samples of this type. This PhD work started with the development of a 6-minute fast supercritical fluid chromatography method for the analysis of lignin-derived phenols from alkaline cupric oxide oxidation, which demonstrated for the first time the potential of supercritical fluid chromatography in the lignin type phenol analysis. As a continuation of this work to enable analysis of more model compounds with structural elucidation, a supercritical fluid chromatography with quadrupole-time-of-flight mass spectrometry method was developed.

Considering the complexity of processed lignin sample, a two-dimensional liquid chromatography × supercritical fluid chromatography system was constructed with trapping column assisted modulation. The system exhibited high degree of orthogonality. A trapping column interface also significantly shorten the run time with higher detectability of the compounds compared with a traditional loop based interface.

Large volume and water injection in supercritical fluid chromatography often lead to poor separation and peak shape, due to strong solvent effect and viscous fingering. Multiple injection technique was investigated in this PhD study to enable the injection of relatively large volume to enhance the detectability. The signal-to-noise ratio enhancement was found to be strongly dependent on retention. Multiple injection provided better enhancement of signal-to-noise than one-time injection of large volume, with comparable repeatability and reproducibility. Water as sample diluent was investigated to study its effect on retention and peak shape. The influence of water in sample diluent varies with varying stationary phases, injection volumes and sample diluent water contents. The study proved that it can be advantageous to use sample diluent containing water, especially when polar stationary phases are used.

Monitoring a complete dynamic extraction process is not possible with any set-up reported so far, but coupling extraction with chromatography with a parallel sampling interface can be a potential solution. As a proof of concept, an on-line pressurized hot water extraction-liquid chromatography system with parallel sampling was built imitating the two-dimensional chromatography philosophy. The system was used in a kinetic study of extraction of curcuminoids from turmeric. Compound-specific extraction curves were obtained and thermal degradation of curcumin could also be demonstrated.

Key words: Analyte focusing, interface, supercritical fluid chromatography, sample diluent, two-dimensional liquid chromatography

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Monitoring a complete dynamic extraction process is not possible with any set-up reported so far, but coupling extraction with chromatography with a parallel sampling interface can be a potential solution. As a proof of concept, an on-line pressurized hot water extraction-liquid chromatography system with parallel sampling was built imitating the two-dimensional chromatography philosophy. The system was used in a kinetic study of extraction of curcuminoids from turmeric. Compound-specific extraction curves were obtained and thermal degradation of curcumin could also be demonstrated.

Popular Science Summary

Analytical chemists work to analyze samples that could contain a large number of different compounds. Different liquid chromatography (LC) techniques are often used to separate these compounds before detecting them with a certain detector. The compounds will be carried by a flow of solvents (mobile phase) and pass through a column filled with immobilized stationary phase of certain chemistry. The separation of the compounds is the consequence of different compounds having different affinities towards the mobile phase, as well as the stationary phase. When a band containing one or multiple compounds reaches the detector, it is usually manifested as a peak by the detector. Based on the mobile phase nature and the relative polarity of the mobile phase and stationary phase, liquid chromatography can be classified into many different categories.

When hydrophobic stationary phases are used, this type of liquid chromatography is often called reversed-phase liquid chromatography (RPLC). RPLC is predominantly used in various types of analysis at the moment, there are several drawbacks of the technique that need to be improved. The first one is efficiency, which is to a large extent determined by the speed of mass transfer in the mobile phase. As the commonly used mobile phases in RPLC are mixtures of water and organic solvent, the only way to enhance the mass transfer is to use high temperature to decrease the viscosity of the mobile phase, which would ruin the separation or even destroy the column. Secondly, the use of RPLC limits the use of stationary phases to relatively non-polar ones and it would be difficult to separate relatively polar compounds with this technique. Moreover, one column chemistry is not enough to resolve all the compounds if the composition of the sample is very complex.

Different from liquid chromatography, supercritical fluid chromatography (SFC) uses compressed carbon dioxide as mobile phase. Supercritical CO_2 has liquid-like density but much lower viscosity than normal liquid solvents, which makes it a good option as mobile phase as it provides solubility of the compounds with enhanced mass transfer properties. Also, the low viscosity enables the use of much higher flow rates compared with those used in RPLC, and the analysis time can be shortened.

The first part of this PhD study focused on the development of SFC methods with different types of detectors for the analysis of degraded lignin samples. Several times faster analysis was achieved with the developed SFC methods than the traditional RPLC methods used to analyze the same type of compounds.

Compared with RPLC, SFC has two major limitations related to how the sample is injected: 1. Smaller injection volume tolerated and 2. Water being considered as

a poor sample solvent to inject with. Therefore, the second part of this PhD project aimed at developing a new injection technique for injecting higher volume of sample to achieve lower detection limit and investigating injection solvent containing water in a systematic way.

In cases where one LC fails to separate all the compounds, the technique which is called two-dimensional liquid chromatography (2D-LC) can be a good option. 2D-LC divided the flow coming out of one LC (as the 1st dimension) into a series of segments. With a properly designed interface, all or selected segments can be sequentially injected into another LC (as the second dimension) and be analyzed. In this way, the compound peaks can be distributed in a two-dimensional space with the help of mathematical tools. One important factor that leads to the success of a 2D-LC set-up is a large difference between the separation mechanisms in the two dimensions. As SFC often uses polar stationary phases with relatively non-polar mobile phases consisting of compressed CO_2 and a small portion of organic solvent, it offers very different separation mechanisms from that of RPLC. One study in the PhD project is to construct a 2D RPLC×SFC system with a specially designed interface. Furthermore, the 2D-LC concept has also been applied in the analysis of extraction processes in a novel set-up.

List of publications

I. Ultra-high performance supercritical fluid chromatography of lignin-derived phenols from alkaline cupric oxide oxidation
Mingzhe Sun, Gunnar Lidén, Margareta Sandahl and Charlotta Turner
Journal of Separation Science, 2016, 39, 3123-3129

II. Ultra-high-performance supercritical fluid chromatography with quadrupole-time-of-flight mass spectrometry (UHPSFC/QTOF-MS) for analysis of lignin-derived monomeric compounds in processed lignin samples
Jens Prothmann*, **Mingzhe Sun***, Peter Spégel, Margareta Sandahl and Charlotta Turner;
Analytical and Bioanalytical Chemistry (ABC), 2017, 409, 7049-7061
*Shared first authorship

III. Influence of water in sample diluent on modern supercritical fluid chromatography
Mingzhe Sun, Yu Rui, Charlotta Turner and Margareta Sandahl
Manuscript

IV. Signal Enhancement in Supercritical Fluid Chromatography-Diode-array Detection with Multiple Injection
Mingzhe Sun, Charlotta Turner and Margareta Sandahl
Manuscript

V. Comprehensive on-line two-dimensional liquid chromatography × supercritical fluid chromatography with trapping column-assisted modulation for depolymerised lignin analysis
Mingzhe Sun, Margareta Sandahl and Charlotta Turner
Journal of Chromatography A, 2018, 1541, 21-30

VI. Dynamic extraction coupled on-line to liquid chromatography with a parallel sampling interface – a proof of concept for monitoring extraction kinetics
Mingzhe Sun, Said Al-Hamimi, Margareta Sandahl and Charlotta Turner
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Author's contributions

I. The author came up with the idea of the project, conducted the lab work and wrote the first draft of the manuscript.

II. The author participated in developing the idea of the project, conducted part of the lab work and wrote one part of the draft of the manuscript.

III. The author came up with the idea of the project, conducted part of the lab work and wrote the first draft of the manuscript.

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V. The author participated in developing the idea, conducted the lab work and wrote the first draft of the manuscript.

VI. The author came up with the idea of the project, conducted most part of the lab work and wrote the first draft of the manuscript.

Publications not included

I. Enhanced kinetics of liquid-liquid extraction using CO_2 expanded liquid
Larissa P. Cunico*, **Mingzhe Sun***, Yu Rui, Semhar Guirmai, Markus Enekvist, Simon Lundegard, Margareta Sandahl, Charlotta Turner
*Shared first authorship
Submitted
Personal contribution:
The author conducted part of the experimental work, and wrote one part of the manuscript.

II. Biological valorization of low molecular weight lignin
O.Y. Abdelaziz, D.P. Brink, J. Prothmann, K. Ravi, **M.Z. Sun**, J. García-Hidalgo, M. Sandahl, C.P. Hulteberg, C. Turner, G. Lidén, M.F. Gorwa-Grauslund
Biotechnology Advances 2016, 34, 1318-1346.
Personal contribution:
The author wrote one part of the manuscript.

III. Highly enantioselective epoxidation of olefins by H_2O_2 catalyzed by a non-heme Fe(II) catalyst of a chiral tetradentate ligand
Mainak Mitra, Olaf Cusso, Satish K Bhat, **Mingzhe Sun**, Miquel Costas, Ebbe Nordlander
Submitted
Personal contribution:
The author conducted one part of the lab work.

IV. Combination of continuous base-catalyzed depolymerisation and membrane filtration of black liquor retentate
Kena Li, Basel Al-Rudainy, **Mingzhe Sun**, Ola Wallberg, Christian Hulteberg, Per Tunå
Manuscript
Personal contribution:
The author conducted one part of the lab work and wrote one part of the manuscript.

Abbreviations

1-AA	1-Aminoanthracene
2D	Two-dimensional
2-PIC	2-Picolylamine
ACN	Acetonitrile
APCI	Atmospheric pressure chemical ionization
BEH	Ethylene bridged hybrid silica
BPR	Back pressure regulator
DAD	Diode array detection
DEA	Diethylamine
DOE	Design of experiment
ESI	Electrospray ionization
FP	Fluoro-Phenyl
GC	Gas chromatography
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High-performance liquid chromatography
IEC	Ion-exchange chromatography
LOD	Limit of detection
LOQ	Limit of quantification
MS	Mass spectrometry
MP	Mobile phase
NPLC	Normal-phase liquid chromatography
PHWE	Pressurized hot water extraction
Q-Tof	Tandem quadrupole time of flight mass spectrometry
RPLC	Reversed-phase liquid chromatography
SEC	Size-exclusion chromatography
SFC	Supercritical fluid chromatography
SP	Stationary phase
UHPLC	Ultra-high-performance liquid chromatography

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1. Introduction

1.1. Background

Chromatography is one of the most important tools to achieve compound separation in chemical analysis. The two most widely used chromatography techniques are gas chromatography (GC) and liquid chromatography(LC). While GC is the technique of choice for volatile compounds, LC can be used for a larger range of compounds regardless of their volatility with possibility to be coupled with a variety of different types of detectors.

In terms of the performance of the technique, LC generally strives for highest possible efficiency and shortest possible time with good separations. Instrumental revolution and advances in column technology have contributed hugely towards these goals. For example, traditional high performance liquid chromatography (HPLC) systems are gradually replaced by ultra-high performance liquid chromatography (UHPLC) with elevated maximum pressure allowed and decreased extra band broadening [1]. This also enabled the use of sub-2 μm particles in the column, which offers high efficiency in much shorter time, compared with column technology two decades ago [2].

The past two decades have witnessed the revival of supercritical fluid chromatography (SFC), owing to the modern instrumentation technology [3]. Utilizing compressed CO_2 with a small portion of organic co-solvent as mobile phase, mass transfer is greatly enhanced in SFC compared with traditional LC. The low viscosity of CO_2 in supercritical or subcritical form also allows much higher flow rates to be used than those in HPLC. Consequently, faster analysis with high efficiency can be achieved in SFC than in HPLC. Furthermore, CO_2 is considered as a much greener solvent than the typical organic solvents used in HPLC, which is in line with the "green chemistry" demand from the public [4].

As a good complementary technique to the widely used reversed phase liquid chromatography (RPLC), supercritical fluid chromatography (SFC) has been gaining more and more popularity in various types of analysis [3]. One example is the analysis of depolymerised lignin. Nowadays, harnessing biomass has become a hot research topic worldwide [5]. As one of the three major components of biomass and a large-quantity byproduct from the paper industry, the successful

utilization of lignin is attracting more and more attention [6]. During the depolymerisation process, the large cross-linked aromatic polymer is partially broken down to small monomeric, dimeric or oligomeric phenolic compounds [7]. Some of these compounds are of high value in themselves and some of them can be used as building blocks for value-added chemicals [8]. **Paper I** and **II** describe SFC methods developed for the analysis of monomeric lignin-derived phenolic compounds with Diode Array Detection (DAD) and Mass spectrometry (MS) detectors respectively. Different column stationary phase chemistries were explored for maximum selectivity. Different additives were tested to improve peak shape and ionization in MS.

