Addressing the challenges of lignin oligomer analysis by liquid-based separation techniques

From universal quantification to multidimensional separations

Papp, Dániel

2024

Addressing the challenges of lignin oligomer analysis by liquid-based separation techniques

From universal quantification to multidimensional separations

DANIEL PAPP | CENTRE FOR ANALYSIS AND SYNTHESIS | LUND UNIVERSITY

Addressing the challenges of lignin oligomer analysis by liquid-based separation techniques

Addressing the challenges of lignin oligomer analysis by liquid-based separation techniques

From universal quantification to multidimensional separations

Daniel Papp

LUND
UNIVERSITY

DOCTORAL DISSERTATION
Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Science at Lund University to be publicly defended on 5th June at 13.00 in B Hall, Department of Chemistry, Kemicentrum
Faculty opponent
Sebastiaan Eeltink, Vrije Universiteit Brussel

Organization: LUND UNIVERSITY

Document name: Doctoral thesis **Date of issue:** 2024.06.05

Author(s): Daniel Papp **Sponsoring organization:**

Title and subtitle: Addressing the challenges of lignin oligomer analysis by liquid-based separation techniques: From universal quantification to multidimensional separations

Abstract: Lignin is the second most abundant biopolymer on Earth after cellulose, found in virtually all plants where it is responsible for rigidity, hydrophobicity and mineral transport. For a long time, it has been considered a waste in the paper production process and incinerated for its energy content. However, with the depletion of crude oil reserves, it has been found to have a large potential to become a candidate to alleviate the transition to a more sustainable economy by replacing oil as a raw material in many industrial processes including the production of fine chemicals, fuels and plastics. This discovery induced a rapidly increasing interest in lignin valorization. At the same time, the development of existing and new procedures to exploit lignin requires reliable chemical analysis to identify and quantify phenolic compounds along the whole stream. Lignin samples have proven to be challenging to analyze since they contain hundreds of phenolics of vast complexity regarding size, functionality and concentration. On the top of this, reference materials are commercially available only for monomers, further hindering the research of oligomers, especially in terms of quantitative analysis. To improve the quality of the data by providing selectivity to the analysis method, separation techniques are critical components of lignin analysis prior to detection. In this thesis work, liquid-based chromatographic techniques were employed to address the difficulty of separating technical lignin samples. Efforts were centered around applying supercritical fluid chromatography and gel permeation chromatography.

Supercritical fluid chromatography was utilized to achieve a high-resolution separation of lignin compounds prior to detection. This was particularly important when a universal detector was employed, which lead to the proposal of a single-calibrant approach to quantify lignin dimers. This way, our methodology addressed the challenge of the lack of dimer standards to build calibration curves. Furthermore, insights into the retention of lignophenolics were collected regarding the selectivity differences between various stationary phases and the role of analyte functionality.

Gel permeation chromatography was thoroughly investigated in terms of the trueness of the molecular weight determination. Combining results from diffusion-ordered NMR, mass spectrometry and partial least squares regression it was found that although it yields in a considerable error for monomers, linear polystyrene still provides reliable results for a large part of the molecular weight distribution curve. Additional observations regarding the stability of lignin solutions and repolymerization were recorded.

Multidimensional chromatography, the ultimate answer to the separation challenge of complex samples, was applied to characterize lignin compounds in technical lignins. A combination of GPC and SFC, not reported before, was explored, with specific attention to compatibility, modulation and the achievable peak capacity. Conventional hyphenation utilizing stationary-phase assisted modulation did not yield a practically useful method. Hence, valve-based solutions were employed to expand the separation space and combined with ion mobility mass spectrometry, it increased the selectivity of the method, allowing for the analysis of large lignin oligomers which have been less in the focus of earlier works. Furthermore, the creative use of valves also addressed a few hindrances identified in the literature regarding the expansion of orthogonality and increasing the peak capacity.

Keywords: lignin, supercritical fluid chromatography, gel permeation chromatography, multidimensional chromatography

Classification system and/or index terms (if any) Supplementary bibliographical information

Language: English **ISSN and key title**:

ISBN: 978-91-8096-044-1

Recipient's notes **Number of pages**: 79

Price Security classification

Signature Date 2024-04-18

Addressing the challenges of lignin oligomer analysis by liquid-based separation techniques

From universal quantification to multidimensional separations

Daniel Papp

LUND
UNIVERSITY

Cover designed by Josep García Martínez

Faculty of Science
Department of Chemistry

ISBN 978-91-8096-044-1 (print)
ISBN 978-91-8096-045-8 (digital)

Printed in Sweden by Media-Tryck, Lund University
Lund 2024

Where other men blindly follow the truth,
Remember, nothing is true.
Where other men are limited by morality or law,
Remember, everything is permitted.
We work in the dark to serve the light.

Table of Contents

Abstract

Lignin is the second most abundant biopolymer on Earth after cellulose, found in virtually all plants where it is responsible for rigidity, hydrophobicity and mineral transport. For a long time, it has been considered a waste in the paper production process and incinerated for its energy content. However, with the depletion of crude oil reserves, it has been found to have a large potential to become a candidate to alleviate the transition to a more sustainable economy by replacing oil as a raw material in many industrial processes including the production of fine chemicals, fuels and plastics. This discovery induced a rapidly increasing interest in lignin valorization. At the same time, the development of existing and new procedures to exploit lignin requires reliable chemical analysis to identify and quantify phenolic compounds along the whole stream. Lignin samples have proven to be challenging to analyze since they contain hundreds of phenolics of vast complexity regarding size, functionality and concentration. On the top of this, reference materials are commercially available only for monomers, further hindering the research of oligomers, especially in terms of quantitative analysis. To improve the quality of the data by providing selectivity to the analysis method, separation techniques are critical components of lignin analysis prior to detection. In this thesis work, liquid-based chromatographic techniques were employed to address the difficulty of separating technical lignin samples. Efforts were centered around applying supercritical fluid chromatography and gel permeation chromatography.

Supercritical fluid chromatography was utilized to achieve a high-resolution separation of lignin compounds prior to detection. This was particularly important when a universal detector was employed, which lead to the proposal of a single-calibrant approach to quantify lignin dimers. This way, our methodology addressed the challenge of the lack of dimer standards to build calibration curves. Furthermore, insights into the retention of lignophenolics were collected regarding the selectivity differences between various stationary phases and the role of analyte functionality.

Gel permeation chromatography was thoroughly investigated in terms of the trueness of the molecular weight determination. Combining results from diffusion-ordered NMR, mass spectrometry and partial least squares regression it was found that although it yields in a considerable error for monomers, linear polystyrene still provides reliable results for a large part of the molecular weight distribution curve. Additional observations regarding the stability of lignin solutions and repolymerization were recorded.

Multidimensional chromatography, the ultimate answer to the separation challenge of complex samples, was applied to characterize lignin compounds in technical lignins. A combination of GPC and SFC, not reported before, was explored, with specific attention to compatibility, modulation and the achievable peak capacity. Conventional hyphenation utilizing stationary-phase assisted modulation did not yield a practically useful method. Hence, valve-based solutions were employed to expand the separation space and combined with ion mobility mass spectrometry, it increased the selectivity of the method, allowing for the analysis of large lignin oligomers which have been less in the focus of earlier works. Furthermore, the creative use of valves also addressed a few hindrances identified in the literature regarding the expansion of orthogonality and increasing the peak capacity.

Populärvetenskaplig sammanfattning på engelska

The decreasing amount of crude oil available for humanity is one of the most burning issues that our generation must resolve. Without this resource, the production of many items used in everyday life may become more expensive or even impossible. That is why alternative sources for the chemical industry must be sought out. One of the promising candidates is lignin, a large molecule in plants, especially in trees, where it is mainly responsible for making the trunk rigid. Currently, lignin is underutilized and some even consider it a waste in paper production. Lately however, a lot of research has been carried out to prove its potential to replace oil in several industrial processes. So far, a few pioneer applications, such as vanillin production, have made it to industry scale, and further development is ongoing. To further pave the way for this, lignin-related samples and products must be thoroughly characterized for their properties and contents.

The chemical analysis of complex samples such as lignin almost always has some kind of separation before the detection step. The more compounds there are in the sample, the better the separation methods must be to ensure that the signal, measured by the detector, corresponds to only one compound in the sample. Separation of these compounds in the case of lignin is quite difficult because one single sample may contain hundreds of them which vary greatly in their properties. The initial development of the field was mostly focused on analyzing the smallest of these, called monomers. However, with the emerging new directions in lignin research, there is more and more demand to characterize the larger constituents, known as oligomers, as well.

This thesis work focuses on the application of liquid-based chromatographic methods for lignin samples. Chromatography is one of the most commonly used techniques to separate mixtures into the respective components based on their interactions with a suitable surface. While the main efforts were centered around the development of actual separation methods, a considerable attention was devoted to detection techniques, method characterization and technical solutions as well.

The concentration measurement of certain lignin oligomers called dimers is particularly difficult because the standard materials for these are not available, however, they are crucial to build calibration curves. To overcome this, a specific

detector was employed which gives a similar output signal regardless of what kind of compound it detects. Combining this with a powerful separation method, we demonstrated how the calibration curve of only one standard can be used for concentration measurements of other dimers. We showed the practical usefulness of the strategy on the example of a real-life sample as well.

Another challenge addressed early in this work was the accuracy of the size-based separations of lignin. These techniques are commonly used to determine the average molecular weight of the sample, which is one of the key properties. This is usually done by comparing the sample to a standard which has a known molecular weight, however, the ones used for lignin may not fully be appropriate. To investigate this, we applied several different techniques to determine the molecular weight of our samples based on different principles. The results agreed well between techniques, except for the smallest lignin compounds, indicating that although theoretically the standards are not perfectly suitable, they still fit well for the purpose.

We devoted a significant effort to investigate the combination of size-based and polarity-based separations to analyze the whole of lignin samples. First, practical considerations were addressed since this particular combination has not yet been explored. The hindrances encountered were overcome by the creative use of valves, which also addressed some problems from other fields of science as well. This separation was designed to be used in connection with mass spectrometry enabling the analysis of large lignin oligomers which have been less in the focus of studies.

List of Papers

Paper I

Single-Standard Quantification Strategy for Lignin Dimers by Supercritical Fluid Chromatography with Charged Aerosol Detection

Daniel Papp, Thanya Rukkijakan, Daria Lebedeva, Tommy Nylander, Margareta Sandahl, Joseph S M Samec*, and Charlotta Turner*

Anal. Chem. 2023, 95, 2, 1436–1445, doi.org/10.1021/acs.analchem.2c04383

Paper II

A Complementary, Multi-technique Approach to Assess the Trueness of Molecular Weight Determination of Lignin by Gel Permeation Chromatography

Daniel Papp, Göran Carlström, Tommy Nylander, Margareta Sandahl, and Charlotta Turner*

Revised manuscript submitted to Analytical Chemistry on 19/04/2024

Paper III

Development of an Online Comprehensive Size Exclusion Chromatography × Supercritical Fluid Chromatography Method for the Analysis of Technical Lignin Samples

Daniel Papp, Tommy Nylander, Margareta Sandahl, and Charlotta Turner*

Manuscript in preparation; to be published in itself or together with Paper IV

Paper IV

Expanding the separation space in comprehensive two-dimensional liquid chromatography by a system switch in the second dimension – an application in technical lignin analysis

Daniel Papp, Mynta Norberg, Peter Spégel, Tommy Nylander, Margareta Sandahl, and Charlotta Turner*

Manuscript in preparation

Author's contribution to the papers

Paper I

I contributed to developing the initial research idea with Turner and Sandahl. I performed the development and validation of the analysis method in the article, conducted data analysis and wrote the manuscript.

Paper II

I synthetized the original research idea based on a problem formulated in the PhD research statement by Nylander, Sandahl and Turner. I conducted the experiments, evaluated the data and wrote the manuscript.

Paper III

I synthetized the original research idea based on a problem formulated in the PhD research statement by Nylander, Sandahl and Turner. I conducted the experiments, evaluated the data and wrote the manuscript.

Paper IV

I synthetized the original research idea together with Norberg. I carried out the chromatographic method development myself. I collected and analyzed the data together with Norberg and wrote a major part of the manuscript.

Abbreviations

1-AA	1-aminoanthracene
$_{effective}^{1,2}n$	effective two-dimensional peak capacity
^{1}D	first dimension
2-PIC	2-picolylamine
2D	two-dimensional
^{2}D	second dimension
AmAc	ammonium acetate
ASM	active solvent modulation
BPR	backpressure (regulator)
CAD	charged aerosol detection
DAD	diode-array detection
DEA	diethylamine
DIOL	high-density diol
DMF	N,N-dimethylformamide
DMSO	dimethylsulfoxide
DoE	design of experiments
ELSD	evaporative light scattering detection
FA	formic acid
$f_{coverage}$	coverage factor
FID	flame ionization detection
FP	fluorophenyl
FR	fraction
GC	gas chromatography
GC × GC	comprehensive two-dimensional gas chromatography
GPC	gel permeation chromatography
HILIC	hydrophilic interaction liquid chromatography

IR	infrared
LC	liquid chromatography
LC-LC	single heart-cut two-dimensional liquid chromatography
LC × LC	comprehensive two-dimensional liquid chromatography
LSER	linear solvation energy relationship
MeOH	methanol
mLC-LC	multiple heart-cut two-dimensional liquid chromatography
MS	mass spectrometry
MW	molecular weight
n	peak capacity
NMR	nuclear magnetic resonance
NP(LC)	normal phase (liquid chromatography)
PAH	polyaromatic hydrocarbon
pfg	pulsed field gradient
PH	phenylhexyl
PMMA	polymethyl-methacrylate
PS	polystyrene
PS-DVB	polystyrene-divinylbenzene copolymer
RP(LC)	reversed-phase (liquid chromatography)
sCO2	supercritical carbon dioxide
SEC	size exclusion chromatography
SFE	supercritical fluid extraction
sLC-LC	selective heart-cut two-dimensional liquid chromatography
t	time
TFA	trifluoroacetic acid
THF	tetrahydrofuran
tR	retention time
(U)HPLC	(ultra)high performance liquid chromatography
(UHP)SFC	(ultrahigh performance) supercritical fluid chromatography
UV- Vis	ultraviolet-visible
V	volume
w	peak width

1 Introduction

1.1 Background

Lignin is the second most abundant biopolymer after cellulose on the Earth. Found in most vascular plants in various amounts between 15 and 40%, where it is mainly responsible for rigidity, hydrophobicity and mineral transport[1,2]. In the plant cells, lignin is bound to cellulose and hemicellulose, forming lignin carbohydrate complexes. Currently, the exact structure of lignin is not fully understood and is a subject of debate. Nevertheless, a consensus about its main building blocks called monolignols exists in the scientific community; these being *p*-coumarylalcohol (H-unit), coniferyl alcohol (G-unit) and sinapyl alcohol (S-unit) originating from phenylalanine through the phenylpropanoid biosynthesis[3]. Lately, other compounds such as tricin[4] and hydroxystilbenes[5] as well as acylated units have also been reported and considered monolignols. The biosynthesis of lignin from these monomers has been reviewed in detail by Boerjan *et al.*[2]. The composition of these subunits in a particular sample is characteristic of the source. For instance, in softwoods, the dominant unit is the G-unit, while hardwoods contain more S-units[6].