Most of the commercial SFC systems use fixed-loop injection to avoid cavitation of the metering device from solvent expansion [9]. There have been numerous studies of the influence of sample diluent selection on SFC performance [10-12]. Unfortunately, even though aqueous samples are ubiquitous, the consensus seems to be that water is a bad choice as sample diluent as it often leads to severe peak distortion and pressure spike. **Paper III** introduces a systematic study of the impact of water in sample diluent on SFC performances. The trade-off between detectability and peak resolution by selection of injection volume is also crucial in SFC. Too high injection volumes with fixed-loop injector often result in significant loss of efficiency without much gain in signal intensity [13]. **Paper IV** presents a study of performing multiple injection in modern analytical SFC, aiming at improving the detectability without the need of changing the injection loop and one-time large volume injection.

As has been mentioned above, the selection of stationary phase plays the most important role in liquid chromatography to achieve good separation of compounds [14]. However, satisfactory separation of a comparatively large number of analytes or target compounds in a very complex matrix can still be very challenging in many cases, despite the rapid development of packing technology and stationary chemistry. Two factors that govern the separation of a LC run are resolving power and peak capacity. Resolving power depends largely on how the stationary phase chemistry addresses the physiochemical property differences among the analytes, while peak capacity relies mainly on the space available for separation. When the differences of analyte properties do not fall into one category or the elution window is not wide enough to accommodate all analyte peaks, traditional one-dimension LC (1D-LC) can no longer generate good separations. In such cases, two-dimensional liquid chromatography can be an attractive option.

Due to its unparalleled peak capacity in a reasonable analysis time compared with traditional one-dimension chromatographic techniques, on-line 2D-LC has gained more and more popularity for the analysis of complex samples in recent years [15-17]. The selectivity in both 2D-LC dimensions can be tuned by both the change of

mobile phase and stationary phase [18]. The on-line coupling also eliminates the sample loss or contamination problem often encountered in off-line two-dimensional system [19].

The most important parameter in evaluating a 2D-LC set-up in a specific analysis is its degree of orthogonality [20]. It can be directly reflected by how much theoretical separation space is used. The key to achieve a high degree of orthogonality is to have the two separation dimensions as different as possible addressing different physiochemical properties of the analytes. Although the combination of two reversed phase (RPLC) separation is the easiest to achieve and avoids to a large extent the issue of solvent incompatibility of the two dimensions, it normally does not provide a high degree of orthogonality [21]. In general, the separation mechanism in SFC resembles that of normal phase liquid chromatography with specific selectivity governed by the stationary phase chemistry. The inclusion of SFC in a 2D-LC system is anticipated to provide high degrees of orthogonality. However, there have only been few reports on this type of research [22-25]. An on-line 2D comprehensive LC×SFC system with trapping column-assisted modulation was developed in **paper V**, and its usefulness was demonstrated with the analysis of a depolymerized lignin sample.

1.2. Aim of the project

Lignin can be a high-potential source for future value-added chemical production, provided that effective depolymerization processes are developed. An obstacle for the study of lignin depolymerization is the complexity of depolymerized lignin. One aim of this project is thus to develop 1D SFC and 2D LC×SFC methods for the analysis of depolymerised lignin samples, pursuing high efficiency and resolving power.

The "green chemistry" demand from the society requires the consumption of organic solvents that are harmful to the environment to be cut down. SFC using compressed CO_2 as the major part of the mobile phase is considered as a good supplement for the traditional LC techniques that use a large quantity of organic solvents. One of the limitations of SFC is a low tolerance of high injection volume and aqueous samples. Therefore, this PhD project aims to conduct a study on sample diluent containing water and technical innovation of traditional loop-based SFC injection to enlarge the sample range and improve the detectability.

Extraction is a widely-used technique from making tea in the kitchen to sample preparation in an analytical chemistry lab. On-line chromatographic analysis of the whole process of a dynamic extraction is difficult to achieve with analytical

systems reported so far. One aim of this PhD study is to apply the concept of 2D-LC in the analysis of dynamic extraction processes.

2. Supercritical Fluid Chromatography

2.1. Supercritical fluids

Although not universally known to the public, the phenomenon of supercritical fluids was discovered already 200 years ago, and has been subject to much research interest [26]. The supercritical fluid form is reached by elevating the temperature and pressure above its critical point, which can be illustrated by a Pressure-Temperature phase diagram (PT diagram). Figure 2.1 shows a PT diagram of a pure compound, in which different phases of the compound are clearly defined and separated by nonlinear boundary curves. Two phases can coexist on these boundary curves and the triple point marks the coexistence of the three commonly known phases: solid, liquid and gas. As the temperature and pressure go up, the liquid-gas phase boundary curve ends at the critical point, where the gas phase and liquid phase have the same density. Above the critical point, liquid and gas exist as one phase. This special state of matter of the compound is called supercritical fluid.

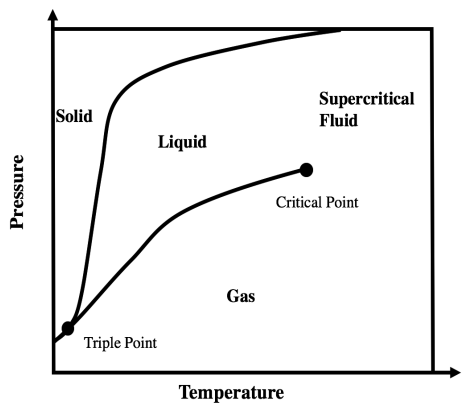

Figure 2.1. PT phase diagram of a pure substance.

Table 2.1 displays the comparison of density, diffusivity and viscosity of gases, liquids and supercritical fluids in order of magnitude. The most unique feature of a supercritical fluid lies in the fact that its properties are intermediate between those

of gases and liquids. In another word, it can diffuse like a gas and still be able to dissolve compounds like a liquid. Owing to the combination of diffusivity and solubility, supercritical fluid can be potentially used in many fields including analytical chemistry. In theory, all substances can be transitioned into their supercritical fluid state, but very few are applicable from a practical point of view. The usage of many substances as supercritical fluid is accompanied by risk of combustion and toxication [27].

Table 2.1 Properties of supercritical fluid in comparison with gas and liquid.

	Density (g/mL)	Diffusion (cm^2/s)	Viscosity (g/cm*s)
Gas	10^{-3}	10^{-1}	10^{-4}
Supercritical fluid	10^{-1} - 1	10^{-4} - 10^{-3}	10^{-4} - 10^{-3}
Liquid	1	$<10^{-5}$	10^{-2}

The most widely used supercritical fluid in analytical chemistry is supercritical carbon dioxide ($scCO_2$). Compared with other components used as supercritical fluid, $scCO_2$ holds several advantages, such as relatively mild supercritical conditions, low toxicity and high miscibility with many organic solvents. In the field of sample preparation, supercritical fluid extraction (SFE) has become an important technique, especially for nonpolar and slightly polar analytes [28]. Easy solvent removal and elimination of analyte oxidative degradation are another two added attractive features of SFE. As a sample analysis technique, supercritical fluid chromatography (SFC) utilizes compressed liquid CO_2 as the major component of its mobile phase. Due to enhanced mass transfer and elevated mobile phase flow rates in SFC, this technique is characterized by high separation efficiency with short analysis time, compared with traditional liquid chromatography techniques. For example, **paper I and II** show SFC applications where traditional HPLC suffers from relatively long analysis times. What should be clearly pointed out is that pure $scCO_2$ only exhibits polarity similar as a non-polar solvent e.g., hexane, which greatly limits its use in various applications involving medium and highly polar compounds. In order to alter the polarity of $scCO_2$, a polar co-solvent (often called "modifier", in most cases an alcohol) is usually added in most of the applications of SFE and SFC [29, 30]. In many applications (e.g., **paper II**), the percentages of co-solvent are so high that the mixture no longer remains strictly supercritical under the common temperature and pressure ranges used in SFE and SFC. Nevertheless, "supercritical" is still the word predominantly used in most of the applications.

2.2. Mobile phase

In the very early stage of SFC, neat CO_2 was utilized as the mobile phase in most of the research performed, together with capillary columns and flame ionization detectors [31]. As the elution strength of neat CO_2 is largely dependent on the density of the fluid, gradient elution was achieved by altering the temperature and pressure of the mobile phase.

Despite the changes in elution strength introduced by the change of density, compressed neat CO_2 is in general a solvent of low polarity. In order to expand the compound range that SFC can analyze, an organic modifier is in most cases added into compressed neat CO_2. The addition of modifiers can significantly increase the elution strength of the mobile phase and favors the compound solubility [32]. Moreover, modifiers can adsorb on the active sites of the stationary phase and reduce peak distortion [33, 34].

Methanol is often considered as a strong organic modifier and the first choice in most SFC applications. In both **paper I and II** methanol was used as the modifier. Addition of methanol can decrease the mobile phase compressibility and lead to changes in density [35]. If a significant amount of modifier is added, the contribution from mobile phase density variation to the change of elution strength of the mobile phase becomes less important than those from modifier type and amount. The addition of different modifiers can introduce different selectivities to the separation, as the hydrogen-bonding and dipole-dipole interactions vary with modifier type [36]. For example, the replacement of methanol with acetonitrile as organic modifier in a SFC run with a polar stationary phase often results in increases in retention and peak width and decrease in peak symmetry. This is the consequence of a lack of hydrogen donating capability of acetonitrile and its insufficiency in shielding the residue silanol groups in the stationary phase [37, 38].

Under most circumstances with SFC, the modifier is much more polar than the compressed CO_2, which leads to modifier clustering. The modifier molecules can cluster with each other and at the same time form layers around the polar analyte molecules [39]. If polar stationary phases are used, polar modifier molecules can also adsorb onto the stationary phase surface and form a modifier layer, which alters the nature of the stationary phase [40].

What is also worth mentioning is that the combination of alcohol modifier and CO_2 creates an acidic mobile phase environment, as alkylcarbonic acid is formed [41]. The mobile phase acidity can alter the ionization states of both the analytes and the stationary phase, causing changes to the corresponding interactions [42].

Two of the most important factors to evaluate a chromatographic separation are

the retention factor of the analyte and the separation efficiency of the column. Studies have shown that these are closely connected with the compound solubility and diffusivity in the mobile phase [43]. When SFC is operated with very little or no modifier added, both the compound diffusivity and solubility are strongly influenced by the density of the mobile phase. Under these conditions, small variations in pressure and temperature can lead to large variations of the density of the supercritical fluid, which can cause significant change in the retention and separation. The use of isopycnic plot (isopycnic lines plotted against pressure and temperature as Y and X axis) can be of great assist in understanding the consequences of adjusting pressure and temperature [44]. In general, an increase of pressure can decrease the retention factors of the analytes as higher density leads to higher solubility, which was also one of the observations during method development in **paper I**. However, this effect was insignificant for late-eluting compounds in **paper II** (Figure 2.2), which was caused by the low mobile phase compressibility with relatively high modifier percentages.

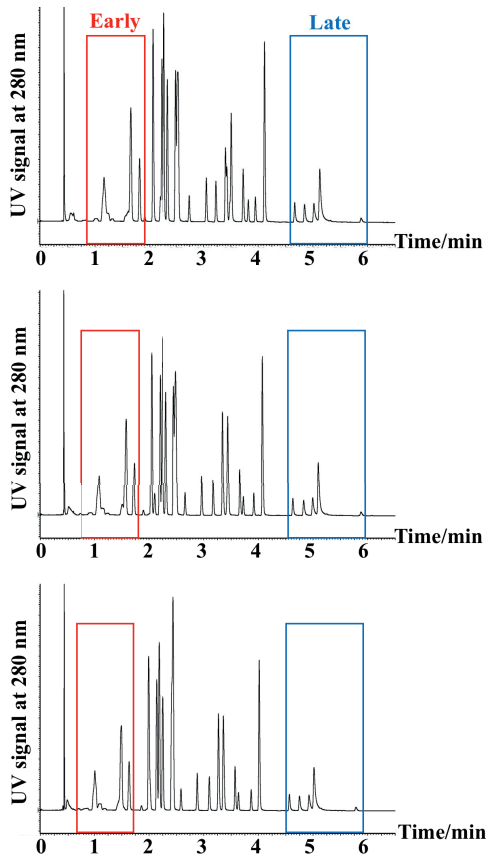

Figure 2.2. The influence of different backpressure. A DIOL column was used. Experimental conditions: see Materials and methods part in **paper II**. Figure from **paper II**, reprinted with permission.

The effect of temperature variation on the retention depends largely on how the mobile phase density is: an increase of temperature can cause a drop of fluid density, which decreases the analyte solubility and increases the retention (observed in both **paper I** and **II,** demonstrated in Figure 2.3). On the other hand, when the mobile phase is already within the high-density range, a decrease in analyte retention can be expected with an increase of temperature since the compressed CO_2 under these conditions have liquid-like solubility behaviors [42].

Many acidic, basic and amphoteric compounds of high polarity exhibit strong retention or bad peak shape when the mobile phase only comprises modifier and CO_2. This entails the addition of a third element into the mobile phase, which is called the additive. Acidic additives such as formic acid, trifluoroacetic acid and citric acid are usually added to improve the elution of acidic analytes, which was demonstrated in **paper I**. While the peak shape of basic compounds can benefit from the addition of basic additives, such as isopropylamine, ethyldimethylamine and trimethylamine. It is also common to add ionic additives, such as ammonium hydroxide and ammonium acetate [32]. The functions of additive addition involve the following three main mechanisms: suppression of analyte ionization, shielding the active sites on the stationary phase and forming ion-pair with analytes in ionic form to improve the retention and peak shape [45]. For example, formic acid was studied as additive in **paper II**. It not only suppressed the ionization of the phenolic acids, but also blocked a large part of the active silanol sites on the stationary phase. The combinational effect is that the retention of the phenolic acids became shorter. In recent years, water has also been reported as additive for SFC [46]. The use of small amount of water in supercritical CO_2, bridged by alcohol modifier can improve the mobile phase solvating power. Besides, it also introduces a HILIC-like mechanism into the separation, with the substantial participation of stationary phases as well [47]. It was proposed in **paper III** that even water introduced into the column by sample diluent might adsorb on the polar stationary phase, which leads to a temporary HILIC-like mechanism.

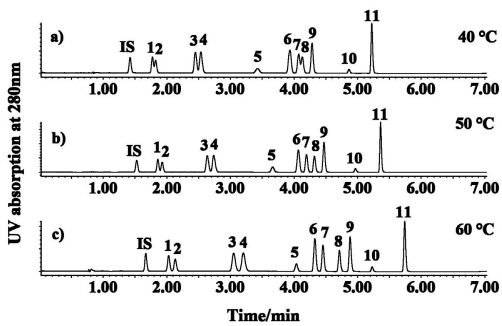

Figure 2.3. SFC chromatograms of 11 lignin-derived phenolic compounds under column temperature a) 40 °C, b) 50 °C and c) 60 °C, all at backpressure 135 bar. For peak labels and chromatographic conditions, see **paper I**. Figure from **paper I**, reprinted with permission.

2.3. Stationary phase

The pursuit for higher separation efficiency has always been one of the main themes of research in the field of any chromatographic techniques. The use of sub-2 μm particles in SFC has become the dominating trend in recent years [32]. The use of sub-2 μm particles can effectively decrease the peak broadening resulted from mass transfer resistance in the Van Deemter equation. Thanks to the development of SFC instrumentation, commercial SFC systems nowadays can provide the high pressure needed to pump the mobile phase through the column packed with these small size particles, as well as minimizing the extra-column band broadening [32]. It has been proven that the use of these small packing particles can decrease mass transfer resistance under gradient conditions, even though the increase of modifier percentage unavoidably causes an increase in mobile phase viscosity and a decrease in compound diffusivity [48]. What is worth mentioning is that the combination of sub-2 μm particle and modern instruments gave birth to the concept of Ultra-high-performance supercritical fluid chromatography (UHP-SFC). Even though SFC is used throughout this thesis, all relevant work can actually be categorized as UHP-SFC.

Following the advances in packing material technologies, recent years have witnessed an increasing use of core-shell (or superficially porous) particles in different UHPLC and SFC applications, where high efficiency with high speed and relatively low pressure drop is demanded [49, 50]. The lower back pressure results from the use of a relatively large particle, which is a combination of the solid core and the outer porous shell. In the meantime, enough surface area still exists in the porous shell for the separation to happen as it occupies a high proportion of the whole particle size. Additionally, column coupling is possible to further increase the separation efficiency with the low back pressure feature. However, it has been pointed out that strong solvent effect could be more profound in SFC separations with core-shell particles [9].

One of the most attractive features of modern SFC is the broad range of stationary chemistry that can be selected. In theory, all stationary phases developed for HPLC use can be directly adopted in SFC applications [51]. Compared with hydrophobic stationary phases like C18, the use of polar stationary phases in SFC is more common. The general separation mechanism with polar stationary phases resembles that of NPLC, as CO_2 in sub- or supercritical state has very low polarity [52]. Specific selectivities of different stationary phases depends on the contributions from various interactions between the compounds and the stationary phases, such as dipole-dipole interaction, electrostatic interaction and hydrogen bonding [53-57]. As the number of different stationary phases that can be utilized in SFC keeps growing, systematic characterization of all major stationary phases becomes important for chromatographers to save time and energy in selecting

stationary phases for specific applications. Lesellier and coworkers in recent years have developed a column classification system based on a solvation parameter model [58, 59]. A spider diagram was generated in an initial attempt with five descriptors depicting different molecular interactions between the stationary phase and analytes: charge transfer, dipole-dipole, H-bond acceptor, H-bond donor and dispersive force [58]. Recently, a new 7-branch spider diagram taking into consideration interactions with ionizable species was plotted [59].

Seven columns (Figure 2.4) were screened for the separation of 40 lignin-derived phenolic monomers in **paper II**. The 1-AA column offered enhanced π-π interaction and provided reasonable retention and separation of the most non-polar lignin phenols. The high-density DIOL column showed a hydrogen-bonding based separation mechanism, which depends on the number and availability of hydroxyl and carboxylate groups on the analytes. Phenolic acids showed relatively stronger retentions in the 2-PIC and DEA column, likely due to ion-dipole interaction between the analytes and the stationary phase.

A resolution level graph was also used in **paper II** to access the overall resolution achieved for the screened columns (Figure 2.5). This type of graph shows the cumulative number of peaks with resolution higher than the labeled values on the X-axis. Both Figure 2.4 and 2.5 convey a clear message that bonding technology and functionalization of chromatographic particles improved greatly the resolving power. DIOL, 1-AA, DEA and 2-PIC columns exhibited better resolution than the other three columns, C18, FP and BEH.

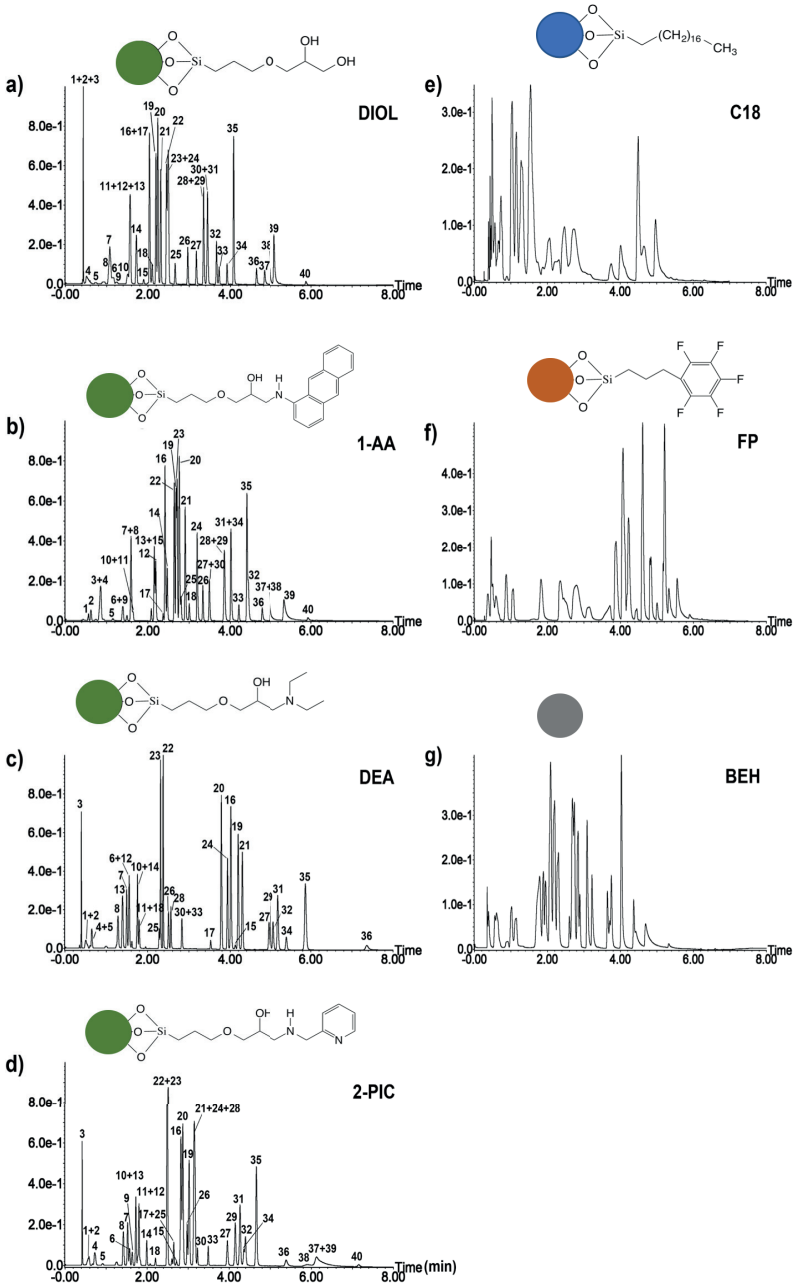

Figure 2.4. UHPSFC-DAD chromatograms of 40 lignin-derived compounds on seven different columns: a) DIOL; b) 1-AA; c) DEA; d) 2-PIC; e) C18; f) FP; g) BEH. For SFC conditions for different columns and peak identities, see **paper II**. Figure from **paper II**, reprinted with permission.

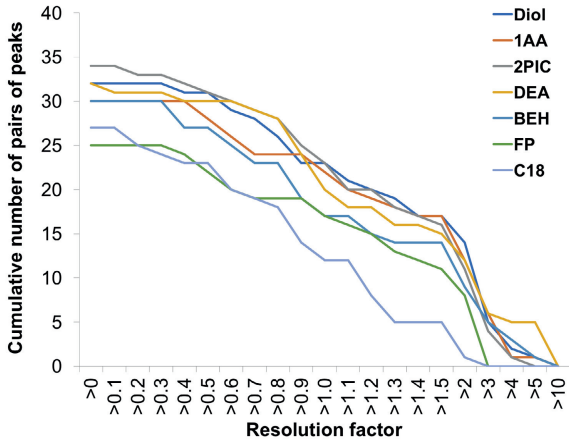

Figure 2.5. Resolution level graph for column screening (data from **paper II**). Columns are represented by different colored lines. The X axis shows a series of different resolution levels, while the cumulative number of peaks with a resolution qualified for each level are on the Y-axis. Figure from **paper II**, reprinted with permission.

In the recent two decades, SFC has also become a popular choice for analytical and semi-preparation separation of chiral compounds [60, 61]. Compared with traditionally used normal phase chiral HPLC separations, SFC offers much more rapid analysis, shorter equilibration time and easy removal of solvents, together with less impact on the environment [62]. Commonly used chiral SFC stationary phases include polysaccharide-based stationary phases, cyclic oligosaccharides, Ion exchange chiral stationary phases, macrocyclic glycopeptide chiral stationary phases and pirkle or brush type chiral stationary phases [61].

2.4. Instrumentation

SFC and HPLC have many similarities in terms of instrumentation. Figure 2.6 shows a schematic diagram of a common SFC system. The two unique components that are specially required for an SFC system are a cooling and pre-compression module before the accurate delivery of CO_2 flow and mixing with modifier flow, as well as a back-pressure controlling module to maintain the system pressure [42].

With modern commercial SFC systems, both gaseous and liquid CO_2 can be drawn, then sequentially cooled efficiently by peltier cooling and pre-compressed for accurate delivery. The major benefit of usage of CO_2 in gaseous state is associated with the elimination of possible contaminants dissolved in liquid CO_2.

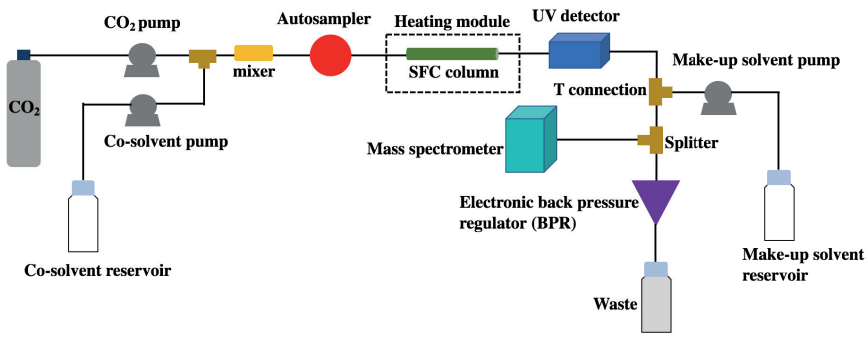

Figure 2.6. Schematic diagram of a typical SFC system.