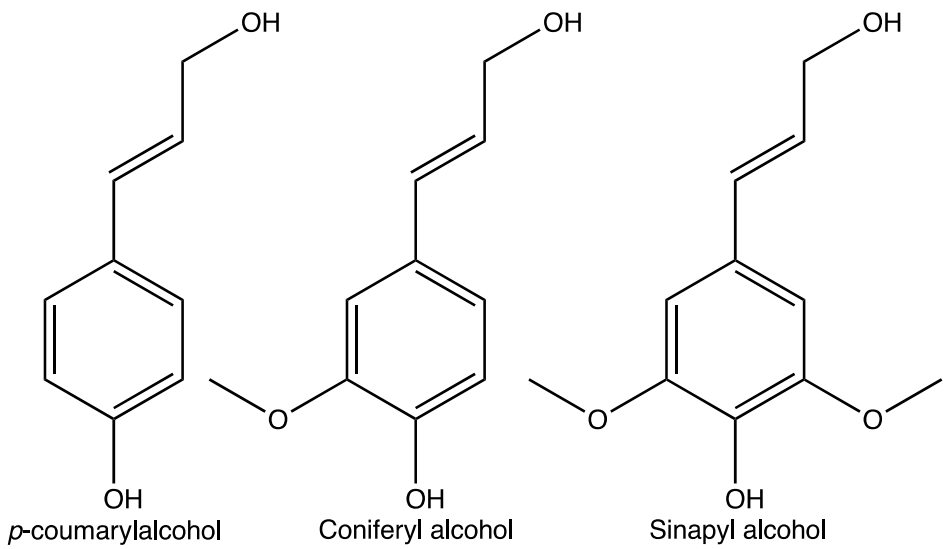

Figure 1. Structure of the three main monolignols, *p*-coumarylalcohol (H-unit), coniferyl alcohol (G-unit) and sinapyl alcohol (S-unit)

During the lignification process, the monolignol units are linked by various carbon-carbon and carbon-oxygen bonds, in a radical polymerization reaction involving enzyme-catalyzed oxidation. Due to the random element in the biosynthesis, lignin does not have a repeating unit unlike other natural or synthetic polymers, instead, it forms a complex three-dimensional macromolecule. Between the monolignols, carbon-carbon and carbon-oxygen bonds are formed, with β-O-4 being the most common one[2]. Figure 2 shows a tentative structure of a softwood lignin, highlighting typical linkage structures.

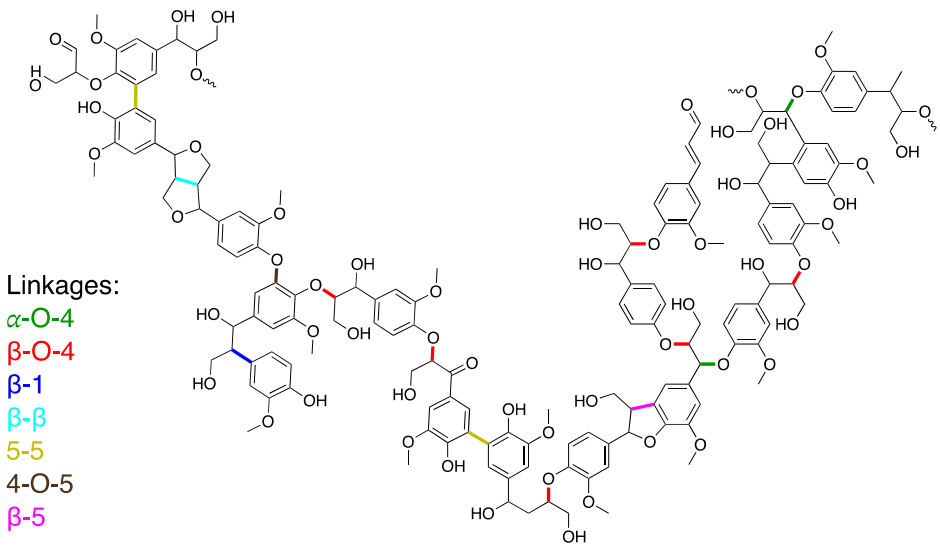

Figure 2. A proposed structure of softwood lignin. Figure adapted from Windeisen et al.[7] with permission from Elsevier.

Lignin has been generated as a by-product in paper production and has so far been incinerated for its energy content to power pulping mills. In the pulping industry, sulfur-containing and sulfur-free processes are routinely used to separate lignin from cellulose, with kraft pulping being the industry-leading technology. This procedure involves cooking the wood chips in an aqueous solution of NaOH and Na_2S under pressure at an elevated temperature of 150-170°C. Among these circumstances, base-catalyzed hydrolysis cleaves C-O bonds and carbohydrates are separated from lignin. Further details of the reactions can be found in a number of reviews[8,9]. The final product of the process is the so-called black liquor, containing kraft lignin, which is of decreased molecular weight and rich in phenolic and aliphatic hydroxyl functionalities and can be precipitated by acidification. Apart from the kraft process, other extraction procedures, such as the sulfite, soda and organosolv processes have also been developed, although they are used to a lesser extent. Naturally, these technologies yield lignins with properties much different from the kraft process; for instance, sulfonate lignins are well-soluble in water, the

opposite of kraft lignin in this regard. Furthermore, though not fully exploited, biorefineries have been developed to produce lignin via various hydrolytical treatments, as detailed by Wang et al in their review[10]. Lignins recovered from these technologies are called technical lignins. Recently, the valorization of technical lignins as a renewable source for the production of value-added chemicals, fuels and plastic additives has gained interest since it is rich in phenolic moieties[11], cheaply available and can be produced in high quantities[10]. Examples of the already implemented processes include the production of guaiacol by selective catalytic fractionation, catalytic oxidation targeting syringaldehyde and alkaline oxidative depolymerization for vanillin production from sulfonate lignin. During extraction and valorization processes the composition of the mixture is further modified by de- and repolymerization, including thermal degradation, hydrolysis and cracking[12,13]. As a result, lignin samples in the industry are extremely complex materials with possibly hundreds of compounds, which may differ greatly in terms of concentration, functional groups, linkage types and physicochemical properties, such as hydrophobicity, solubility, acidity and molecular weight. Nonetheless, the understanding of the ongoing reactions and the development of existing and future conversion methods requires knowledge about the contents of the feedstock, reaction mixture and the product. Thus, with the increased interest in utilizing lignin, the demand for analytical techniques targeting lignin has grown greatly. Over the years, a multitude of analytical techniques have been developed to address various research questions, each with its own benefits and shortcomings.

Due to the mentioned complexity of lignin samples, lignin analysis most often includes a separation step, even though there are techniques for bulk characterization. For instance, ultraviolet-visible (UV-Vis) spectroscopy[14], infrared (IR) spectroscopy[15,16] and nuclear magnetic resonance (NMR) spectroscopy[17–20] are commonly used to determine various functionalities in the sample. Yet, these techniques do not yield information about the identity or the concentration of individual lignin monomers or oligomers, which are among the main interests of the lignin researchers developing new applications. Such research questions require increasing the selectivity of the analytical method, which is commonly achieved by including a separation technique prior to detection.

Lignin monomers have traditionally been analyzed by gas chromatography (GC) either with mass spectrometric[21–24] or flame ionization detector[21,24,25]. However, gas chromatography is only directly applicable for sufficiently volatile analytes, thus larger oligomers must undergo derivatization prior to analysis[26], otherwise they get pyrolyzed[22,27]. This makes the procedure more complex and time-consuming while increasing the risk of introducing a bias. Circumventing this limitation of the technique, liquid chromatography (LC) has been successfully applied to lignin samples. Most commonly, reversed-phase liquid chromatography is employed. Kiyota *et al.* reported the use of ultrahigh-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC/MS/MS) to qualitatively analyze

lignin oligomers up to tetramers in a sample originating from sugar cane[28]. Song *et al.* and Prothmann *et al.* both applied similar UHPLC/MS/MS methods and elucidated the structures of lignin oligomers up to tetramers[29,30]. Methods using diode-array (HPLC-DAD)[31,32] or electrochemical detection[33] have also been reported and DAD is still an integral part of LC instrumentation even though the MS signal is mainly utilized. With the wider commercialization of the technique, supercritical fluid chromatography (SFC), a greener and more efficient alternative to LC has also been reported in several studies[34–37]. In addition, although the research purpose is completely different from the above techniques, size exclusion chromatography is also part of the lignin analysis toolbox to monitor depolymerization[38,39] and assess the molecular weight profile of lignins[40].

Nevertheless, due to the extreme complexity of lignin samples, the purity of chromatographic peaks remains an issue, complicating identification and quantification. Addressing this, two-dimensional chromatography has been employed in lignin analysis. The major improvement provided by these techniques is a superior resolving power and consequently, increased peak capacity compared to one-dimensional approaches. Comprehensive two-dimensional gas chromatography (GC × GC) has been widely employed for various challenging samples to identify and quantify monomers[41,42]. However, similarly to one-dimensional GC, the separation of non-volatile oligomers must be preceded by either pyrolysis[43,44] or derivatization[45]. Although the technique is well-established in other fields, liquid-based two-dimensional chromatography for lignin is less explored. Currently, offline coupling of the two separation dimensions is more common than online two-dimensional LC as summarized by van der Hurk *et al.*[46] as well as Tammekivi *et al.*[47] in their recent reviews. Nevertheless, the commercialization of two-dimensional LC-specific equipment and the increased knowledge of the technique mainly originating from biopharmaceutical analysis has made online 2D-LC more accessible for the lignin community, yielding a few pioneer applications featuring RP×RP[48] and RP×SFC[49,50] separations.

As implied above, most of the research on the analysis of lignin has been centered around qualitative aspects, ranging from relatively simple molecular weight determination to tentative identification of oligomers up to tetramers. On the other hand, there is a clear gap in terms of quantitative studies, which are almost exclusively targeted at lignin monomers. The quantification of oligomers, nevertheless, remains problematic, which mostly stems from the lack of commercially available standards, crucial to establish a relationship between the detector signal and the concentration.

1.2 Aim of the thesis

This thesis work aims to tackle the challenging task of separating and analyzing lignin oligomers in technical lignin samples. Since a single lignin sample may contain hundreds of these analytes in varying sizes, physicochemical properties and concentrations, their characterization requires selective chromatographic methods to ensure as pure zones as possible for detection. Being nonvolatile compounds, gas chromatographic methods cannot directly be applied for this purpose, *ergo* liquid-based techniques were employed. Efforts were centered around using of supercritical fluid chromatography and gel permeation chromatography for specific applications. The focus on supercritical chromatography originated from the fact that its kinetic efficiency and stationary phase selectivity are superior to conventional liquid chromatographic methods applied for lignin analysis; in addition, it is also considered a green separation technique. The retention mechanisms of lignophenolics on various SFC stationary phases, possible method development strategies and the assessment of detection methods for specific purposes received special attention. In parallel, the systematic error of GPC was evaluated for size-based separations, which is considered a workhorse technique in the lignin field. Eventually, the two techniques were hyphenated in an online two-dimensional system to be coupled to high-resolution mass spectrometry, having the potential of serving as a basis for a comprehensive analysis platform for lignophenolics of all sizes in technical lignin samples.

The main research questions are summarized below:

1. Can charged aerosol detection coupled with SFC be used for the quantification of lignin dimers using only one or a few calibration standards? Can a one-dimensional chromatographic method provide the necessary selectivity when the detector is nonselective?
2. Are linear polystyrene standards, commonly used as calibrants in gel permeation chromatography of lignin, appropriate in terms of the trueness of the determined molecular weight? What is the magnitude and the source of the error?
3. What is the practical use of the online hyphenation of gel permeation and supercritical fluid chromatography? How can valve-based solutions be applied to expand the resolving power and applicability?

2 Supercritical fluid chromatography

2.1 Supercritical fluids as chromatographic solvents

The IUPAC Gold Book defines a supercritical fluid as a compound, mixture or element above its critical pressure and temperature[51]. As such, supercritical state can be understood as another state of matter next to solid, liquid and gaseous. In the hundred-year history of the study of supercritical fluids various substances have been investigated, summarized in Table 1 together with their critical parameters. Out of these, carbon dioxide (CO_2) has been the most widely utilized supercritical fluid for industrial and research processes since it requires relatively mild conditions to bring it into supercritical state, additionally, it is nontoxic, chemically stable and inert[52].

Table 1. Critical parameters of selected substances used in supercritical fluid research. Adapted from Clifford *et al.*[53]. Reproduced with permission from Springer Nature.

Substance	Critical temperature /°C	Critical pressure /bar
Carbon dioxide	31	74
Water	374	221
Ethane	32	49
Ammonia	133	114
Nitrous oxide	37	72

The application of supercritical fluids in chromatography stems from their special properties, *viz.* liquid-like density and gas-like viscosity. What is more, as illustrated in Figure 3, these properties can be easily altered by changing the pressure and the temperature of the system, meaning that the density and viscosity of supercritical CO_2 can be tuned to fit to the purpose. Figure 3 also shows that supercritical fluids are the most sensitive to pressure and temperature changes near the critical point, which can be traced back to the compressibility of the fluid.

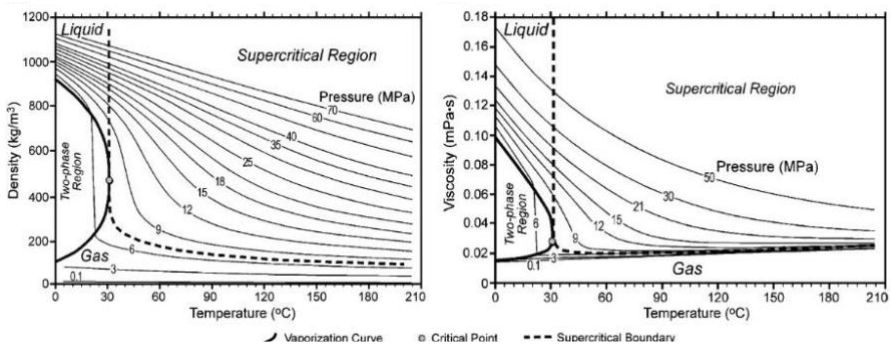

Figure 3. Changes in the density and viscosity of supercritical carbon dioxide as functions of temperature and pressure. Figure from Nordbotten *et al.*[54]. Reproduced with permission from Springer Nature.

Low, gas-like viscosity makes supercritical fluids especially interesting for chromatographic applications since it is linked to the diffusivity through the Stokes-Einstein equation. In comparison to conventional liquids, diffusion coefficients in supercritical fluids are typically one order of magnitude higher, yielding faster mass transfer processes. This leads to a higher kinetic efficiency[55] and increased peak-to-peak resolution. Furthermore, this is also the main reason why the development of supercritical fluid chromatography (SFC) methods focuses more on the selectivity aspect instead of kinetic optimization. In addition to the enhanced diffusivity, low viscosity also ensures lower pressure drops over the chromatographic column. Combining this with the low mass transfer resistance, SFC methods can be run at higher linear velocities compared to liquid chromatography without a substantial loss of kinetic efficiency.

The relative static permittivity of the supercritical fluid, which is a measure of polarizability[56], changes in parallel with density when altering the pressure and the temperature[57]. In other words, density can be correlated with the elution strength of the mobile phase in chromatography. This allows designing density gradients in SFC and adjusting retention through varying the pressure[58].

Pure supercritical CO_2 near the critical point has a relative static permittivity comparable to that of hexane[59] thus, it is usually treated as a nonpolar solvent in the chemical industry. While this in itself might prove beneficial for certain purposes such as separating hydrophobic analytes[58,60,61], its practical usefulness is limited. At the same time, the application area can be further expanded by adding a so-called cosolvent to increase the polarity of the solvent and enable running solvent gradients. Various alcohols, esters and acetonitrile are commonly used for this purpose.

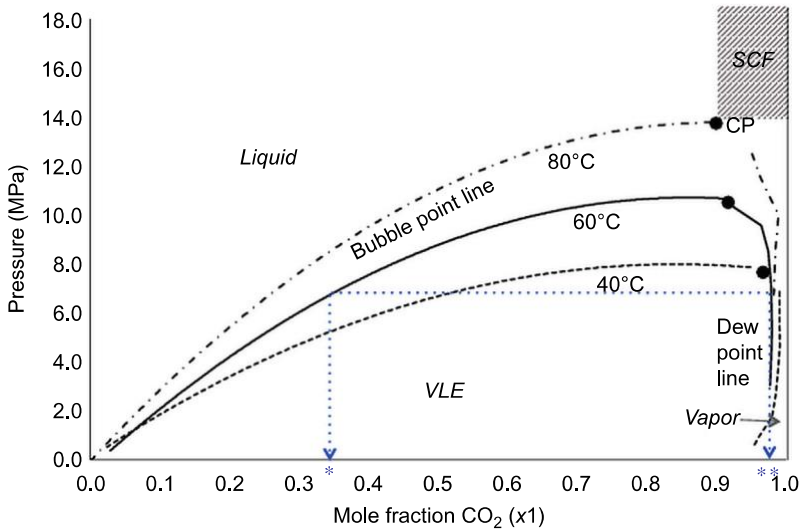

Figure 4. Pressure-composition phase diagram of a carbon dioxide-methanol system at various temperatures between 40 and 80°C. Figure reprinted from Cunico et al.[62] with permission from Elsevier.

Figure 4 shows the pressure-composition (p-x) phase diagram of a binary CO_2-MeOH system. With the addition of the cosolvent, the composition of the mixture appears as a new variable having an effect on the properties of the solvent. First of all, it is important to note that binary systems may end up in the two-phase envelope in a vapor-liquid equilibrium (VLE); however, in chromatographic practice, this is not desirable. According to Figure 4, the size of this region increases with temperature, thus it is imperative to ensure the necessary pressure to keep the mixture in one phase. Secondly, mixing an organic solvent with sCO_2 brings in limitations regarding compressibility: notably, with a higher cosolvent percentage the mixture becomes less compressible[63], accordingly, the tunability of density and viscosity decreases. Lastly, it has to be noticed that the supercritical region *per definitionem* is only in the top right corner above the critical point and all other states are subcritical. However, this is not always reflected clearly in speech and the term "supercritical" is often used for chromatographic methods where the mobile phase is actually in a subcritical state.

2.2 Stationary and mobile phase chemistries in SFC

In their critical review, Lesellier and West bring attention to the fact that during an SFC method development, not all possible system variables have equal impacts on the separation[64]. In fact, selectivity, which is considered a key parameter to

improve chromatographic resolution in SFC, is governed by the identities of the stationary and mobile phases. For this reason, stationary and mobile phase screening is an essential part of the development procedure.

2.2.1 Stationary phase

Classically, due to the nonpolar nature of supercritical carbon dioxide, packed SFC stationary phases tended to be more on the polar side such as bare silica with diol or amine modifications[64]. However, technically all HPLC columns can be used in an SFC system as long as they withstand the pressure requirements. The evaluation of various stationary phases has been carried out in multiple studies in West's group in a series of papers focusing on alkyl-bonded[65], aromatic[66], HILIC[67] and polar phases[68] with their solvation parameter models. Later works from the same group classified SFC stationary phases based on linear solvation energy relationship (LSER) models using Abraham descriptors. These results not only emphasize how SFC stationary phases complement each other in terms of retentivity but also highlight similarities between them. For instance, it is interesting to observe that the four stationary phases offered as a method development package by Waters (Figure 5) do not actually offer significantly different selectivity of the probe analytes covering a wide polarity range[64,69]. However, lignophenolics are rather polar phenolic compounds. Hence, in such a case, expanding the column screening to apolar stationary phases is not advisable; instead, the minor retentivity differences between polar phases should be exploited and targeted in the screening process. This prompts chromatographers to consider the chemistry of the possible analytes in the sample and select stationary phase accordingly.

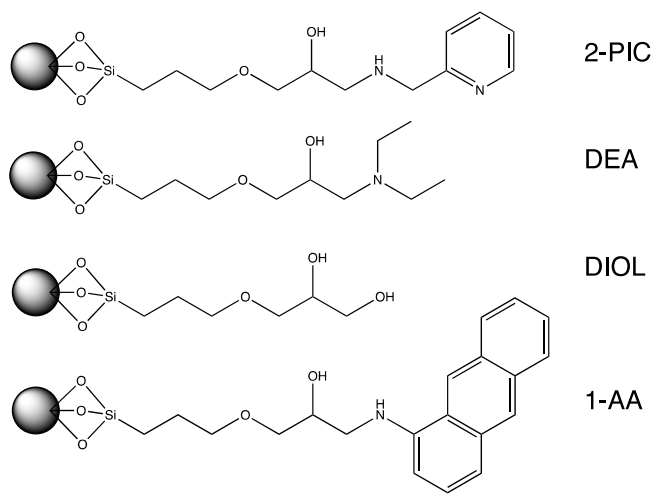

Figure 5. Stationary phases in the Waters Torus column screening kit. 2-PIC: 2-picolylamine; DEA: diethylamine; DIOL: high density diol; 1-AA: 1-aminoanthracene. Figure adapted from **Paper I**.

Particularly in the lignin field, high density diol (DIOL)[35] and 1-aminoanthracene (1-AA)[36,37,70] have been found useful. Similar results were obtained in **Paper I**, where lignin dimers were targeted for quantification by a universal detector, thus maximizing chromatographic selectivity was of utmost importance. Using a carbon dioxide-methanol gradient, four stationary phases (DIOL, 1-AA, diethylamine (DEA) and 2-picolylamine (2-PIC)) were compared based on the cumulative number of resolved peak pairs (Figure 6). 1-AA was observed to separate all but one peak pair of the investigated dimeric and monomeric standards and was chosen as the optimal stationary phase for this application. 1-AA and 2-PIC stationary phases have additional π-interactions besides dipolar interactions with the analytes, proving to be particularly useful to separate lignin-derived phenolics. At the same time, as shown by Gros *et al.*[69], these stationary phases cover a small part of the total available selectivity space, which is indicated by the fact that the retention order did not change between stationary phases. This conclusion was reached by Prothmann *et al.* as well who exploited the mentioned π-π interactions further to separate monomer and dimer classes in a lignosulphonate sample[36]. Class separation, although observed, was less prominent in **Paper I** with all stationary phases, which might be because only a limited number of lignin standards were evaluated. In particular, two monomer analytes with an aliphatic hydroxyl functionality eluted much later in comparison to monomers that did not contain such a group.

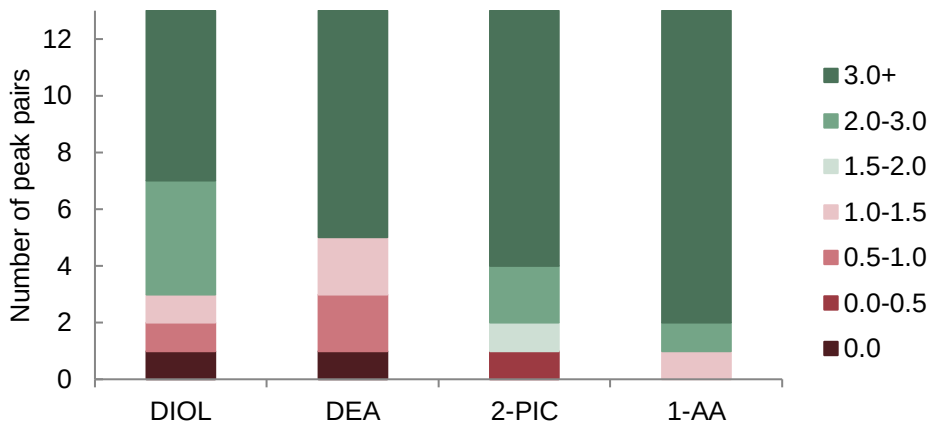

Figure 6. Cumulative resolution between lignin-related monomers and dimers using various stationary phases. Figure taken from **Paper I.**

A more in-depth investigation of stationary phase selectivity was conducted in **Paper III**. Five stationary phases were screened with various mobile phases to separate 42, mostly monomeric lignin model compounds with the aim of using the method as the second dimension of an online two-dimensional system. For this reason, in this application not only peak-to-peak resolution but adequate retention

was also considered to avoid so-called wraparound peaks. Figure 7 reveals substantial differences between the different phases in terms of retentivity. First of all, it is evident that the number of detected peaks differs on different columns. Further experiments uncovered that a few analytes required a higher percentage of cosolvent and a longer time to elute than what was allowed in the screening procedure. This effect was the most pronounced for the DEA stationary phase, which retained multiple compounds too strongly. Considering the structures, it appeared that all of the affected analytes possess an acidic group capable of forming strong hydrogen bonds and therefore interact well with the stationary phase. Possibly similar strong H-bonds were the reason why some of these analytes were excessively retained on the 2-PIC and 1-AA phases as well, while these interactions are weaker on the DIOL and BEH phases, which do not contain a strong acceptor nitrogen atom.

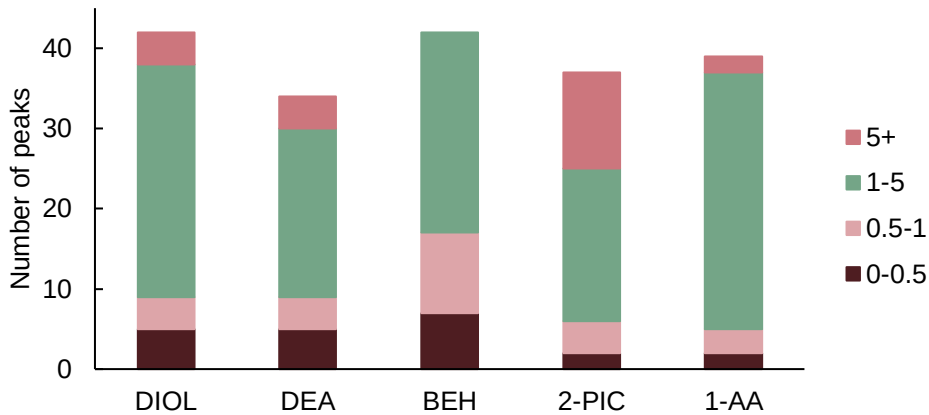

Figure 7. Apparent retention factors of lignin model compounds on various stationary phases. Chromatographic conditions: mobile phase: carbon dioxide-methanol gradient, 130 bar BPR; column temperature: 50°C; detector: UV-Vis @254 nm. Figure from **Paper III**.