Compared with HPLC, SFC also demands special injection designs. The classical variable-loop auto-sampler is not appropriate for SFC use as the metering device can be cavitated by the expansion of mobile phase [9]. Instead, fixed-loop injection is applied in SFC so that the metering device is isolated from the loop and rotor. However, partial-loop injection with this injection mode can lead to relatively worse RSD of peak area than that of retention time [45].

2.4.1. Sample diluent in SFC

In HPLC analysis, the rule-of-thumb is to dissolve the sample in a solvent that is as similar as possible to the mobile phase starting composition. This is apparently not applicable in SFC as compressed CO_2 is not possible to be used as sample diluent. In most of the SFC applications, sample diluent is one or a mix of organic solvents, which unavoidably leads to strong solvent effect on poorly retained compounds. Some work on the influences of different properties of various organic sample diluents on the chromatographic performance of SFC have been reported in recent years [11-13, 63, 64]. As water being a strong solvent with many polar stationary phases and its miscibility with supercritical CO_2 being very limited, sample diluent containing water seems to be excluded from the category of appropriate SFC sample diluents. However, beneficial effects of water injection have been pointed out in several studies [65-68]. **Paper III** presents a comprehensive study of the impact of water-containing injection on SFC. In general, peak distortion became more severe with higher injection volume and higher water content in the sample diluent. However, it can be beneficial for certain compounds to be injected in water-rich sample diluent, especially on polar stationary phases. Another interesting finding is that the immiscibility of water and stationary phase may cause the injected water to perform like a compound band with certain retention in the column and result in a noise peak when it is eluted.

2.4.2. Injection techniques in SFC

When SFC is used in trace analysis, one simple way to improve the detectability of the compounds is by increasing the volume injected. In SFC with fixed-loop injection, the injection volume is adjustable by applying injection loops of different sizes. However, a relatively large injection volume can cause severe peak distortion and loss of efficiency and resolution [13, 64]. Also, pressure spike can take place after the injection if the sample diluent has a relatively high viscosity, which has been demonstrated in **paper IV** and **V**. **Paper IV** investigated the possibility of performing multiple injection in analytical SFC to circumvent the change of injection loop and the problem with pressure spike. As shown in Figure 2.7, a certain number of injections were made in a continual way with low-elution strength mobile phase before the final gradient is started for analysis. With proper analyte accumulation on the column head, signal to noise ratio can be enhanced compared with a single injection.

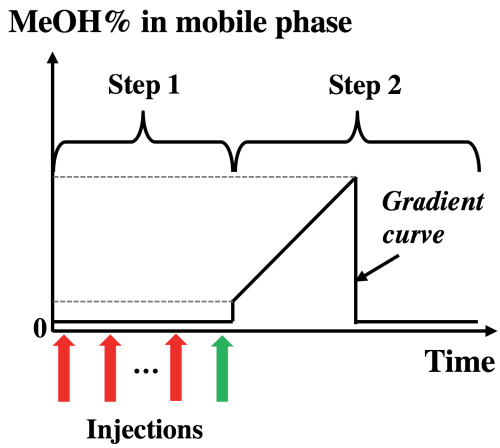

Figure 2.7. Scheme of multiple injection approach in SFC.

The study revealed that signal enhancement for a certain compound varies greatly with change in stationary phase chemistry and sample diluent. The signal enhancement ratio is strongly dependent on the retention of the analyte. Compared with one-time large volume injection of the same mixture, the multiple injection approach showed similar repeatability and reproducibility in terms of peak area and retention time. The application potential of this approach was demonstrated with the analysis of sulfanilamide spiked honey acetonitrile extract (Figure 2.8) and diclofenac spiked ground water sample.

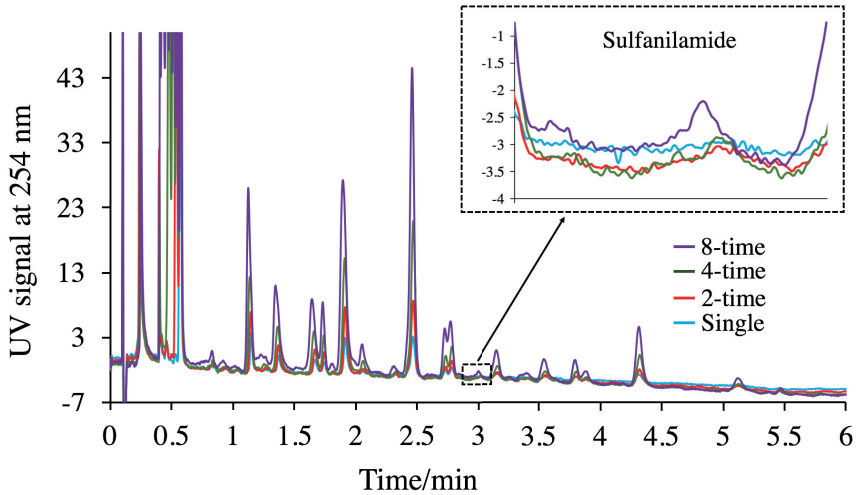

Figure 2.8. Multiple injection of sulfanilamide-spiked (500 ng/mL) honey extract in ACN.

2.4.3. Gradient delay volume

For a typical LC or SFC system, gradient delay volume refers to the volume between the solvent mixing point and the column inlet. Before the start of a gradient run, this volume is usually filled with the mobile phase of the starting composition after equilibration is performed. Now that modern SFC enables high throughput fast separation, gradient delay volume is also a critical parameter as it adds a certain unnecessary isocratic period depending on its magnitude [32, 69]. Compared with state-of-art UHPLC instruments, commercial SFC systems have significantly larger gradient delay volume [48, 70]. This mainly results from the need of a relatively larger mixing chamber than those used in UHPLC in order to effectively mix compressed CO_2 with modifier at various ratios. This problem can be partially remedied by the high mobile phase flow rates commonly used in SFC in one-dimension analysis. However, special gradient settings are needed if SFC is used as the second dimension in a two-dimensional chromatography system. **Paper V** gives such an example.

2.4.4. SFC column dimensions

Another aspect of instrumentation is the dimension of the SFC columns. Although there is no absolute distinction between columns specially designed for SFC and for UHPLC use, the selection of an SFC column requires more considerations than that of a UHPLC column. The choice of column dimensions is one such case. Columns with 2.1 mm inner diameters (i.d.) are commonly used in all types of UHPLC applications. However, 2.1 mm i.d. columns shorter than 400 mm are not

suitable for SFC application using current commercial systems, as the extra-column band broadening cause a large portion of efficiency loss [32]. As was stated by Fekete and coworkers that the extra-column band broadening should be less than 11% of band broadening in the column in order to be considered negligible [70]. Compared with state-of-art UHPLC systems, current commercial SFC system have significantly higher extra-column band broadening due to many factors, such as the use of special connections and high pressure diode array detector (DAD) flow cell [71]. As a remedy of this issue, the use of larger i.d. columns are preferred with less contribution of efficiency loss outside the column. What should be pointed out is that optimum flow rates for columns with wider i.d. are not always possible to be meet, especially with high modifier percentages in the mobile phase, as current SFC systems do not offer as high pressure as those with UHPLC instruments [32]. Consequently, 3 mm×100 mm columns become the most popular choice when taking efficiency loss and solvent consumption into consideration.

2.4.5. Detectors coupled with SFC

Diode array detectors are the most used detector in SFC up to now. In general, the achievable LOD and LOQ are usually higher with SFC-DAD than those with UHPLC-DAD. The major cause of this phenomenon is the pressure fluctuation. As refractive index is closely related to the density of the liquid, small variations of pressure of the supercritical fluid from the pump stroke or BPR can cause fluctuations in density and consequently RI and UV signal [72]. In addition, recent research has also linked part of the UV noise with the mismatch of mobile phase temperature coming out the column and the temperature of the DAD flow cell [73].

Open cell detectors such as evaporative light scattering detector (ELSD) and charged aerosol detector (CAD) have also been successfully coupled with SFC in various applications [74-77]. Both ELSD and CAD rely on the formation of analyte aerosols after nebulization and solvent evaporation, which limits their detectability to only non-volatile compounds [78, 79]. CAD has been reported to provide better quantitative performances than ELSD due to its higher sensitivity for low concentrations [75]. As a quasi-universal detector, the use of CAD in SFC analysis can be advantageous when chemical standards are not available for the targeted compounds [80]. The use of SFC-CAD in semi-quantitative analysis of lignin type small phenols have been attempted during the PhD study, but all efforts made have been regretfully in vain. This is not totally unexpected as lignin-derived phenolic monomers are normally far too volatile to be analyzed by CAD, making an universal calibration nearly impossible. Nevertheless, these experiments led to some interesting findings which might become the inspiration for future research. For example, the influence of mobile phase composition on CAD response in SFC

seemed to be less profound than that in HPLC. Also, chemometric approach appeared to be promising in semi-quantitative analysis of the lignin-type monomeric compounds, which deserves further investigation in the future.

The coupling of SFC with mass spectrometry (MS) has become gradually more and more popular as MS can provide another dimension of analysis in complement to the retention time and UV absorption of the analytes [81]. Two types of interface are currently dominantly used in the coupling of SFC and MS: full-flow and split-flow [82, 83]. Full-flow interface enables the whole flow to be directed to the MS with the addition of a make-up solvent flow between the column outlet and BPR to wash off precipitation and enhance ionization. Split-flow mixes the flow from the column outlet with a make-up solvent flow and then the mixed flow is directed to a splitter where a certain proportion of the total flow goes to MS and the rest of flow goes back to BPR for maintaining the pressure. **Paper II** presented a SFC-MS method development for the analysis of lignin-derived small phenolic compounds. Split-flow interface was used for the coupling of SFC with MS.

Like LC-MS, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are the most commonly used ionization techniques in the coupling of SFC and MS. Normal ESI sources are more compatible with split-flow type interface as what was used in **paper II**, but full-flow interface is also possible to use with modern advanced ESI sources [84]. Compared with ESI, APCI is more suitable for accommodating higher flow and ionizing less polar compounds. For example, polycyclic aromatic hydrocarbons were analyzed with SFC/APCI-MS with pure CO_2 as mobile phase more than 20 years ago [85]. When it comes to mass analyzer, most of the commonly used ones in LC-MS can also be used in SFC-MS besides single quadrupole [86-89]. In the work presented in **paper II**, quadrupole time-of-flight mass analyzer was used. ESI source settings such as source temperature, cone voltage, capillary voltage, desolvation gas flow and temperature were optimized using a D-optimal design of experiment (DOE).

The choice of additive is another important factor to consider during the development of a MS method. Volatile additives such as formic acid, ammonium formate and ammonium hydroxide are more preferred than additives with relatively low volatility. What is also very important to be pointed out is that column life time and chromatographic reproducibility can be compromised if additives are not properly used [45]. A good practice of additive usage can be applying enough equilibration before starting a SFC run and carefully washing the column before storage to ensure complete removal of the residue additives. If the addition of additive is only for MS ionization enhancement, it can take place after the column with a make-up flow as what was done in **paper II**. The make-up flow solvent and flow rate, together with the identity and concentration of the additive were optimized with the help of DOE in **paper II**.

As has been mentioned above, changes in mobile phase pressure lead to mobile phase density changes and consequently fluctuations in DAD signal. A dynamic BPR with a wide pressure range and precise pressure control is essential for modern SFC systems. In addition, heating is usually applied on the BPR to prevent formation of frost due to cooling from CO_2 expansion. For example, BPR temperatures were both set to 60 °C in the studies reported in **paper IV** and **V**.

2.5. Applications

Compared with GC, SFC holds the advantages of being capable of analyzing non-volatile compounds, offering better selectivity and shorter analysis time. The advantages of SFC over LC are faster mass transfer, lower organic solvent consumption and a wider range of stationary phases. While polar and non-polar compounds have poor retentions in RPLC and NPLC respectively, they can both be analyzed in SFC. As long as the targeted compounds have sufficient solubility in CO_2-modifier mobile phase, SFC is highly likely to be applicable [90].

SFC is becoming more and more popular in pharmaceutical and biological sample analysis, even though liquid chromatography (especially RPLC) still dominates this type of application [91]. It offers orthogonality, thus unique complementarity to LC, especially in chiral analysis. As pure CO_2 or CO_2 with very little modifier resembles a non-polar solvent, the applicability of SFC in analysis of relatively non-polar lipids, steroids and vitamins have been investigated and proven by various research groups [92-97]. When CO_2 in supercritical or sub-critical state is mixed with substantial amount of modifier, it can also be used as mobile phase for comparatively polar compound analysis in SFC together with various types of polar stationary phases. There have been numerous papers published covering SFC analysis of polar pharmaceuticals and biological metabolites [98-100]. Another very important task in pharmaceutical analysis is to achieve fast and efficient chiral separation of enantiomeric drugs. As has been briefly discussed before, SFC emerged as a good solution and its suitability and usage in chiral pharmaceutical analysis has been widely reported both in analytical and preparative scale [61, 101].