This development also allowed to explore the retention of lignophenolics based on their functionality as illustrated by a retention map shown in Figure 8. As apparent, on the DIOL stationary phase there is a clear difference in the retention of analytes with and without a mobile proton in their structure. That is to say, acids and alcohols stay longer in the stationary phase due to their ability to establish hydrogen bonds with the surface OH-groups of the DIOL phase. A similar trend was observed for the other stationary phases as well, indicating the importance of H-bonds in retention in SFC. Unfortunately, due to the lack of available standards, such observations based on functional groups could not be made for the dimer standards, which seemingly covered the entire retention range. It has to be noted however that with the increase of the size of the molecule, the relative strength of hydrogen bond donating capacity will decrease and simultaneously, the molecular

surface area available for π-interactions will increase. As a result, for the separation of larger lignin oligomers a stationary phase with aromatic modification such as phenyl or larger PAH modification might be more suitable as found by Prothmann *et al.*[36,37]; however, a more thorough investigation of this is yet to be carried out.

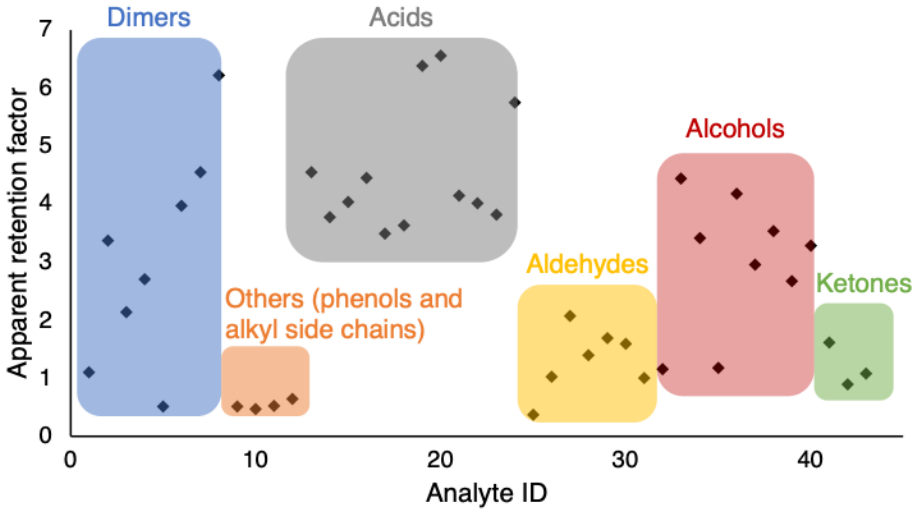

Figure 8. Retention map of selected lignin-related phenolics on a DIOL stationary phase. Analyte ID is an arbitrary number to denote the analytes from 1 to 42. Chromatographic conditions: mobile phase: carbon dioxide-methanol gradient (10-35% cosolvent), flow rate: 3 mL/min, 130 bar BPR; column temperature: 50 °C, detection wavelength: 280 nm.

While the retention order of analytes was very similar for all the tested stationary phases, systematic differences between different groups can be observed in Figure 9. For instance, the influence of the surface density of interaction sites on retention is apparent when comparing the BEH (bare silica) stationary phase with the high-density DIOL particle (Figure 9A). In this case, retention occurs via polar interactions with the surface OH-groups of the stationary phase, available in a higher surface density on the DIOL phase. As a consequence, lignophenolics are retained stronger on the DIOL column. This effect is the most noticeable for analytes which contain a mobile proton (framed in grey and red) and are retained by hydrogen bonds with the stationary phase, resulting in an approximately two-fold increase of apparent retention factors.

The effect of additional π-interactions on the retention of lignin-related compounds can be seen in Figure 9B. First of all, it is apparent that the retention mechanism on the 1-AA stationary phase is not purely based on π-interactions, since if this was the case, dimers would be consistently more retained than all monomers. In fact, polar monomers such as acids and alcohols stay long on the column and largely overlap with the retention range of dimers, a finding also described in **Paper**

I. This again points towards the conclusion that polar interactions, especially hydrogen bonds are more significant when it comes to selectivity. However, additional type of interactions such as the ones offered by the 1-AA phase may prove useful when improving the retention of analytes, since any kind of interaction alters the absolute retention. Here, the change was found to be the largest for the lignin dimers (framed in blue) in comparison to monomers, which can be explained by dimers having a larger molecular surface area for π-interactions with the 1-AA ligand.

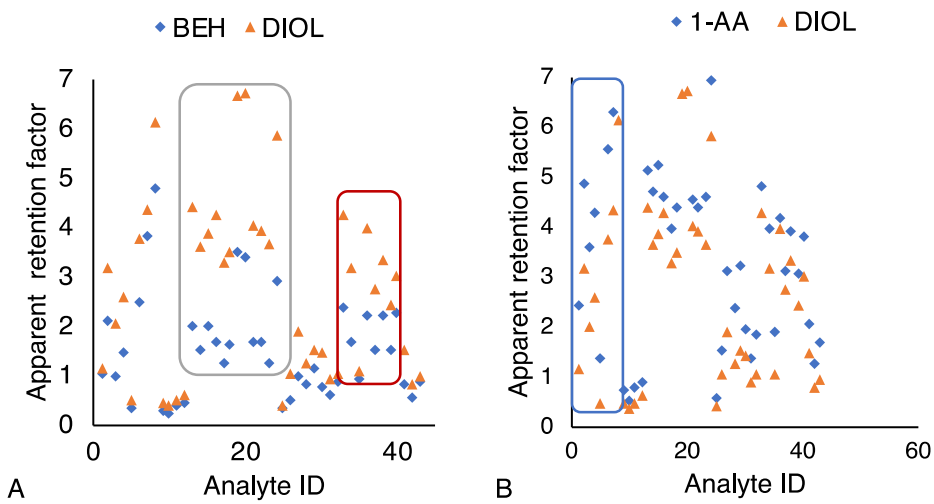

Figure 9. Comparative retention maps of selected lignin-related phenolics on stationary phases BEH vs DIOL (A) and DIOL vs 1-AA (B). Analyte ID is an arbitrary number to denote the analytes from 1 to 42. Chromatographic conditions: mobile phase: carbon dioxide-methanol gradient (10-35% cosolvent), flow rate: 3 mL/min, 130 bar BPR; column temperature: 50 °C, detection wavelength: 280 nm.

2.2.2 Mobile phase

The main component of the SFC mobile phase is liquid or supercritical carbon dioxide, which, as discussed above, is nonpolar *per se*. To increase the polarity of the mobile phase and thus make it applicable for a wider array of analytes, polar modifiers such as alcohols or acetonitrile[71] can be used to create solvent gradients typically up to 50 V/V% analogously to liquid chromatography[55]. Lastly, it is possible to use minor amounts of additives (up to a few percent) mixed in the cosolvent. Examples of regular additives are acids, bases, salts and water. These may have multiple effects on the separation, including changing selectivity by altering the apparent pH of the mobile phase, improving peak shape by masking secondary interaction sites on the stationary phase or contrarily, being adsorbed to the surface to establish retention sites[64].

The investigation of the effect of different cosolvents on the SFC of lignophenolics has not received much attention in published works. Without exception, all published works[34–37,70] used methanol, despite the fact that studies from other fields have shown the usefulness of trying other cosolvents such as acetonitrile or isopropanol to optimize the selectivity[72]. On the other hand, the development of gradients is a crucial part of optimization, especially since, unlike in LC where retention modeling[73] and expert softwares[74] are available to aid the work, SFC gradients are still developed by a meticulous trial-and-error process. Most importantly, this can be traced back to the wider variety of stationary phases compared to LC. Prothmann *et al.*[36] conducted an extensive stationary phase screening for the separation of lignin oligomers and dimers. Their study included a gradient development for all investigated stationary phases, ending up with different settings among different columns. An example they point out in their discussion is the low retention of lignophenolics on the fluorophenyl (FP) stationary phase, which steered the gradient development towards flatter profiles, while other columns required steeper gradients compared to the common starting point. In **Paper I**, the same approach was followed to fairly assess all the screened stationary phases and the best-performing gradient for each column was compared in Figure 6. After choosing the column to continue the development with, the gradient was kept the same during the fine-tuning of the flow rate, pressure and temperature of the method. However, it is possible that due to the changes introduced in the fine-tuning step minor modifications to the gradient would have been beneficial.

On the other hand, additives and their role in the separation have received quite considerable attention in SFC. Similarly to the cosolvents, the effect of additives on the retention of analytes also depends on the whole system including the stationary phase, cosolvent percentage in the mobile phase and naturally, the type of analyte. Ovchinnikov *et al.* evaluated trifluoroacetic acid (TFA), diethylamine (DEA), ammonium acetate (AmAc) and water on various stationary phases. They found that while TFA generally increases the retention of the test analytes, it remains mostly unaltered in case of using DEA or AmAc, which seemingly had similar effects. The behavior of water was found to be more complex and harder to interpret[75]. In addition to altering the retention of analytes, additives are often used to improve the peak shapes, especially for basic analytes[76,77]. On the other hand, the effect of additives on the SFC of lignophenolics has been found to be minor. Prothmann *et al.* investigated the influence of formic acid and ammonium formate in two different concentrations but reported no improvement of the peak shape[35]. In contrast, 20 mM citric acid was found to improve the shape of tailing peaks[34]. Besides formic acid and ammonium formate, water was tested in **Paper III**. While the concentration effects were not investigated, a few qualitative conclusions could be drawn. Compared to pure methanol, using 0.1% formic acid did not change the retention significantly on any of the studied stationary phases. Nonetheless, analytes with acidic functionality were retained more when ammonium formate was added to the cosolvent while having much less effect on the retention of other analytes. This

effect was the largest on the DIOL and bare silica phases and might be explained by the formation of ion pairs with the analytes. Water exhibited no or minor increase of retention without any discrimination regarding analyte structural elements, even though Soukup *et al.*[78] hypothesized an adsorbed water layer on the surface of the stationary phase, allowing a HILIC-like retention mechanism to take place and yielding higher retention for polar analytes.

2.3 Fine-tuning of an SFC separation

Although the main focus of the method development in SFC is the screening of stationary and mobile phases, multiple system parameters are to be optimized after finding the right combination. Temperature is one such setting commonly optimized in LC, however, in SFC the system pressure has a significant effect on the separation as well.

In SFC, pressure and temperature have multiple roles. Imperatively, they must be chosen in a way that the mobile phase is always in the one-phase region (Figure 4). In routine applications, this is rarely a problem, however, it still must be of concern when running wide gradients. Furthermore, the density of the mobile phase can be varied via temperature and pressure, thus it can be fine-tuned for a particular application. Decreased temperature and/or increased backpressure yield elevated density and solvent power therefore the retention of analytes is decreased. At the same time, these effects are tied to the compressibility of the mobile phase, which in turn depends on the composition[79]. In our experience, the boundary lies around 10 V/V% methanol in carbon dioxide, below which the mobile phase is easily compressible and changes in temperature and pressure have a large influence the solvent power. Importantly, for the above reason, flow rate also has an influence on retention as it changes the pressure drop on the column.

Since the influence of the flow rate, backpressure and temperature are fundamentally related through density, it appears reasonable to kinetically tune a method by means of design of experiments (DoE). Yet, multiple difficulties were found with this approach in **Paper I**. In that study, a Box-Behnken design was generated to model and optimize the separation conditions to resolve four difficult-to-separate peak pairs in the middle of the chromatogram. In this model, the peak-to-peak resolutions between the adjacent peak pairs were chosen as responses to be maximized. Even in such a simple case it was found that neither multilinear regression, nor partial least squares regression yielded optimal conditions to separate all peaks to the baseline. This is easy to recognize when one considers the following: if one peak moves away from its left neighbor, it moves closer to its right one; consequently, by improving the resolution between one particular peak pair, it worsens between others. Furthermore, this choice of responses did not address

possible peak shape issues. Moreover, as mentioned above, the modified retention may have rendered the already developed gradient suboptimal. This also prompts the inclusion of gradient parameters in the design; however, describing even simple linear gradients would introduce multiple additional parameters into the model, resulting in further complications. Overall, currently the best approach is to investigate the influence of flow rate, pressure and temperature separately, as it was conducted in published works[34,35].

2.4 Detection techniques in SFC and their use for lignin applications

In principle, SFC detectors do not differ from those used in liquid chromatography, nonetheless, a few technique-specific adaptations should be pointed out. First of all, detectors that are placed before the backpressure regulator unit are under high pressure. These are typically non-destructive detectors. In SFC, almost exclusively UV-Vis absorbance detectors are used at this position[80,81], however, pressure and temperature changes as well as the mechanical noise from the BPR induce a noise level typically higher than in LC[82]. Furthermore, a necessary modification in this case is that detector cells are to withstand high pressure of 100-150 bar. Detectors coupled post-BPR, such as a mass spectrometer[35,37,77,83] do not have such restrictions. Nonetheless, the expansion of CO_2 in the connecting tubing might cause precipitation of the analytes. This is normally prevented by introducing a makeup flow pre-BPR. Common makeup solvents are based on methanol, acetonitrile or other ordinary solvents and may contain additives to enhance detectability. In their study, Prothmann *et al.* investigated the influence of the type of makeup solvent and a few additives on the ionization efficiency of lignin monomers using a DoE approach. The authors found the superiority of methanol over isopropanol and proved that ammonia is a suitable additive for negative mode ionization[35]. On the contrary, Andrianova *et al.* concluded that positive mode ionization was more effective, detecting lignin oligomers up to 9000 Da while studying the effects of various makeup additives[84].