Another major application field of SFC is the analysis of natural and food products [102, 103]. The traditional focus of SFC application in foodstuff and natural product has been lipid analysis, owning to the good solubility of lipids in supercritical or sub-critical CO_2, especially triacylglycerols [103]. Meanwhile, the addition of polar modifier and additives makes more polar lipids and other types of compounds amenable for SFC analysis. SFC has been utilized for analysis of both different lipid classes and specific lipid species in the same class [104, 105]. Besides lipid analysis, SFC also finds wide applications in the analysis of fatty

acids [106, 107], carotenoids [108, 109]and vitamins [28, 110].

One promising challenge for SFC to tackle is the analysis of products from ligno-cellulosic biomass conversion. As believed to be a high-potential source of future fuel and value-added chemicals, ligno-cellulosic biomass conversion is currently a hot research area that receives world-wide attention [111]. Unfortunately, the conversion of this type of biomass often lead to a very complex mixture including monomeric and oligomeric compounds derived from both cellulose and lignin [5]. Although being a highly efficient and rapid separation technique, SFC has very rarely be reported as the analysis tool in this area [112-114]. As the first attempts of applying SFC in lignin-derived compound analysis, **paper I** and **II** demonstrated the great potential of SFC in this field.

3. Two-dimensional liquid chromatography

3.1. Working principle of two-dimensional liquid chromatography

Strictly speaking, two-dimensional liquid chromatography (2D-LC) is not a new concept with an origin traced back to about 40 years ago [115]. As the sample being tackled by analysts become more and more complex and the analysis needs keep expanding, more and more attention has been drawn to 2D-LC in recent years [15]. The blossom of scientists' interests in 2D-LC has also led to revolutionary advances in instrumentation development.

Evolving from 1D-LC, 2D-LC adds one interface to collect fraction(s) of one LC after the separation and transfer the collected fraction(s) into another LC for a 2^{nd} dimension separation. In general, there are two main modes in which 2D-LC can be operated in: heart-cutting and comprehensive. Figure 3.1 illustrates the working principles of these two modes. In a heart-cutting 2D-LC run (LC-LC), one selected fraction of the 1^{st}-dimension eluent is collected and transferred into the 2^{nd}-dimension column for another separation. By using different instrumental set-up, it is possible to collect more than one fraction from the 1^{st}-dimension flow and have them analyzed in the 2^{nd} dimension. This specific mode of heart-cutting 2D-LC is more accurately described as multiple heart-cutting 2D-LC (mLC-LC) [116]. In a comprehensive 2D-LC analysis (LC×LC), the 1^{st}-dimension eluent is divided into a series of segments of the same length in time. All segments will then be collected and transferred into the 2^{nd} dimension. Modern instrumentation also allows comprehensive mode to be applied only for a specific fraction of the 1^{st}-dimension eluent, which lead to the relatively new concept of selective comprehensive 2D-LC or sLC×LC [117]. Given that comprehensive 2D-LC is by far the most widely used 2D-LC technique and covers all the work included in this thesis, the following discussion addresses only comprehensive 2D-LC unless indicated otherwise.

The 2^{nd}-dimension analysis can be done either on-line or off-line. Interestingly, the 2D-LC scientific community seems to have developed an unequivocal preference

of on-line 2D-LC over their off-line counterparts. Although some controversy can be stirred by this statement, the rarity of the word "off-line" in the titles of published off-line 2D-LC work is an obscure indication of its rightness. Nevertheless, both on-line and off-line 2D-LC combined with either heart-cutting mode or comprehensive mode have their own advantages and disadvantages, which have been very well summarized [118]. The work included in this thesis mainly focuses on on-line comprehensive 2D-LC studies.

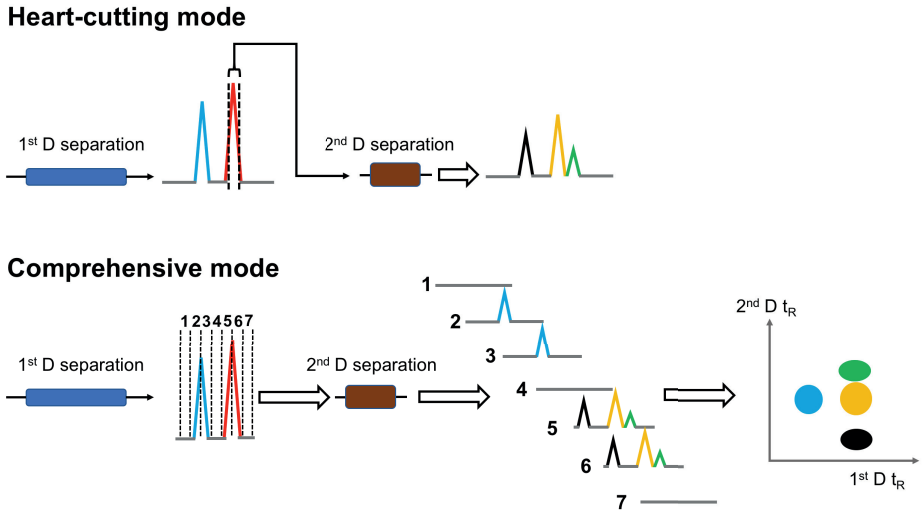

Figure 3.1. Working principles of heart-cutting and comprehensive 2D-LC.

Figure 3.2A shows the scheme of a typical on-line comprehensive 2D-LC system. Both of the two dimensions operate in the same way as a one-dimensional LC. After the 1st-dimension detector, the eluent is directed into an interface which is usually a switching valve with two collection loops. One collection loop (A) collects the 1st-dimension flow with the other one (B) being flushed by the 2nd-dimension mobile phase. After a certain period of time, the valve is switched (Figure 3.2B) and the collected fraction in loop A is then flushed into the 2nd-dimension column and loop B is now used for 1st-dimension eluent collection. The valve is switched back and forth at a certain interval, allowing all fractions of the 1st-dimension flow to be collected and sequentially transferred into the 2nd dimension for further analysis. The time between two valve switches is often called modulation time.

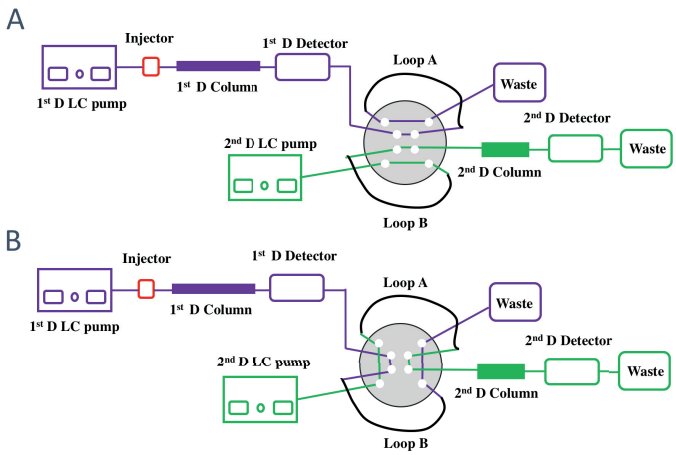

Figure 3.2. Scheme of an on-line comprehensive 2D-LC system. A: the eluent from the 1st dimension is being collected by loop A and the content in loop B is being flushed into the 2nd-dimension column for analysis. B: vice versa. Figure from **paper V**, reprinted with permission.

The major incentive for applying comprehensive 2D-LC instead of one-dimensional LC is the significantly increased peak capacity. As in any one-dimensional LC techniques, the peak capacity of any of the two dimensions in comprehensive 2D-LC is defined as the maximum number of peaks that can be fitted between the retention time of the first and last peak, with a resolution of 1.0 between all pairs of peaks [119]. It is then obvious that gradient elution is preferred in both dimensions for pursuing high peak capacity, as gradient elution lead to more uniform peak widths despite the elution time differences.

In a gradient elution, peak capacity (n) can be estimated with equation 1.

$$n = 1 + \frac{\Delta t_r}{W_{ave}} \quad (1)$$

Δt_r stands for the retention time difference between the first and last peak. W_{ave} is the average 4σ peak width of all peaks. In a well-designed gradient elution starting from time 0, the first peak usually elutes around the dead time (t_0) and the last peak around the end of the gradient. Considering the magnitude of the second term in equation 1, it can then be simplified into the following form:

$$n = \frac{t_g}{W_{ave}} \quad (2)$$

In this equation, t_g symbolizes the length of the gradient in time.

From equation 2, a general observation is that the peak capacity can be increased by either increasing the gradient time or decreasing the peak width. However, for a column of specific dimensions, the impacts of the two approaches may differ significantly depending on the flow rate the separation is operated at. In **paper V** it was demonstrated that when the 1st-dimension separation had a very low flow rate, rather than lengthening the gradient time, higher increase of peak capacity could be achieved by a rise in flow rate, which effectively decreased the peak width.

If the two-dimensional separation space of comprehensive 2D-LC is pictured as a square or rectangular car parking yard, it is very straightforward to figure out that the maximum number of cars allowed to be parked is the product of the number of cars that can fit in a horizontal line and that in a perpendicular line. Similarly, theoretical peak capacity of a comprehensive 2D-LC separation is then the product of 1st-dimension peak capacity and 2nd-dimension peak capacity as described by equation 3.

$$n_{2D} = {}^{1}n \times {}^{2}n \tag{3}$$

${}^{1}n$ and ${}^{2}n$ are the theoretical peak capacities of the first and second dimension respectively.

However, the maximum theoretical peak capacity can only be achieved in an ideal separation under two conditions: 1. The separation of the compounds in a sample is governed by two orthogonal and complementary mechanisms in the two dimensions, and the peaks are distributed all over the 2D separation space. 2. All separations achieved in the 1st-dimension are preserved in the 2nd dimension [120]. There are two main sources of deviations from this ideal situation in real applications: the re-mixing of separated compounds in the 1st dimension occurring when entering the 2nd dimension and the incomplete coverage of the 2D separation space [121, 122].

Due to the complexity of real samples, there is always high probability that compounds that have similar 1st-dimension retention times have the same 2nd-dimension elution time. With a certain sampling rate, there will be re-mixing of the compounds to different extents depending on how well the compounds are separated in the 1st dimension when the collection starts and their respective peak widths. The following scenario serves as a relative extreme case of this type of issue: suppose that three compounds are separated in the 1st dimension with resolution 1.0 between the first compound and the second compound, and the second compound and the third compound. These three compounds also have the

same elution time in the 2nd dimension. All three peaks have 4σ peak widths of 0.3 min and the modulation time is 1.0 min. If the collection started from the beginning of the first peak, then all three peaks will be collected in one modulation and only one peak will be recognized from the final 2nd-dimension chromatograms. A more detailed simulated demonstration of how 1st-dimension peak capacity is affected by the sampling rate has been made in reference [123].

As this undersampling issue causes loss of peak capacity and information in the 1st dimension, it should be minimized as much as possible in a comprehensive 2D-LC run. It is very clear that an increase in sampling rate can be beneficial as peaks will then be collected in a higher number of modulations. One guideline proposed by Murphy, Schure and Foley for minimizing undersampling in the 1st dimension was that "3 to 4 samples in the 2nd dimension must be taken across the 8σ width of the 1st-dimension peak" [124].

A more quantitative expression of undersampling was deducted and reported by Davis and co-workers for 2D peak capacity correction [125, 126]. They developed an average peak broadening factor $<\beta>$ (equation 4) and used it as a correction factor on the theoretical 2D peak capacity equation (equation 5).

$$<\beta> = \sqrt{1 + 3.35(\frac{t_s}{W_1})^2} \quad (4)$$

t_s is the collection time that equals to the modulation time. W_1 is the 1st dimension average 4σ peak width.

$$n_{2D} = ({}^1n \times {}^2n)/<\beta> = ({}^1n \times {}^2n)/\sqrt{1 + 3.35(\frac{t_s}{W})^2} \quad (5)$$

Considering W_1 can be deducted using equation 2 if a proper 1st-dimension gradient is used, equation 5 can be further converted to equation 6.

$$n_{2D} = ({}^1n \times {}^2n)/\sqrt{1 + 3.35(\frac{t_s \times {}^1n}{t_{g1}})^2} \quad (6)$$

t_{g1} is the 1st-dimension gradient time.

As the 2nd-dimension separation has to be reasonably long to allow decent resolution and column equilibration, almost all comprehensive 2D-LC analysis is

performed with severe undersampling in the 1st-dimension [119]. Accordingly, equation 6 can be simplified to equation 7.

$$n_{2D} \approx ({}^{2}n \times t_{gl})/1.83t_s \quad (7)$$

Surprisingly, the total 2D peak capacity corrected for undersampling in the 1st dimension is not influenced by the 1st-dimension peak capacity. A practical implication of this calculation was shown in **paper V**. Although higher 1st-dimension peak capacity was achieved by the usage of trapping columns in replacement of loops on the interface, no gain of total 2D peak capacity was observed for trapping column-assisted modulation.