Although mass spectrometry is the most commonly used detector for SFC for lignin analysis, other, less widespread detectors can be used for specific applications. In particular, charged aerosol (CAD)[85–87] and evaporative light scattering detectors (ELSD)[88] can be used for universal quantification purposes when appropriate standards are scarcely available. Both these detectors generate an aerosol of the SFC effluent, dry it and measure either the charge on the surface of the dried particles[89] or the scattered light[90]. In **Paper I**, the application of charged aerosol detection was explored to quantify lignin dimers in a complex sample. The response of eight in-house synthetized lignin dimer compounds (D1-D8) was

evaluated, ultimately aiming to find out if one of the dimer standards can be used as a universal calibrant. The acquired data is summarized in Figure 10, where the mentioned single-standard approach was tested. Figure 10 reveals that although the CAD is often assumed to be a universal detector, significant differences between the responses can be observed. Eventually, the study found D2, D4 or D5 to be suitable calibrant for the investigated dimers. The mean absolute error using D2 as calibrant was 12.8%, comparable to the reproducibility of the method. In contrast, using D8 as a calibrant would result in serious overquantification of D1. While the developed single-standard quantification approach was successfully applied to a real complex sample, several shortcomings of the work have been recognized, stemming from the fact that the study was conducted using only eight, in-house synthetized standards. To start with, this introduced a relatively large uncertainty to the signal uniformity investigations since the purity of the standards varied between 90 and 99%, clearly having an impact on the results. Furthermore, being limited to eight compounds rendered the establishment of structure-response relationships unfeasible. A similar study conducted by Robinson *et al.* on pharmaceuticals suggested that the response is dependent on the surface area of the dried particles rather than on the injected mass of the analytes[91]; however, this could not be confirmed in our work. In addition to the above, the effect of the makeup solvent, which supposedly has a meaningful effect on the response, was not sufficiently studied.

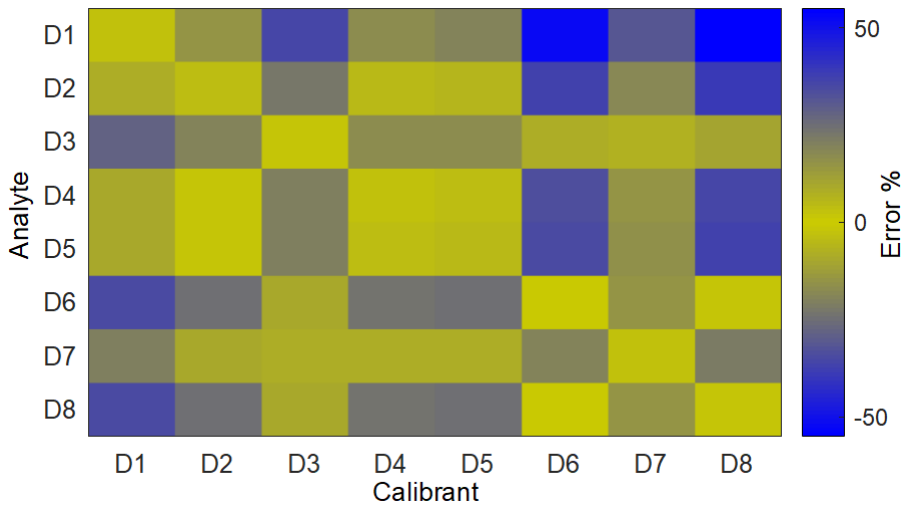

Figure 10. Errors of quantification for lignin dimers (D1-D8), using the single-standard calibration approach. Analyte structures and chromatographic conditions are found in **Paper I**.

Another major finding in **Paper I** was the importance of dealing with post-column zone broadening, which is crucial in the case of a quasiuniversal detector.

This had a detrimental effect on the resolution in the CAD chromatogram compared to the DAD chromatogram (Figure 11A and B) rendering peak area measurements inaccurate. To resolve this problem, post-acquisition signal arithmetics were applied[92,93]. These techniques change the raw signal from the detector, which is the main reason why they are viewed with a certain degree of concern. In accordance with what was found by Hellinghausen *et al.*[94], power transformation of the CAD signal had the largest effect on the apparent resolution. Furthermore, it was shown that modification of the signal did not introduce major errors of quantification while yielding a better-looking chromatogram and made peak area measurements less affected by uneven baseline or peak overlaps (Figure 11B and C).

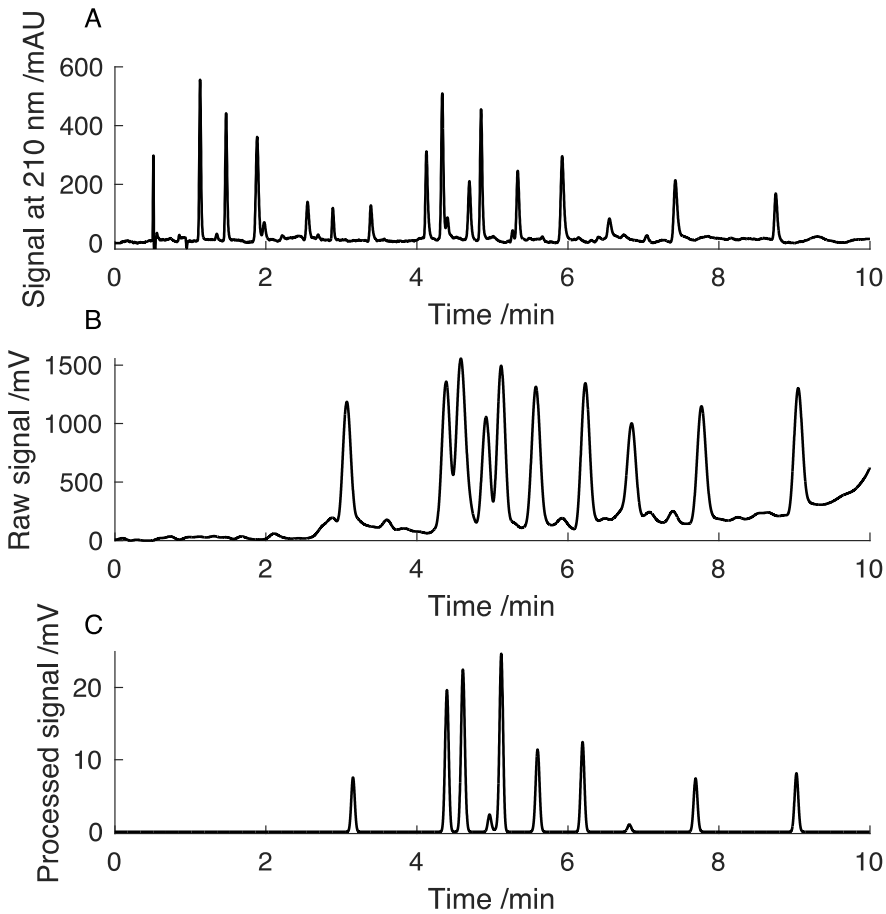

Figure 11. DAD (A) and CAD (B-C) chromatograms of selected lignin standards. DAD detection was carried out at 210 nm and the signal was blank corrected Chromatogram B shows the raw CAD signal, while chromatogram C illustrates how the apparent resolution enhancement techniques improved the quality of the chromatogram. Chromatographic conditions are found in **Paper I**.

3 Gel permeation chromatography of lignin

3.1 The principles of gel permeation chromatography

Gel permeation chromatography (GPC), also known as organic size exclusion chromatography (SEC), is the go-to technique in the industry to determine the molecular weight distribution of various natural and synthetic polymers for half a century. The separation process occurs in a porous gel material, governed by the availability of the pores to the sample components. Briefly, the smaller the molecule is, the more pores it penetrates and the later it elutes from the column. To keep the separation purely entropy-based, interactions between the stationary phase and analyte are not desired in GPC[95]. Instead, the separation process is to be governed exclusively by the hydrodynamic volume of the analyte, which in turn depends on its molecular mass, conformation and branching degree[96]. Therefore, the behavior of the investigated sample in the eluent greatly influences the trueness of the molecular weight determination.

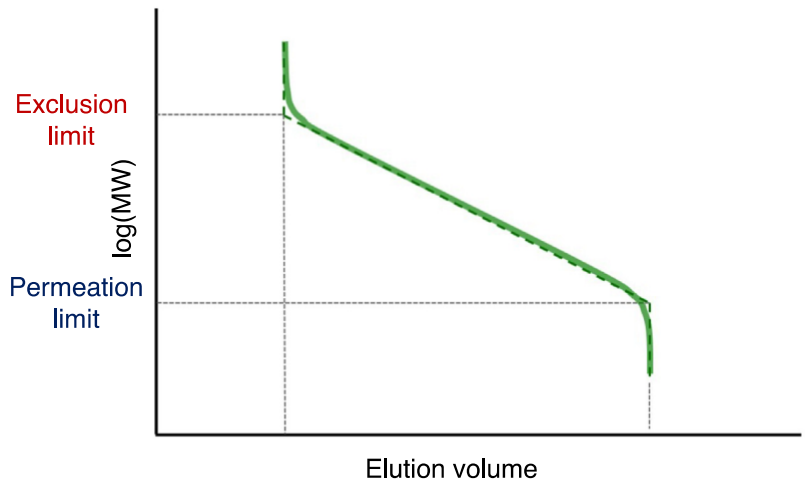

Figure 12. A typical calibration curve in size exclusion chromatography. Figure adapted from Lubomirsky *et al*.[96] with permission from Elsevier.

The conversion of the elution volume of the separated polymer fractions to molecular weight is carried out by calibrating the system with standards of known molecular weights, as shown in Figure 12. Although it might seem trivial, it is of utmost importance that these calibrants behave exactly the same way as the sample molecules. In the case of most synthetic polymers and proteins, this is not a limitation; nevertheless, pertinent calibration standards might not be available for polymer blends or natural polymers such as lignin and consequently, the determined molecular weight from the calibration curve may be erroneous. These errors originate from either aggregation (positive bias) or adsorption (negative bias), which the calibrant does not account for. In such cases, a more complex detection system can remedy the problem, such as viscometric[97] or light scattering detectors[98]. These provide information on the molecular weight without relying on primary standards, on the other hand, they require further assumptions and data fitting to obtain results.

3.2 Lignin as a solute in organic and aqueous solvents

As pointed out above, the GPC separation in an ideal case is governed purely by exclusion effects from the pores, depending on the size of the analyte molecule, which in turn can directly be converted into molecular weight using Equation 1[99].

$$r_h = \sqrt[3]{\frac{3*M*\bar{\bar{v}}}{4*\pi*N}} \qquad (1)$$

where r_h is the hydrodynamic (Stokes) radius, M is molecular weight, $\bar{\bar{v}}$ is partial specific volume and N is the Avogadro-number.

Whereas this expression establishes a simple relationship between the hydrodynamic volume and molecular weight, it is only valid for spherical molecules. This restriction highlights the importance of studying the conformation and aggregation behavior of analytes in a particular solvent system. Neutron scattering studies in the literature reported rod-like lignin aggregates in DMSO-d_6[100,101], NaOD[102,103] and D_2O[104] with sizes ranging from 50 to 200 nm. These structures are well-known to be formed in solution[39,105–108]. Studies have reported a connection between the degree of aggregation and the amount of aliphatic hydroxyl[101], carboxyl[109] and phenolic hydroxyl groups[110], noting that π-π interactions also play a key role[101]. However, a quantitative model linking molecular properties such as ring-double bond equivalent (RDBE), acidity, polarity to the degree of aggregation is lacking. While this could not be fully addressed, in **Paper II,** considerable attention was devoted to investigating the behavior of low-MW lignophenolics. That is to say, monomer and dimer standards were run in gel permeation chromatography setup calibrated with linear polystyrene standards and the error of molecular weight was calculated. The loadings of a fitted partial least

squares regression model clearly indicated the significance of the hydrogen-bond donating capacity with respect to the relative error. In addition, the effect of functional groups could be directly studied, an advancement in comparison to other works, where quantitative estimations for various functionalities were carried out for complete lignin samples by two-dimensional NMR spectroscopy[101,111]. Figure 13 reveals that, as suggested earlier, monomers featuring an aliphatic OH-group *viz.* alcohols and acids exhibit a higher relative error than those with more nonpolar functionalities, implying that hydrogen bonds are important in the aggregation process. It is also interesting to notice the decreasing trend between H-, G- and S-units, possibly caused by the fact that additional substituents on the benzene ring may alter the acidity of the hydroxyl groups thus reducing the degree of aggregation. It has been found that such aggregates can be disrupted by adding a salt to the eluent[112] or derivatization[105,113].