Another factor that the theoretical maximum peak capacity should be corrected for is the partial coverage of the 2D separation space. This spatial coverage is decided by the interaction of the set of compounds in a sample with the two stationary phases at the two dimensions. It is closely related to the term "degree of orthogonality", which describes how different the two separation mechanisms are in a comprehensive 2D-LC system [122]. Figure 3.3 displays different degrees of orthogonality depicted by simple 2D chromatograms.

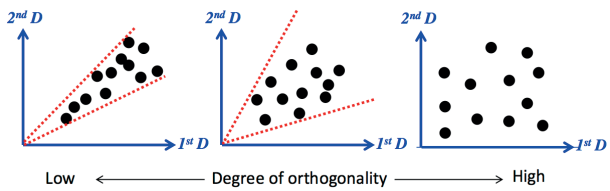

Figure 3.3. Different degree of orthogonality in comprehensive 2D-LC shown in 2D plots.

Spatial coverage or degree of orthogonality is not only dependent on the column chemistry. The analyte types also play an important role. For example, the combination of size exclusion chromatography (SEC) and RPLC (SEC × RPLC) is expected to provide very orthogonal separation. This is for instance the case with the separation of a traditional Chinese medicine, as the compounds differ both in size and polarity [127]. However, if the same system is used for the analysis of small phenolic compounds derived from lignin depolymerisation, the peaks highly likely will not spread over the 2D space, due to the narrow range of size encompassed by the sample. So, the key factor that decides the spatial coverage and the degree of orthogonality in a comprehensive 2D-LC is whether the sample possess two wide ranges of different physio-chemical properties. If this condition is met, high spatial coverage and degree of orthogonality can then be possibly realized by selection of two column chemistries that specifically respond to the two compound properties respectively.

Quantification of the actual coverage of the 2D separation space is essential for the correction of the theoretical peak capacities. For this purpose, different approaches have been developed including bin-counting strategies, ecological home-range theory, fractal mathematics and Asterisk Equations metric [15]. As each of these approaches have strengths and weaknesses, no consensus has been reached on the best choice. The bin-counting approach proposed by Gilar and modified by Davis was adopted in **paper V** for the correction of theoretical peak capacity [122, 128]. As is illustrated in Figure 3.4 (data retrieved from **paper V**), all peaks with UV absorption higher than a set certain level were plotted in a two-dimensional plane, with their retention times on the two dimensions as coordinates. Then the total plane area was divided into a series of rectangular time bins by casting a grid on the surface. A rectilinear outline was drawn to surround all occupied bins and a fractional coverage factor f_{cov} was obtained as the ratio between the number of bins surrounded and total number of bins.

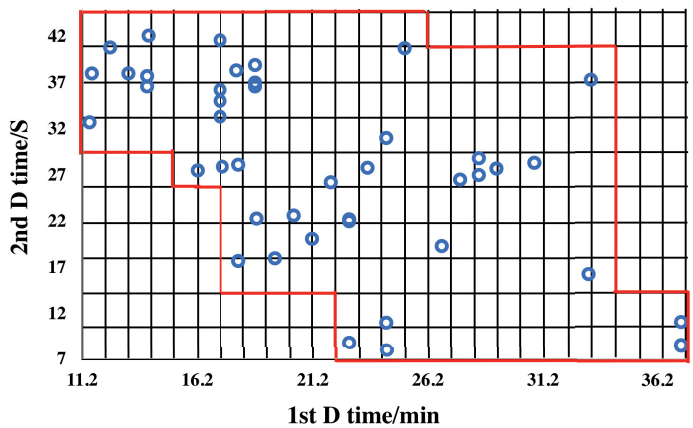

Figure 3.4. Illustration of bin-counting approach for coverage quantification.

A two-dimensional LC system is much more complicated than the simple combination of two stand-alone one-dimensional systems. Also, 2D-LC systems are far more expensive than 1D LC systems. So as peak capacity is usually the main incentive to apply comprehensive 2D-LC instead of one-dimensional separation techniques, it is crucial to have a fair comparison between the peak capacity offered by comprehensive 2D-LC and those by one-dimensional LC systems before adopting the 2D approach. Thus, a more realistic estimation of the 2D peak capacity is essential. If the correction method for undersampling in the 1st dimension in equation 4 and the spatial coverage metric proposed by Gilar and modified by Davis are adopted [122, 128], the practical comprehensive 2D-LC peak capacity can be calculated with equation 8.

$$n_{2D} = ({}^{1}n \times {}^{2}n \times f_{cov})/<\beta> \quad (8)$$

3.2. Aspects of the design of a 2D-LC system

3.2.1. Separation mechanism selection

Similar to one-dimensional separation, the development of a 2D-LC method often starts with choosing the general separation modes in the two dimensions. Accordingly, specific stationary phases, column dimensions and mobile phases are selected. Considering the number of different separation modes in liquid chromatography, the theoretical number of mode combinations is very high [129]. However, only a minority of the combinations have applicability as the rest suffer heavily from one or more following defects: low orthogonality, low peak capacity, solvent incompatibility and instrumentation difficulties [119]. Among those feasible combinations, RPLC × RPLC is the most frequently adopted one for various applications. This combination provides easy system assembly, high peak capacity and minimal solvent incompatibility. The only concern about this set-up is the difficulty to obtain high degree of orthogonality of the two RPLC separations. One promising solution is to apply different pH in the two dimensions to keep the analytes in different ionization states in different dimensions [122]. Another promising combination that has been proven is HILIC with RPLC. In terms of the coupling of HILIC and RPLC, RPLC is usually placed in the 2nd dimension as HILIC does not have the fast re-equilibration feature required [130, 131]. Besides RPLC × RPLC and HILIC × RPLC, several other applicable combinations of separation modes have also been studied in recent years, such as SEC × RPLC, IEC × RPLC and NPLC × RPLC [132-137].

Despite that the combination of two reversed phase modes is still the most commonly used 2D-LC system so far, the high potential of SFC being used as either of the two dimensions in 2D-LC has been proven in several publications in recent years [22, 23, 25, 138, 139]. With trapping column interface with make-up water flushing, SFC × RPLC was demonstrated to provide higher degrees of orthogonality than RPLC × RPLC, because of the uniquely normal phase like separation mechanism displayed by SFC [138, 139]. To avoid the complicated interface design and better harness the efficiency of SFC, an on-line multiple heart-cutting LC-SFC system was built and used for achiral-chiral analysis of drug formulations [22]. **Paper V** describes the building and performance evaluation of a 2D RPLC × SFC system using solid trapping collectors on the interface. The system showed relatively high degree of orthogonality for all SFC columns screened. Coverage factors ranged from 0.64 to 0.72 for a mixture of 40 compounds and 0.79 for a depolymerised lignin sample with the selected SFC

column (Figure 3.5).

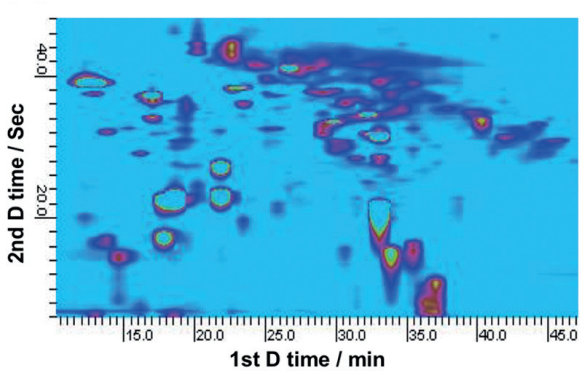

Figure 3.5. 2D RPLC×SFC separation of a lignin depolymerised sample with an interface using two trapping columns. See experimental section in **paper V** for chromatographic conditions. Figure from **paper V**, reprinted with permission.

3.2.2. Gradient and gradient delay time

Gradient elution is usually applied in one or both dimensions in a 2D run. There are several reasons behind this: 1. to increase peak capacity; 2. to shorten analysis time; 3. to ensure complete elution of all compounds in every modulation; 4. to facilitate analyte focusing on the 2nd-dimension column head at the beginning of every modulation [140]. For the 2D RPLC×SFC system described in **paper V**, it was demonstrated that flow rate increase can lead to higher 1st-dimension peak capacity increase more than an increase in 1st-dimension gradient time, when the 1st-dimension LC flow is kept relatively low.

In all 2D-LC systems, there is always a certain time difference between the theoretical programmed gradient curve and the real mobile phase composition curve on the column head [123]. This delay time is finite as it is decided by the system volume between the pump head and the column head, which usually includes a solvent mixer and some connection capillaries. The gradient delay time can be easily deducted from the flow rate F and the delay volume Vol_d, as shown in equation 9.

$$t_d = Vol_d / F \tag{9}$$

In **paper V**, the 2nd-dimension gradient was set to start already in the previous modulation, compensating for the comparatively large gradient delay volume of the system (~700 µL).

3.2.3. Sample dilution

When a certain analyte band goes through any chromatography separation, it is unavoidable that the band will be diluted at the outlet of a column compared with at the inlet. Sample dilution in 2D-LC is much more severe than in traditional 1D-LC as the transferred sample from the 1st-dimension column end will experience another dilution in the 2nd dimension [141]. Besides our work in **paper V**, on-line comprehensive RPLC×SFC has previously been reported with two interfacing loops [23]. However, with this design only a very low 1st-dimension flow rate can be used considering the injection volume limitation on the 2nd-dimension SFC. Slow 1st-dimension flow rate resulted in high dilution factor and lengthened analysis time. **Paper V** demonstrated that trapping column assisted modulation can effectively collect and concentrate the sample from the 1st-dimension eluent, which enabled relatively higher 1st-dimension flow rate than those used in the previous literature [23]. Consequently, the system offers higher detectability and shorter analysis time compared with RPLC×SFC system using traditional loop interface.

What is also worth mentioning is that special attention must be paid for peak area comparison with a concentration-dependent detector used at the end of the 2nd-dimension separation, such as single-wavelength UV or diode array detector. Under these circumstances, the peak areas for the same analyte in the same sample plug may appear to be very different depending on the flow rate used [142].

3.2.4. Solvent incompatibility and advanced interfacing design

One of the most challenging tasks in developing a 2D-LC method is to minimize the solvent incompatibility issue [143]. For example, HILIC×RPLC was shown to provide high degrees of orthogonality as the two separation mechanisms are to a large degree complementary. However, the pitfall is usually with the negative impact on RPLC separation of transferring relatively ACN rich HILIC eluent to a RPLC column. This issue is especially problematic in coupling RPLC and SFC, as the 2nd-dimension SFC can not tolerate a relatively large volume of water being injected. In **paper V**, a study was carried out to assess the influence of transferring water containing eluent to the 2nd-dimension SFC. As can be seen in Figure 3.6, injections containing relatively high amount of water surprisingly led to better peak shape, especially when the injection volume was comparatively high. This later became the inspiration for the study conducted in **paper III**. However, a high pressure spike was found to occur at the beginning of the run when the water content in the injected plug is high. When the injection volume reached a certain level, this spike started to cause an extremely noisy baseline and even system over-pressure shut down.

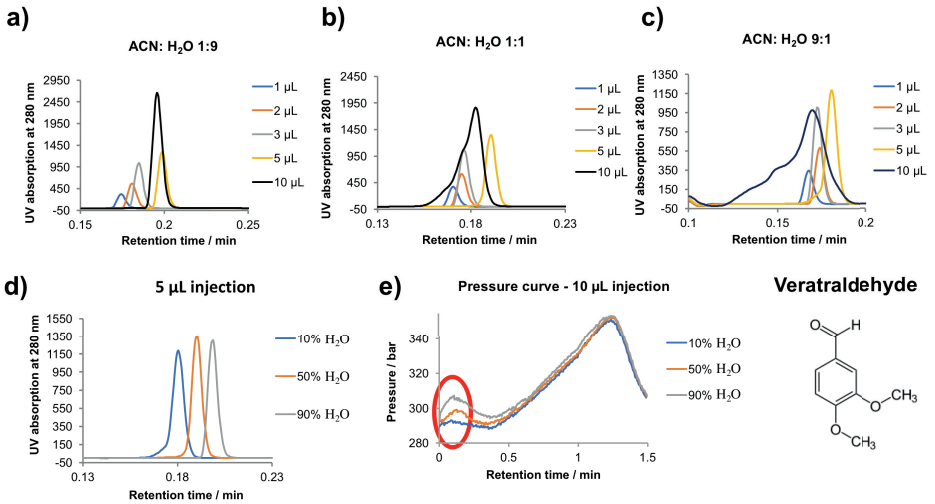

Figure 3.6. Influence of injection volume and water content on SFC separation performance. a): veratraldehyde in ACN:H_2O 1:9, different injection volumes; b): veratraldehyde in ACN:H_2O 1:1, different injection volumes; c): veratraldehyde in ACN:H_2O 9:1, different injection volumes; d): veratraldehyde in different ACN:H_2O mixture, 5 µL injection; e): pump pressure curves of different injections. See **paper V** experimental section for chromatographic conditions. Figure from **paper V**, reprinted with permission.

In conventional loop-based interface design, the only possibility is reducing the size of the sampling loops. Instead, there are two major techniques reported in recent years that have been proven to effectively remedy this problem. The first one involves a dilution process of the 1st-dimension eluent with a much weaker solvent before the transfer [144]. This can be done by adding another pump providing the weak solvent flow and mix with the 1st-dimension eluent or more recently by a new valve design that allows one fraction of the 2nd-dimension mobile phase to flush the collected fractions and simultaneously using the rest of the 2nd-dimension mobile phase to dilute the sample plug before it reaches the 2nd-dimension column head. This technique is often referred as "active solvent modulation" [145]. Another technique addressing the solvent incompatibility issue can be generally described as "solid-phase- or trapping column-assisted modulation" [146, 147]. This is the technique adopted in the work described in **paper V**. Two identical small trapping columns are used on the two positions on the interface. By selecting appropriate stationary phases, small trapping columns can effectively retain and focus the targeted analytes, as well as reduce the transfer volume. One improvement of this technique introduces the concept of active solid phase-assisted modulation [148]. The 1st-dimension eluent is mixed with a flow of weak solvent before entering the traps to reduce the elution strength of the flow and achieve better analyte focusing in the trap. Both trapping-column assisted modulation and more advanced active modulation techniques involving sample pre-concentration in a solid trap before the 2nd dimension come with performance compromises. Thus, trapping capacity must be evaluated as a very important

parameter. Trapping capacity is strongly dependent on the volume, so traps with larger volume may increase the trapping capacity. However, this also adds more gradient delay time. Moreover, traps with small internal diameter and particle size can introduce another significant pressure drop besides the one through the 2nd-dimension column and further reduce the 2nd-dimension flow rate tolerated. Experiments performed in **paper V** showed that trapping columns of relatively small volumes tested had limited trapping capacities, which not only limited the increase of the 1st-dimension flow rate but also undermined the repeatability and reproducibility of the trapping column assisted RPLC×SFC system. Besides solvent dilution and solid phase assisted modulation, solvent reduction has also been proven to be effective in minimizing the solvent incompatibility. It is usually done by thermal or vacuum evaporation of the 1st-dimension eluent before the transfer [149, 150].

3.2.5. Column dimension selection

The selection of the 2nd-dimension column dimension (column length, inner diameter and particle type and size) must take into consideration several major criteria: first of all, the choice of column dimensions should strive for high column efficiency, which is closely related to particle size, linear velocity and column length [151]. Secondly, the maximum pressure drop through the column must not surpass the upper pressure limit of the pump. Thirdly, the flow rate tolerated must be high enough to decrease the gradient delay time to a negligible value compared with the gradient time. Fourthly, the column diameter combination must tolerate injections of the transfer volume. Additionally, it is also preferred to lower the organic solvent consumption when possible for the consideration of cost and environmental protection [152]. Due to these considerations, column i.d. lower than 2.1 mm is not preferred as the flow rate is too low, which leads to a relatively high gradient delay time and column void time. Small particle sizes are always preferred for better resolution, but it may not be affordable for normal HPLC systems [153].

Compared with the choice of 2nd-dimension columns, there is not many limiting factors in the selection of the 1st-dimension column. One important aspect that must be taken into consideration is that columns with i.d. lower than 2.1 mm are generally used as they lead to reasonable transfer volume into the 2nd dimension with reasonable linear flow rates [140].

3.2.6. 1st-dimension flow rate and 2nd-dimension injection volume

Perhaps the most complicated issue that one encounters during the construction of a 2D-LC system are associated with the large number of parameters that should be

selected. Only the combination of two LC separation dimensions can generate twice the number of experimental variables as those in a one-dimensional LC. Aside from these, some parameters should also be optimized for a well-functioning interface. What makes things more complicated is that some of those variables are connected with each other with interactive effects on the method performance [119]. For example, the selection of modulation time is usually determined by the minimum time required for a sufficient 2^{nd}-dimension separation and the column regeneration time. When this parameter is fixed, the selection of 1^{st}-dimension flow rate becomes crucial. A comparatively low flow would lead to less severe undersampling and lower 2^{nd}-dimension injection volume, which would be beneficial when the two dimensions face solvent compatibility issue. However, low 1^{st}-dimension flow rate also results in long analysis time and long gradient delay time that jeopardizes the separation efficiency. As has been shown in **paper V**, a decrease in flow rate when the flow rate is already in a low range could lead to significant decrease of the 1^{st}-dimension peak capacity. Besides, the 2^{nd}-dimension injection volume would be too low in some cases for low-concentration compounds to be detected. In contrast, a too high 1^{st}-dimension flow rate can greatly shorten the analysis time, decrease the gradient delay volume in the 1^{st} dimension and reduce the sample dilution between the two dimensions. But it can also lead to severe undersampling and destructive effect on the 2^{nd}-dimension peak shape [119], which was also demonstrated in **paper V**: when the 2^{nd}-dimension separation could not be done rapidly enough, undersampling became more severe and even led to a drop in practical peak capacity in combination with the increase of the 1^{st}-dimension peak capacity brought by the increase of flow rate.

3.2.7. Detectors

The most commonly used detectors for 2D-LC applications are UV-Vis and MS. Even though in most of the cases only the chromatograms from the 2^{nd}-dimension are utilized for both qualitative and quantitative study, it is still a good practice to connect a UV detector right after the 1^{st}-dimension column to gain more information about the separation, provided that it would not introduce too much extra peak broadening and can handle possible short pressure spike caused by the turning of the valve [119]. The 2^{nd}-dimension detector used in the 2D RPLC × SFC system in **paper V** is a special case, where the 2^{nd}-dimension DAD detector has to be equipped with a high-pressure compatible flow cell in order to handle at least the pressure at the back pressure regulator. Besides adding as low volume as possible, high sampling frequency is another important requirement for detectors used in the 2^{nd} dimension. As the 2^{nd}-dimension separation is usually completed in less than one minute, peaks with half width lower than 500 ms are not uncommon. Accordingly, the sampling frequency of the detector has to be able to reach higher

than at least 20 or preferably 40 Hz. In order to remedy the sample dilution in the 2nd dimension and obtain more structural information, MS detection is also widely used as the 2nd-dimension detector. However, it should be noted that some MS detectors may not afford a sampling frequency high enough to cater for the narrow 2nd-dimension peaks [123]. Moreover, the high flow rate in the 2nd dimension can be problematic for some ionization technique, for example electrospray ionization [123].

3.2.8. On-line coupling of extraction with chromatography

As chromatography is one of the most frequently used technique for sample analysis, extraction is certainly among the most important sample preparation processes. In most cases, the two analytical steps are done separately, or in a so called "off-line" fashion. The extract is collected with one instrument and then the chromatographic analysis is performed with another. As sample preparation is still considered as the bottleneck for the whole analytical procedure, coupling extraction with chromatography on-line is a potential way to improve the analytical sensitivity and accuracy. Numerous efforts have been made in hyphenating these two process with various types of highly automated systems [154]. Extraction can generally be performed in two different ways: static and dynamic. While the static mode requires less complicated instrumentation and less amount of solvent, the dynamic mode enables faster mass transfer and less undesired compound reactions under harsh extraction conditions [155]. Many studies have been conducted on coupling a wide variety of dynamic extraction processes with chromatographic analysis [156-160]. Most of these works utilize a two-position switching valve as the interface, with one loading module installed in one position for extract collection and a short capillary connection in the other position for the mobile phase of the chromatography to flow by during the extract collection. When a selected fraction of the extract is collected, the valve is switched to allow the collected extract to enter the chromatographic pathway for analysis. This type of set-up can be used for two major purposes: analyzing the composition of the extract during certain periods of extraction; and calculating the total extraction yield if all the targeted compounds can be effectively trapped in the loading module. However, continuous monitoring of a real dynamic extraction process is not possible, as the extraction solvent flow must be temporarily halted to avoid loss of unanalyzed extract fractions when the loading module is switched off the extraction path. This challenging task can be fulfilled by coupling extraction and chromatography with a parallel sampling interface as described in **paper VI**, mimicking the on-line comprehensive 2D-LC concept. A pressurized hot water extraction - ultra-high-performance liquid chromatograph system (PHWE-UHPLC) was built (Figure 3.7). As proof of concept, this system was utilized to

study compound specific extraction kinetics of curcuminoids from turmeric powder.

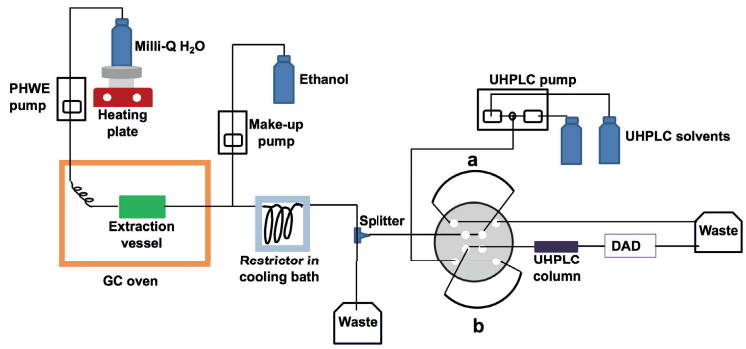

Figure 3.7. Scheme of the on-line PHWE-UHPLC system with parallel sampling. At the current position, the PHWE extract under continuous flow condition is being collected by loop **a** and the content in loop **b** is being flushed into the UHPLC column for analysis.

Figure 3.8. shows how the data obtained from the hyphenated system can be compiled to yield the extraction curve of a specific compound. From the results of curcuminoids extraction (Figure 3.9), it is very clear that the initial extraction was solubility-controlled (extracted amount is dependent on flow rate) at low temperature and mass transfer-controlled (extracted amount changes very little with flow rate) at high temperature. Also, curcuminoids thermal degradation can be observed by comparing the final extracted amounts obtained with different temperatures.

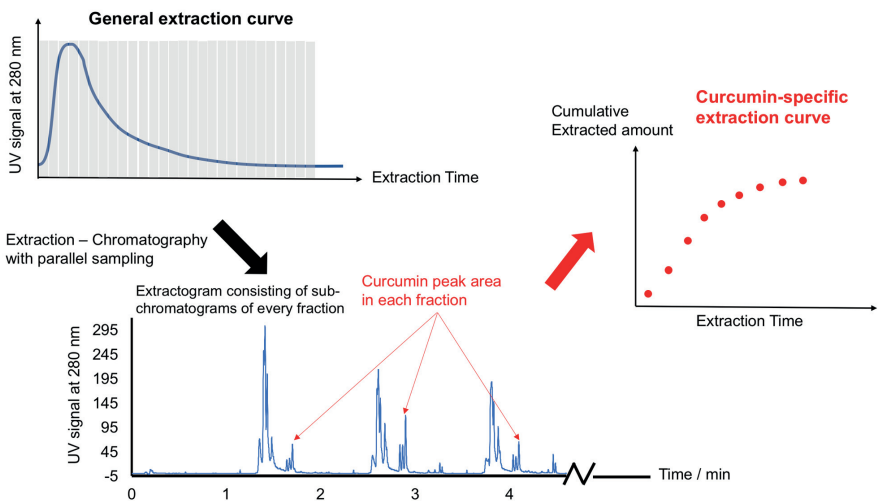

Figure 3.8. A scheme demonstrating how UHPLC chromatograms in a PHWE-UHPLC run are used to generate a compound specific extraction curve.