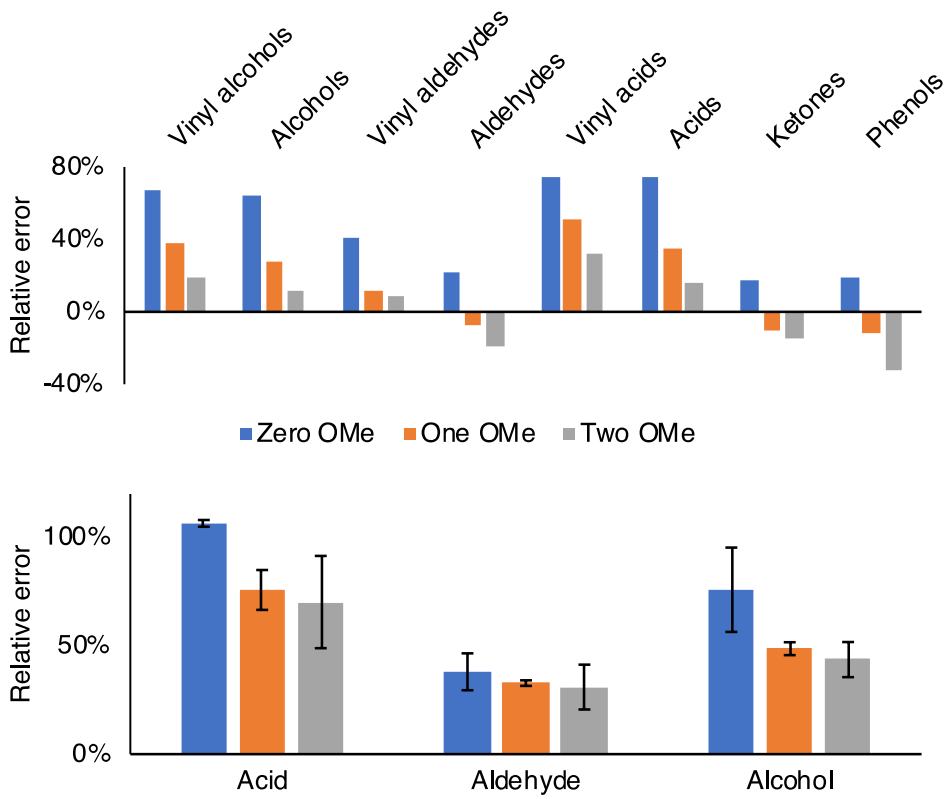

Figure 13. Relative errors of lignin monomers compared to the true molecular weight, as determined by gel permeation chromatography (A) and pulsed field gradient nuclear magnetic resonance spectroscopy (B). Relative error is calculated as the difference between the measured and the true molecular weight of the particular analyte, divided by the true molecular weight. Figures taken from **Paper II**.

Although it is commonly overlooked and taken for granted, the solubility and solution stability of lignin must be discussed when it comes to solution behavior. Naturally, only the dissolved fraction of lignin can be analyzed by GPC, thus the results correspond to this particular fraction. Notably, the solubility of lignin varies greatly in both organic and aqueous solvents depending on its origin, extraction method and possible further treatments[114–119]. Overall, short-chain alcohols[119], glycols[120] as well as mixtures of various organic solvents and water[118] have been found useful to dissolve higher amounts of lignin, implying the importance of hydrogen bonds in the dissolution process. Lately, novel solvent systems based on ionic liquids[121–123] and deep eutectic solvents[124,125] have been proposed. Furthermore, refinements to the calculation of Hansen solubility parameters for lignin have been suggested[126], facilitating the prediction of solubility in less explored solvents. Even though there is an ongoing discussion about the exact dissolution mechanism, the solubility of lignin is well-studied. However, the stability of solutions has not been addressed. During our GPC experiments in **Paper II**, a well-known but so far undocumented phenomenon was observed. Apparently, fractionated lignin kept at -20°C in solution repolymerized over the course of a month of storage, indicated by the increased average molecular weights measured by GPC in Table 2. In addition, direct infusion mass spectrometry was conducted on each of the fractions, exhibiting spectra with shifted mass center and wider mass distribution than expected from the fractionation. The log-normal profile of the mass spectra suggested that the repolymerization process does not stop after one recombination step. Table 2 also indicates that the stability increases with molecular weight. For instance, there is a 2.6-fold change in MW for the lightest fraction FR I, while for the largest fraction the increase is only 27%.

Table 2. Average molecular weights of isolated lignin fractions, showing repolymerization. All average molecular weights were determined by gel permeation chromatography against polystyrene standards. Data taken from **Paper II**.

Fraction	Average MW in 2023 July /Da	Average MW in 2023 August /Da
FR I	162	424
FR II	370	578
FR III	580	789
FR IV	1000	1679
FR V	2000	2919
FR VI	3000	3816

3.3 The trueness of molecular weight determination in the gel permeation chromatography of lignin

Size exclusion chromatography has been employed in lignin analysis as a fast and simple way of assessing the molecular weight of a sample. However, accurate determination of the molecular weight requires calibration standards that behave exactly the same way as lignophenolics. In GPC systems, which are often run with tetrahydrofuran (THF) as eluent[40,127–130], linear polystyrene[95,130–132] or polymethylmetacrylate[95] are used for calibration. **Paper II** addressed the suitability of polystyrene standards for lignin analysis by a complementary, multitechnique approach. Combining results from offline direct infusion mass spectrometry, pulsed field gradient diffusion NMR (pfg-diffusion NMR) and a conventional GPC setup with THF/PS-DVB, we were able to explore the trueness of the molecular weight determination from various angles, thus obtaining a more complete picture. Results from mass spectrometry corroborated with those from PS-calibrated GPC (Table 3) up to the point when monocharged species could be assumed. In such a case, the measured m/z values can easily be weighed and converted to molecular weight as in the case of fractions FR I to FR III, covering the molecular weight range up to pentamers which corresponds to the commercially most interesting range. Jacobs *et al.* found a reasonable agreement between GPC and mass spectrometry results using a similar approach[131]. However, in our case, there is an exponentially increasing discrepancy between the results from the two techniques for the higher molecular weight fractions. Comparable results were obtained by Andrianova *et al.* on complete lignin samples assuming the same charge state as we did[95]. The increasing disagreement between GPC and MS can be traced back to the fact that in electrospray ionization, lignin oligomers became multiply charged, thus lowering the measured average m/z. Importantly, Jacobs *et al.*, who employed laser-desorption ionization, did not observe such differences. To circumvent the problem of multiple-charged ions, Andrianova *et al.*[84] have developed a method to deconvolute mass spectra, enabling the comparison of molecular weights measured by MS and GPC. It is important to point out that in-source fragmentation may lead to an underestimation of average m/z in MS. However, with the soft ionization settings that we applied this could be avoided, indicated by analyzing a lignin dimer, where more than 95% of the total signal corresponded to the intact molecular ion. Similarly, minimal in-source fragmentation was reported by Önnerud *et al.* for lignin dimers[133]. Nevertheless, this has not been verified for higher order oligomers as the corresponding standards are lacking.

Table 3. Comparison of molecular weights of isolated lignin fractions, obtained by GPC and MS. Data from **Paper II**.

Fraction	Average MW by GPC against PS /Da	Average m/z by MS
FR I	424	454
FR II	578	567
FR III	789	696
FR IV	1679	1171
FR V	2919	1165
FR VI	3816	1192

While the above evaluation of GPC against mass spectrometry of the isolated fractions filled the gap between well-studied monomers and complete lignins, it does not allow for the investigation of which processes cause bias in GPC. As discussed above, the main reasons for an erroneous molecular weight determination are phenomena that change the elution time, either by altering the apparent hydrodynamic volume *e.g.* by aggregation, or by interacting with the column packing thus delaying elution[95]. Pfg-diffusion NMR, however, is only influenced by interactions occurring in solution[134] and as such, it can be utilized to decouple the influence of possible analyte-packing interactions. In **Paper II**, both lignin monomer standards and complete lignin samples were analyzed by Pfg-diffusion NMR and THF-based GPC with linear PS calibration. The comparison of determined molecular weights suggests that reversed-phase-like retention effects are present in the GPC column, even though they are less prominent than association phenomena. This is indicated by the consistent direction of disagreement between the two techniques shown in both Figure 13 and Figure 14. Interestingly, the magnitude of bias was approximately the same regardless of the type of lignin samples in the range of 300-400 Da.

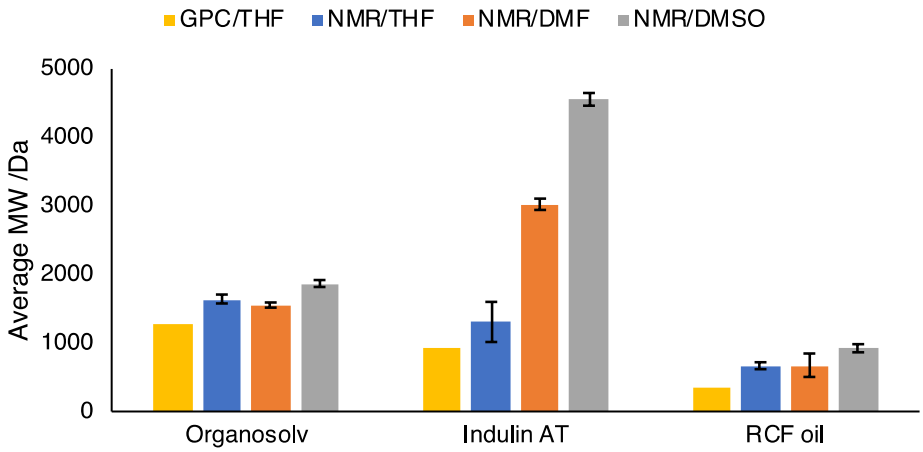

Figure 14. Comparison of molecular weights of different lignin samples determined by pfg-diffusion NMR and GPC against polystyrene standards. Figure taken from **Paper II**.

Our NMR experiments also revealed that serious errors can be introduced into the determination if the GPC and NMR solvents are different; moreover, this error depends heavily on the sample type. For instance, the molecular weight of Indulin AT determined by pfg-diffusion NMR was much higher than the one determined by GPC. This is assumed to be caused by extensive aggregation phenomena, also observed by neutron scattering experiments[101]. Aggregation is possibly further amplified by the fact that salts such as LiCl, commonly used in DMSO-based GPC, were not used in the NMR-experiments. Rönnols *et al.*, although not investigating the matter further, compared pfg-diffusion NMR in DMSO-d_6 and GPC with THF observing differences in the determined molecular weights up to 1100 Da[135]. On the other hand, Montgomery *et al.* found a much better agreement between the two techniques. Similarly to what we have shown in **Paper II**, pfg-diffusion NMR estimated the molecular weight to be higher than GPC, nevertheless, the random errors of the determined molecular weights were quite large[136].

4 Two-dimensional liquid chromatography

4.1 Theory of two-dimensional chromatography

Two-dimensional chromatography has developed as a natural response to the growing complexity of samples in analytical chemistry. That is to say, modern analytical questions target identification and sometimes even quantification of analytes in difficult matrices of various origins[137–141]. Due to their natural complexity, such samples are nearly impossible to sufficiently resolve by conventional one-dimensional methods, prompting the development towards the increase of separation power. This separation power is characterized by peak capacity, defined by Dolan *et al.* In gradient conditions, commonly used in chromatographic practice, this depends on the width of the peaks and the length of the separation window[142].

$$n = 1 + \frac{t_{R-last} - t_{R,first}}{w_{average}} \approx 1 + \frac{t_{gradient}}{w_{average}} \quad (2)$$

where n is peak capacity, t_R is retention time and w is peak width at the baseline.

The concept of two-dimensional chromatography, pursuing higher peak capacities originated from Giddings, who showed that the peak capacities of two orthogonal systems can simply be multiplied together to yield a system with higher separation power according to Equation 3[143].

$${}^{1,2}_{total}n = {}^{1}n \times {}^{2}n \quad (3)$$

Giddings assumes complete orthogonality of the coupled systems as well as the absence of remixing[143]. However, these requirements are rarely fully met in real-world applications, calling for corrective factors, as expressed by Davis *et al.*[144]. Thus, based on Equation 4, the various subsystems in a two-dimensional LC system to be studied when developing a method can be identified.

$${}^{1,2}_{effective}n = \frac{{}^{1}n \times {}^{2}n \times f_{coverage}}{\sqrt{1+3.35*\left(\frac{t_{modulation}}{w_b}\right)}} \quad (4)$$

The pursuit for higher and higher peak capacities can be traced back to the statistical overlap theory, first described by Davis *et al*. Briefly, as chromatographic peaks do not populate the chromatogram according to an even distribution, not all observed peaks are chromatographically pure but contain multiple analytes; instead, Poisson statistics describe the relative amount of pure (singlet), doublet, triplet *etc*. peaks. The extent of peak overlapping was found to be dependent on the so-called peak saturation (Equation 5), which is the ratio of the number of analytes (m) in the sample and the peak capacity of the system. Eventually, more pure peaks are expected to be detected with low peak saturation, in other words, if the system has a much higher peak capacity than the number of components to be separated[145].

$$\alpha = \frac{m}{{}^{1,2}_{effective}n} \qquad (5)$$

To realize such separation, there are multiple possibilities to hyphenate two liquid chromatographic systems. The simplest way to achieve this is probably coupling the two separations in an offline manner[146–148]. In addition to its straightforwardness, offline methods do not suffer from problems arising from the modulation interface typically associated with online coupling, since in this case the two separations operate entirely independently from each other. Nevertheless, automatization is a bottleneck in terms of throughput. On the other hand, online two-dimensional chromatography integrates the two systems more tightly. This introduces a complex network of interconnected system variables affecting the chromatography. At the same time, thanks to the growing number of tutorial works[149–151] as well as the development and commercialization of the necessary interfaces[152] and software the approach became more available and gained popularity to target complex samples[48,138,140]. Besides this classification, two-dimensional LC methods may be grouped based on the coverage of the first-dimensional separation, distinguishing comprehensive (LC×LC) and single (LC-LC), selective (sLC-LC) or multiple heart-cut (mLC-LC) methods[150].

4.2 Considerations around the design of an online comprehensive two-dimensional liquid chromatography system for the analysis of technical lignin samples

The intrinsic complexity of lignin samples, as discussed earlier, makes these samples challenging to analyze. Therefore, chromatographic separation has a major role in chemical analysis of lignin. One-dimensional chromatographic methods, while proving particularly useful for a large number of applications, do not have the

resolving power necessary for comprehensive analysis. Studies targeting depolymerized lignin samples have shown the potential of comprehensive two-dimensional chromatography, however, large lignin oligomers above tetramers have been out of the scope.

4.2.1 Choice of chromatographic modes

Naturally, the choice of chromatographic modes to be hyphenated dictates the effective peak capacity of the system, as shown by Equation 4. Since the main drive to develop a 2D method is to increase the peak capacity of the system, it is logical to select chromatographic modes that contribute to this by having a high resolving power *per se*. In light of this aspect, modes of high resolving power such as reversed-phase[50,139,153–155], normal phase[156–158] and SFC[50,70,146,159] have been explored for various purposes, while ion-exchange[154,160] and size exclusion chromatography[161–165] are more restricted regarding the field of application with a focus on protein and polymer separations.

Nevertheless, it is not merely the peak capacity on the unique dimensions, but the combination of chromatographic modes that gives the true power of two-dimensional chromatography. To capitalize on this, the two modes should separate the sample components based on substantially different principles, in other words, orthogonality should be high. Unfortunately, *a priori* prediction models to estimate the orthogonality between two phases are currently lacking, thus this aspect of the development is usually addressed based on experience. As such, the calculation of orthogonality measures[166,167] is based on the yielded chromatogram *post hoc*. Normal phase × reversed-phase[168], reversed phase × SFC[50,70] and size exclusion × reversed-phase[164] have been reported to exhibit good orthogonality. In particular for depolymerized lignin analysis, Sun *et al.*[50] and Tammekivi *et al.*[70] have published RP × SFC methods with high orthogonality of 0.7, while de Saegher *et al.*, although orthogonality was not explicitly evaluated, used GPC × RP[164,165]. Considering the intention to study not only depolymerized but also technical lignins, a size-based separation was deemed a sound choice to be included in our investigations. Building on the successful offline application and the high expected orthogonality of the two modes, **Paper III** explored the online combination of gel permeation chromatography × SFC for the analysis of technical lignin samples. However, only a mediocre orthogonality of 0.57 was calculated by the convex hull method. While this was not expected based on first principles, both Prothmann *et al.*[37] and **Paper I** have shown that polar stationary phases used in SFC are capable of separating lignophenolics according to the degree of oligomerization, which correlates with the hydrodynamic size, being the basis of separation in GPC.

Lastly, it is worth considering if it is possible to extract useful information from the elution pattern in any of the modes which may aid classification, false positive

detection or further method development. An example of the latter is the separation by Montero *et al.*, who recognized that the polarity-based separation in their first dimension can be exploited when choosing a chromatographic mode for the second dimension. That is to say, early-eluting (very polar) analytes were sent to a HILIC column in the second dimension while compounds eluting later were further analyzed on a C18 phase. This approach with the online stationary phase switch was termed multi-^{2}D LC × LC[169]. An idea inspired by this was expanded in **Paper IV**, where not just the stationary phase but whole systems were switched during the run (Figure 15). This was realized by an alternative use of the commonly employed 2-position/8-port 2D LC valve. In this work, an online GPC × SFC separation was initially developed. However, it was soon noticed that larger lignin oligomers do not elute from SFC due to insufficient solubility in the sCO_2-based mobile phase. This prompted the inclusion of a third separation system, which is capable of keeping the large lignophenolics in solution and providing separation prior to detection. The resulting system featured SFC as the second dimension for the fractions with MW lower than 700 Da and reversed-phase chromatography for the higher molecular weight fractions. This solution expanded the orthogonality of the system to 0.72, comparable to published methods using SFC for lignin applications[50,70]. In addition to the gain of orthogonality, the concept of switching stationary phases or complete separation modes in the second dimension helps to overcome common limitations regarding the modulation and separation in the second dimension, *e.g.* solubility issues, inadequate retention in the second dimension and problems with peak shapes.

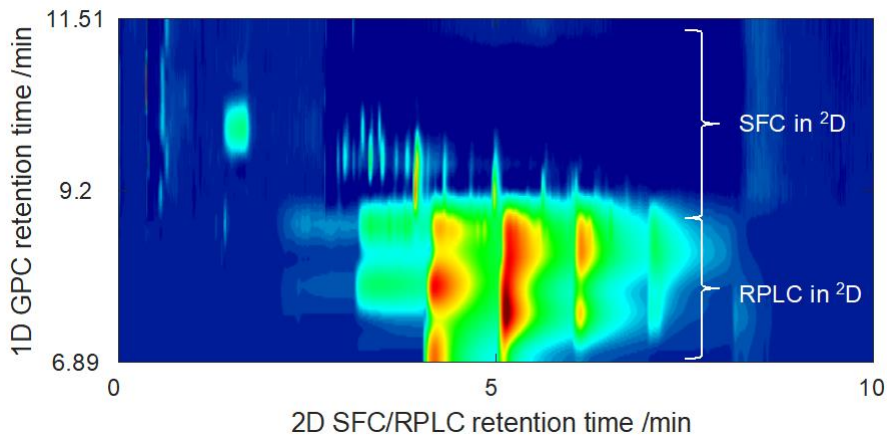

Figure 15. Comprehensive online GPC × SFC/RPLC separation of an Indulin AT sample, showing the essence of the ^{2}D switch concept. Chromatographic conditions: first dimension: gel permeation on Mesopore column (250×2.1 mm, 3 μm) with THF eluent (50 μl/min flow rate, 40°C). second dimension A: SFC separation on DIOL column (100×3 mm, 1.7 μm, 130 Å), sCO_2:MeOH gradient, 100 bar BPR, 40°C, 1.7 mL/min flow rate; second dimension B: RPLC separation on C30 column (100×4.6 mm, 3 μm, 300 Å), water:ACN gradient, 40°C, 1.3 mL/min flow rate. Injection volume: 5 μL. Figure taken from **Paper IV**.

4.2.2 Influence of modulation on the speed of separations

Comprehensive online coupling of liquid chromatographic systems is most easily realized by an 8- or 10-port valve called modulator, equipped with two loops. During the run, repeated back-and-forth valve switches ensure the complete transfer of the ^{1}D eluent to the second dimension by filling one of the loops, while the other serves as an injector loop in the second dimension. The size of these loops introduces several limitations to the system through the volume of the collected fraction from the first dimension described by Equation 6, linking the modulation time and the volumetric flow rate of the first dimension together. Since the flow profile at typical ^{1}D flow rates is parabolic[170], the loop is usually filled up to 60-70% to avoid losses.

$$V_{loop} \geq V_{fraction} = {}^{1}F \times t_{modulation} \qquad (6)$$

Typical loop sizes vary between 20 and 80 µl to avoid overloading the column in the second dimension. This leads to multiple consequences regarding both separation dimensions. First of all, to avoid losses Equation 6 must be upheld prompting a balance between the flow rate of the first-dimensional separation and the modulation time. With the former influencing the peak capacity of the first dimension directly by extending the gradient time, the latter puts a constraint on the second-dimensional separation time according to Equation 7, at the same time affecting the effective peak capacity of the system via the undersampling factor according to Equation 4.

$$t_{modulation} = {}^{2}t_{gradient} + {}^{2}t_{re-equilibration} = {}^{2}t_{cycle} \qquad (7)$$

Equation 7 establishes a relationship between the modulation time and the maximal cycle time of the second-dimensional separation, requiring a fast gradient and re-equilibration. To achieve this, the column length in the second dimension must be relatively short. However, this decreases the kinetic efficiency of the separation via reducing the number of theoretical plates, furthermore, as Equation 2 points out, fast gradients suffer from reduced peak capacity. Also, running such separations is demanding for the instrument due to high pressure drops over the column. With the introduction of UHPLC and UHPSFC instrumentation and improved stationary phase technology of particles with sub-2 µm diameter or core-shell structure, these hindrances have become less of a problem.

Concluding from the above, typical comprehensive online two-dimensional separations with a loop interface employ slow, long separations in the first dimension collecting fractions of the first-dimensional effluent into alternating loops. Then, the collected fractions are continuously analyzed in the second dimension by a fast gradient[149]. This conventional approach was tested for the combination of gel permeation chromatography and supercritical fluid

chromatography for lignin samples in **Paper III**. A major limitation discovered was that since the peak capacity of GPC is inherently low, the majority of the separation power of the system originated from the SFC separation. However, being rendered extremely fast to counterweigh the dead volume of the system and to be able to be accommodated to an arbitrary 1-minute-long modulation time, supercritical fluid chromatography failed to deliver the necessary resolution to apply the method for complex lignin samples. This became especially apparent when it came to the separation of oligomers above dimers in a technical lignin sample (blob around 30-34 minutes in the first dimension of Figure 16).

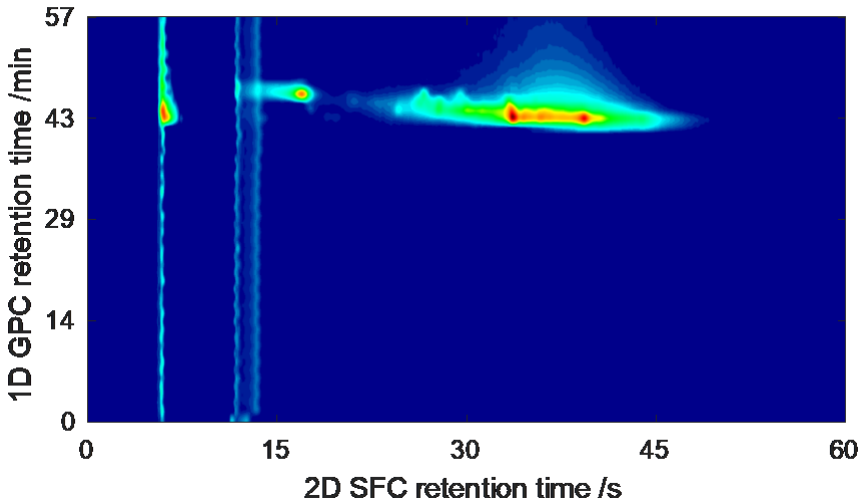

Figure 16. Comprehensive two-dimensional GPC × SFC separation of an Indulin AT lignin sample with DEA trapping columns. Chromatographic conditions: first dimension: gel permeation on Mesopore column (250×2.1 mm, 3 μm) with THF eluent (15 μl/min flow rate, 50°C). second dimension A: SFC separation on DIOL column (100×3 mm, 1.7 μm, 130 Å), sCO_2:MeOH gradient, 130 bar BPR, 50°C, 1.25 mL/min flow rate. Figure taken from **Paper III**.

This limitation of the method pointed out the need to decouple the modulation time from the cycle time of the second dimension if GPC was to be kept as the first dimension. Most simply this can be carried out by operating the two chromatographic systems in an offline manner, such as in **Paper I**, where essentially an offline LC-LC method was applied to the complex lignin oil sample. The need for a two-dimensional separation is noticeable in Figure 17, showing that the offline GPC fractionation removed most of the interfering peaks between 4 and 6 minutes, while the SFC separation, not limited by the modulation time, efficiently separated the lignophenolics in the isolated fractions.

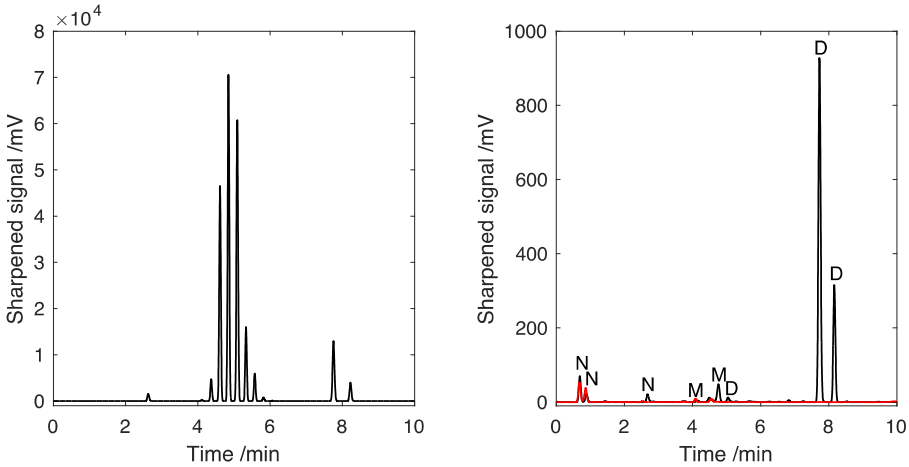

Figure 17. SFC/CAD chromatogram of the complete lignin oil (left), dimer fraction (right, black chromatogram), and monomer fraction (right, red chromatogram) after peak sharpening. N, M and D stand for not identified, monomer and dimer peaks, respectively. Figure reprinted from **Paper I**.

The decoupling of the modulation time and the second-dimensional runtime was further investigated in **Paper IV** in an online configuration. For this, it was recognized that the GPC process, unlike other, enthalpy-driven separations, does not yield discrete peaks populating the elution window but produces one wide, poorly resolved zone. The width of this zone is dependent only the flow rate and has the magnitude of only a few minutes. With this, we scheduled a system which takes fractions and stores them in a peak-parking device. For this purpose, multiple heart-cutting valves were employed, first and described by Pursch *et al.*[171] (Figure 18).

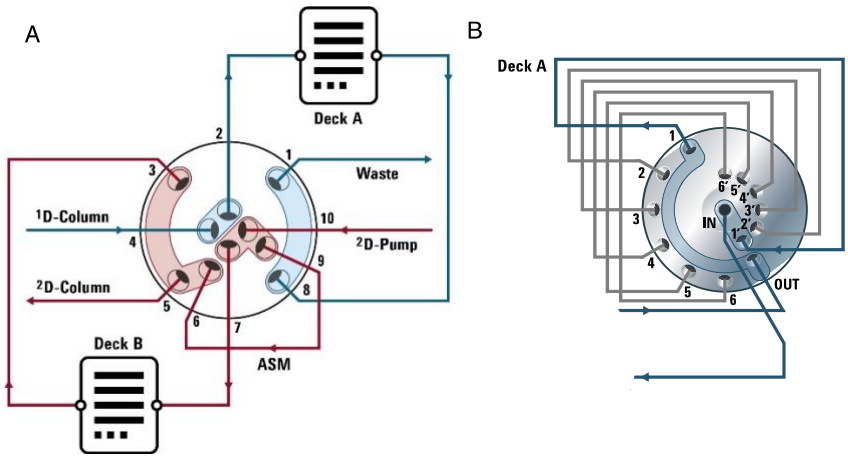

Figure 18. Tubing scheme of the multiple heart-cut modulation with an active solvent modulation valve in the center. A: Full modulation configuration; B: detailed tubing of a multiple-heart cut valve. Figures are properties of Agilent Technologies and adapted with their permission.