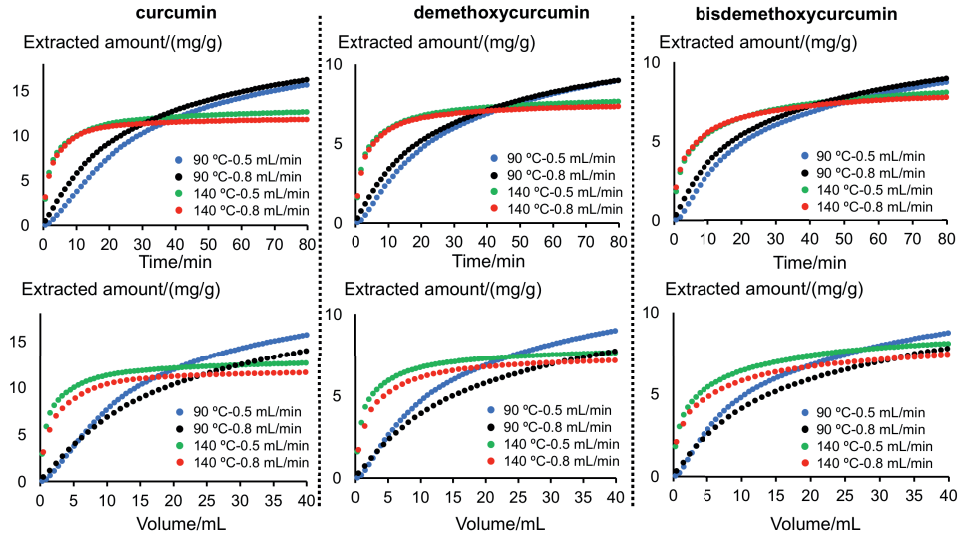

Figure 3.9. Extraction curves for three curcuminoids under different conditions. See experimental section in **paper VI** for specific experimental conditions.

3.3. Fields of applications of 2D-LC

Benefiting from the advances in hardware and software, 2D-LC is currently a hot research topic in chromatography and gradually moves from demonstration examples in academic labs into industrial lab uses. Normally, 2D-LC is used for the separation of complex samples, with comprehensive 2D-LC addressing mainly analyte profiling needs and heart-cutting 2D-LC aiming at one or more targeted compounds.

The biggest advantage that comprehensive 2D-LC holds over one-dimensional liquid chromatography is the unparalleled peak capacity. If the two separation mechanisms of the two dimensions are set to be sufficiently different, separation of high orthogonality can be potentially achieved. This significantly enhanced peak capacity has greatly facilitated a variety of profiling needs. RPLC × RPLC has been widely used in the analysis of bioactive compounds in different types of food, natural products and herbal extracts [161-163]. Although presenting more severe solvent compatibility issues than RPLC × RPLC, HILIC × RPLC generally offers higher degree of orthogonality and has been successfully applied in the profiling of metabolites, phenolic compounds and flavonoids in various matrixes [162, 163].

The combination of SFC and RPLC has also been reported in several studies for the analysis of fruit extract, fish oil and biomass degradation product [23, 138,

139]. Lignin is cross-linked phenolic polymer that is a major component of biomass. Lignin depolymerization can produce value-added phenolic compounds which can replace the traditionally used petroleum-based benzene, toluene and xylene. The complexity of the product poses as one of the major challenges for the development of efficient depolymerization methods. Many small phenols produced have very similar structures and chemical properties. 2D-LC was adopted in **paper V** for the analysis of small phenolic compounds in a depolymerized lignin sample. As the 2D LC × SFC separation offered high degree of orthogonality, a large number of peaks were separated with several compounds tentatively identified.

Pharmaceutical analysis using comprehensive 2D-LC technique is also increasing [164], such as peptide mapping of monoclonal antibodies and the analysis of synthetic dyes [165, 166]. Another attractive application of comprehensive 2D-LC is in the analysis of synthetic polymers. 2D-LC analysis can be effectively performed on polymers by combining two separation modes targeting at different properties of these polymers, such as molecular weight and functional groups. For this purpose, SEC × LC and LC × SEC have both been reported and hold the advantages of higher efficiency in the 2nd dimension and simpler instrumentation respectively [167].

For targeted analysis, the entire 1st-dimension eluent does not need to go through a 2nd-dimension analysis. Heart-cutting 2D-LC allows a specific fraction of the 1st-dimension flow to be collected and then analyzed in the 2nd dimension. The most obvious advantage of heart-cutting 2D-LC over comprehensive 2D-LC is that the 2nd-dimension separation time is less constrained, yielding better resolution of the compounds. If more than one compounds of interest co-elute in an LC analysis, multiple heart-cutting 2D-LC can be adopted to collect multiple fractions from the 1st dimension then analyze them sequentially in the 2nd dimension. When peak purity is checked or the two dimensions face severe solvent incompatibility issue, selective comprehensive 2D-LC can be utilized, where one single peak can be cut and collected in three or more fractions [168]. One of the most important application of heart-cutting 2D-LC is the analysis of pharmaceutical impurities [164, 169]. As the impurities may resemble to a very high degree the structure of the active compound, a 2nd-dimension separation can be used to check if there is any extra impurity co-eluting with the major compound peak in the 1st-dimension LC. Another pharmaceutical application of heart-cutting 2D-LC lies in the spontaneous analysis of both chiral and achiral compounds in drug formulations [170]. The achiral-chiral coupling in the two dimensions can effectively separate the targeted isomeric compounds from the other ingredients and then distinguish the isomers themselves. As discussed in the SFC section in this thesis, SFC is advantageous in chiral analysis compared with traditional NPLC as it leads to faster analysis, easier solvent removal and smaller environmental impact. The

inclusion of SFC as the 2nd dimension in a multiple heart-cutting 2D-LC system has been proven to be successful [22]. Besides pharmaceutical usages, heart-cutting 2D-LC have been reported in many other applications in recent years, such as vitamin D metabolite profiling [171], salt removal in protein analysis [172] and analysis of natural products-derived pesticides [173].

There is also a growing interest in coupling extraction and 2D-LC for on-line analysis [174-176]. These systems are useful in analyzing the whole extract or certain fractions of the extract if the composition is very complex. If these systems can be described as "extraction coupled with a 2D system", **paper VI** presents a novel study of "extraction coupled in a 2D-like system". As has been demonstrated with the PHWE - UHPLC system built, the whole extraction process can be monitored and compound specific extraction curve can be obtained. In this type of system, there is no external sample injected for analysis. Philosophically, the "first dimension" extraction is the sample.

4. Conclusions

4.1. Concluding remarks

This thesis work started with SFC method development for lignin-derived compound analysis. A fast method was developed for the quantitative analysis of 11 phenolic compounds generated from oxidative degradation of humic acid by cupric oxide that can be used for geographical tracing. The method provided more than 5 times shorter analysis time than any reported GC or HPLC methods. Another SFC method with MS detection was developed for the analysis of 40 lignin-derived phenols. 32 of the 40 compounds were partially or baseline separated in approximately 6 min. Bonding technology and functionalization of chromatographic particles improved greatly the resolving power, according to the results from column screening. Compared with the traditional HPLC methods used for this type of analysis, the major advantage of the SFC methods is the potential rapidness with high efficiency. Then the focus of the thesis work shifted to SFC sample diluents and techniques. It was proposed that water injected can hypothetically adsorb on polar stationary phases and form a temporary stationary phase, which leads to temporary HILIC-like separations if the stationary phase has high water absorbing capacity. Sample diluents containing water can also be beneficial in the analysis of some compounds, especially on polar columns. However, it was also seen in this work that the injection of comparatively large volume of water can cause pressure spikes in the beginning of the analysis and huge background noise peaks during the chromatographic run. The proposed hypothesis for the reason behind is that excessive water injected clusters and behaves like a compound band. To avoid the negative effect of increasing injecting volume in fixed-loop injection SFC, multiple injection approach was shown to generate high signal-to-noise enhancement for strongly retained compounds. The potential of the multiple approach was shown in the analysis of sulfanilamide spiked Honey extract and diclofenac spiked ground water sample. Other contributions of this thesis work concern two-dimensional liquid chromatography. Small-volume trapping columns were used and evaluated as collectors on the interface in building a 2D RPLC × SFC system. The use of solid traps instead of collection loops enabled an increase of the 1st-dimension flow rate, which accordingly decreased the total analysis time. However, the use of higher 1st-dimension flow rate was found to result in more severe undersampling. The 2D

philosophy was then extended to a study of extraction kinetics. With the demonstrative on-line PHWE - UHPLC system built with a parallel sampling interface, the whole extraction process of curcuminoids can be monitored. The specific extract component profile can be obtained at any time during the extraction. Compound thermal degradation was also successfully observed.

4.2. Future aspects

The work throughout the whole PhD project has certainly raised some new research questions yet to be solved and at the same time opened up possibilities for project extension or novel research. During the SFC method development for lignin-derived compound analysis in the previous work, solubility issues were not taken into consideration as the targeted compounds are all of relatively low molecular weight. If the analyte range is to be extended to larger lignin-type molecules, it would be very beneficial to perform a study on the solubility of lignin phenols in various SFC mobile phase systems. Splitters are commonly used in SFC for various purposes, such as interfacing SFC with MS and assisting the use of multiple detectors. One interesting and important study would be a thorough investigation of how changes in flow rate, pressure, temperature and composition of SFC flow affect the split ratio and the compositions of the split flows. Analytical SFC peak distortion from sample diluent has been thoroughly studied in terms of the influence of chemical properties of the sample diluent. However, systematic studies on the effects of physical processes (such as de-mixing and viscous fingering) taking place between the flushing of the sample plug by the mobile phase and the sample plug reaches the column head have been very rare. Even though the preliminary work on SFC-CAD was not fruitful, it might pave the way for a more thorough study with chemometric approaches for solving the analytical problem. Active modulation with more efficient solid traps can drastically improve the performance of the interface used in the 2D LC×SFC study. Scaling up the on-line comprehensive 2D extraction × chromatography system with preparative chromatography can potentially enable on-line target compounds extraction-separation and collection. From a personal point of view, the real core value of two-dimensional chromatography lies more in “two-dimensional”, rather than “chromatography”. The processes that can be coupled with chromatography in a 2D-like fashion should not be limited to just chromatography or extraction as demonstrated in the thesis. Although naming such hyphenated systems “two-dimensional” systems can stir controversy, the usefulness of this type of hyphenation is beyond doubt. For example, monitoring an organic reaction usually requires periodic manual sampling and chromatographic analysis. With a properly designed reaction container, it might be possible to achieve on-line multiple

sampling - spontaneous analysis, which would be less laborious but providing more detailed and accurate information. As passing on knowledge has always been greatly valued in the group, hopefully some of these reflections and inspirations may lead to something substantial in the future.

It is undeniable that many loose ends and pitfalls are left due to capability and time limits, as well as resources available. In the work of SFC method development for lignin compounds, chemometric approaches could have been adopted in the chromatographic parameter optimization step, to yield more convincing optimum conditions. More investigation should be carried out to confirm on the source of the background noise peak when water was studied as SFC sample diluent. The multiple injection work failed to elucidate the fundamental cause of the performance difference between multiple injection and one-time injection of similar volume. In the 2D LC×SFC work, the system and method could be much more useful if the system were coupled with any type of mass spectrometer, as available lignin standards are scarce. A systematic study could have been performed in the extraction - chromatography work to find out the best sampling frequency, which allows the longest modulation time possible for better separation while still acquiring sufficient information regarding extraction kinetics.

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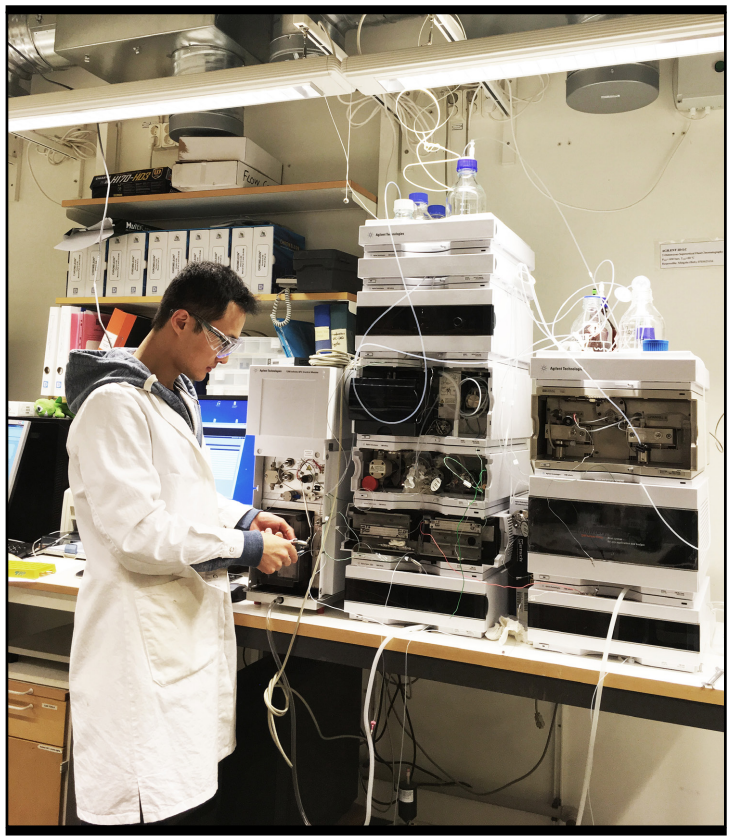

I am very grateful to be given the opportunity to work in the field of analytical chemistry. Hopefully, it is a journey to be continued, even though I do not know yet where the future takes me.

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