With this setup, eleven fractions were taken, covering the whole GPC separation, which were later sequentially analyzed in the second dimension. This approach features several advantages over the conventional loop-based modulations. First of all, Equation 7 does not have to be fulfilled anymore since the fractions are stored in a multiple heart-cut manner. The main consequence of this is that the SFC run in the second dimension can be extended for a longer time, allowing for a higher peak capacity. This also means that running a high-speed separation in the SFC dimension is not a must, making the method more instrument-friendly and robust in terms of operation conditions. Lastly, since the system dead volume does not have a significant role in this setup, a longer column with more theoretical plates can be employed to enhance kinetic efficiency. In addition to the above, since the number of fractions is fixed, modulation times can be shortened as long as Equation 8 is upheld. In other words, provided that the GPC elution zone is sufficiently narrow, high resolution sampling similar to heart-cut methods can be achieved, decreasing the effect of undersampling on the peak capacity of the system.

$$t_{\text{elution zone}} = t_{\text{modulation}} \times \#_{\text{loops}} \quad (8)$$

Compared to a study employing hydrophobic interaction chromatography × size exclusion chromatography[171], our study yielded substantially higher resolving power thanks to the lowered modulation time and the higher peak capacity provided by the long second-dimensional run. Works with a similar approach but realized in an offline manner had comparable performance characteristics to our method in terms of resolving power, analysis time and peak capacity of the individual dimensions[164,165]. Although the offline RP × SFC separation by Tammekivi *et al.*[70] outperformed our method based on sheer numbers, it has to be noted that their focus was on depolymerized soda lignin and they did not detect any oligomers larger than tetramers. In comparison, thanks to the ^{2}D system switch concept explained above, we could transfer high-MW lignin oligomers to the second dimension, which was not possible in earlier studies employing only SFC in the second dimension. As such, our method enables the analysis of large lignin oligomers in technical lignin samples in addition to the extended orthogonality.

4.2.3 Influence of the modulation on the second-dimensional separation

In the classical setup of modulation using two sample loops, the collected fraction acts as the injection plug to the second dimension. This means that the effluent of the first dimension must fulfill all the criteria that are expected from a sample diluent, *viz.* comparable polarity to the starting composition of the mobile phase gradient and sufficiently low volume to achieve an impulse-like introduction to the column. In real-life scenarios, none of the two criteria are easily met. Solvent

incompatibility, which may range from simple solvent strength mismatch to immiscibility is a commonly encountered problem[172]. This combined with the relatively large injection volume to the second dimension may seriously compromise the separation by causing peak shape distortion, retention loss and in extreme cases, complete breakthrough[173]. Several different solutions have been proposed to overcome this hindrance, including stationary-phase or trapping column assisted modulation[50,174–177], thermal modulation[178,179] and evaporation-based interfaces[156,158]. In addition, some approaches aim to dilute the solvent in the fraction to yield a similar composition to that of the second-dimensional gradient. The easiest way to perform this is by post-1D infusion of a weak solvent through a T-splitter[180,181]. Lately, Stoll *et al.* have developed active solvent modulation, a valve-based approach to dilute the fraction from the first dimension with the eluent of the second dimension[152]. Online coupling of THF-based GPC with SFC required careful consideration and choice of these solutions during our work. In **Paper III**, the classic dual loop interface was compared with stationary-phase assisted modulation. Using trapping columns resulted in enhanced detectability in comparison to the loop configuration as analyte zones were compressed in the trapping phase, as illustrated in Figure 19. Peak shapes were also improved, especially when the trapping system was operated in cocurrent mode. Similar findings were reported by Sun *et al.* who analyzed depolymerized lignin samples in a RP × SFC configuration using a phenylhexyl (PH) trapping phase; however, in our case this phase did not trap analytes due to THF being a strong eluent for PH. At the same time, the results of stationary phase screening in the same study proved highly useful when selecting trapping chemistry, leaving DEA and 2-PIC worthy of further testing for breakthrough.

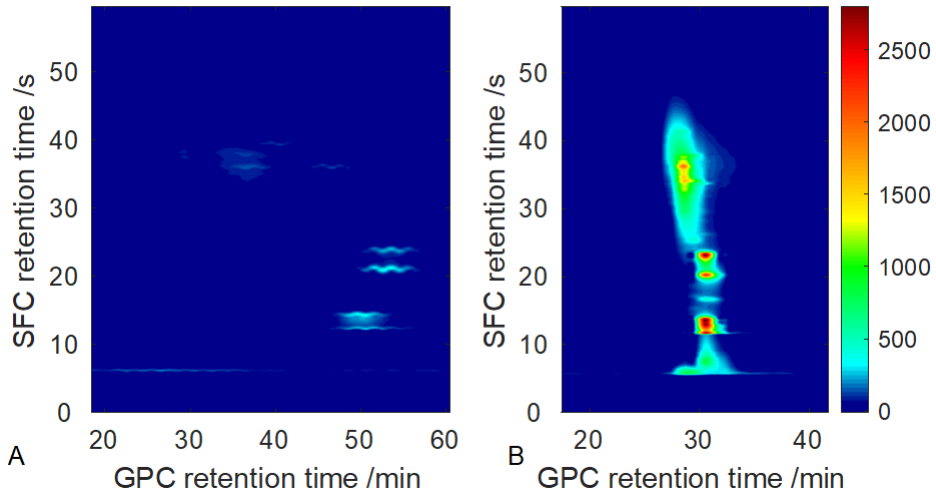

Figure 19. Comparison of dual loop (A) and stationary-phase assisted modulations (B) for GPC × SFC separation of lignophenolics in a reductive catalytic fractionation birch oil sample. Chromatographic conditions: first dimension: THF-based GPC (PS-DVB column, 50°C); second dimension: SFC on DIOL column (50×3 mm, 1.7 μm) with sCO_2:MeOH gradient, 130 bar BPR, 50°C, 2.75 mL/min flow rate.

Breakthrough studies aimed to find the maximum of the 1D effluent volume that can be passed through the trap without prematurely eluting analytes. Contrary to our expectations, a clear maximum of peak volumes was not observed when the 1D effluent volume increased. Instead, the peak volumes of the selected lignophenolics except for ferulic acid exhibited a decreasing trend, as shown in Figure 20. A possible explanation for this is that while the DEA phase retained lignophenolics quite strongly during the column screening experiments with sCO_2-MeOH gradient. In this investigation THF acted as a stronger eluent, resulting in a significant loss of retention. Nevertheless, ferulic acid, being retained by ionic interactions with the amino group was efficiently trapped. Another phenomenon contributing to the loss of trapping capacity is the overloading of the phase, caused by the fact that all monomers elute in the same fraction from the GPC.

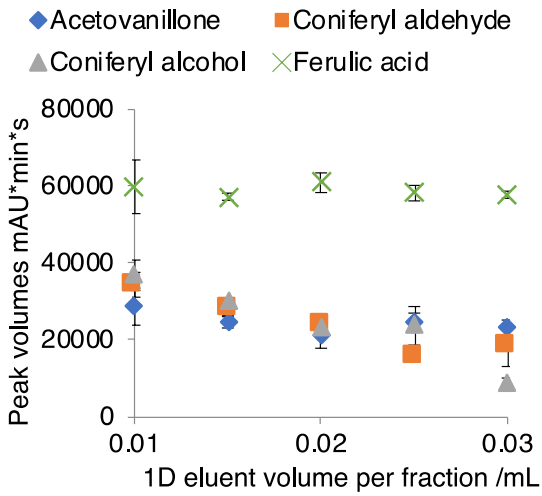

Figure 20. Evaluation of the trapping capacity of the DEA phase for selected lignin monomeric compounds. Chromatographic conditions: first dimension: gel permeation on Mesopore column (250×2.1 mm, 3 μm) with THF eluent (50°C). second dimension A: SFC separation on DIOL column (100×3 mm, 1.7 μm, 130 Å), sCO_2:MeOH gradient, 100 bar BPR, 50°C, 1.25 mL/min flow rate. Figure taken from **Paper III**.

The disappointing result of the breakthrough studies indicated that the modulation between THF-based GPC and SFC requires weakening of the 1D effluent. During the work discussed in **Paper IV**, post-1D infusion of heptane was tested to improve fraction transfer from loops to the SFC dimension, resulting in improved peak shapes and retention. This approach has not yet been reported in two-dimensional systems featuring SFC as the second dimension but has been used with LC × LC setups[169]. On the other hand, including a make-up flow restricted the 1D flow rate further so as not to overfill the loop (Equation 6) and put an additional constraint on the make-up pump to deliver a flow of 5 μL/min heptane. Regrettably, although a proof of concept was shown in Figure 21, such a low flow rate could not be pumped consistently leading to reproducibility issues and loss of analytes.

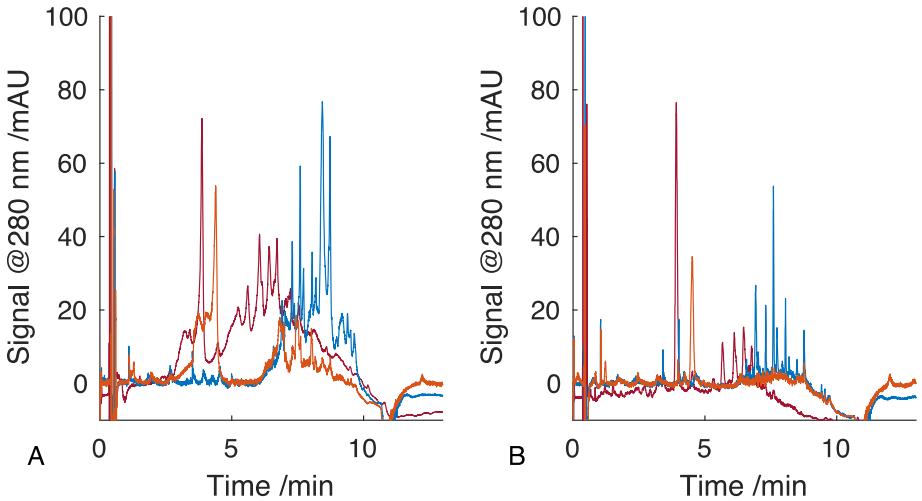

Figure 21. Comprehensive online GPC × SFC separation of an Indulin AT lignin sample without (A) and with (B) post-1D heptane infusion. Traces with the same color in A and B represent the same fractions from GPC. Chromatographic conditions: first dimension: GPC on Mesopore column (250×2.1 mm, 3 μm) with THF eluent (35 μl/min flow rate, 50°C). second dimension A: SFC separation on DIOL column (100×3 mm, 1.7 μm, 130 Å), sCO_2:MeOH gradient, 100 bar BPR, 50°C, 1.25 mL/min flow rate.

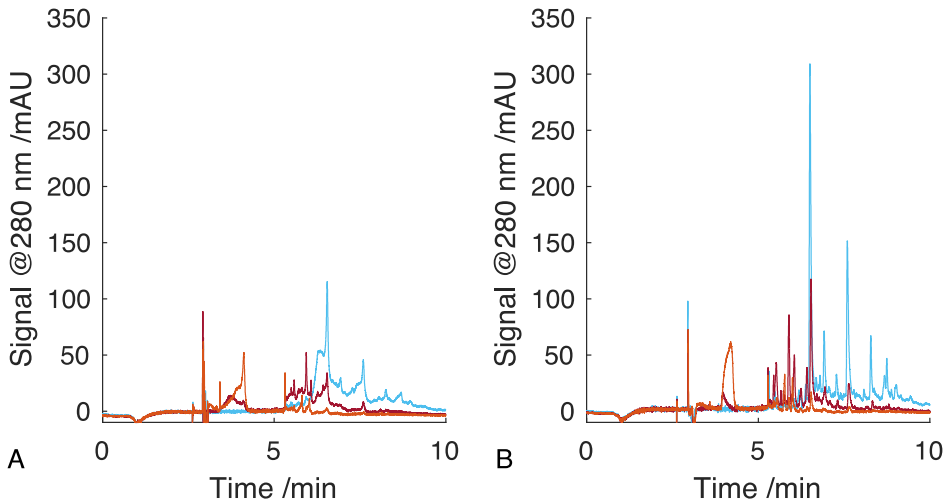

Figure 22. Effect of active solvent modulation (ASM) on isolated SFC slices in two-dimensional GPC × SFC. Chromatogram A was recorded without ASM, while chromatogram B used an ASM capillary with the length of 170 mm and a 30 seconds long ASM phase. Traces with the same color in A and B represent the same fractions from GPC. Chromatographic conditions: first dimension: GPC on Mesopore column (250×2.1 mm, 3 μm) with THF eluent (50 μl/min flow rate, 40°C). second dimension: SFC separation on DIOL column (100×3 mm, 1.7 μm, 130 Å), sCO_2:MeOH gradient, 100 bar BPR, 40°C, 1.7 mL/min flow rate; second dimension. Injection volume: 5 μL. Figure taken from **Paper IV**.

To make the method more robust, active solvent modulation as described by Stoll *et al.*[152] was successfully applied (Figure 22). For the optimization of this modulation, the length of the ASM period and the capillary lengths were considered. However, neither of these factors appeared to have a major influence on the second-dimensional separations. In the LC dimension, this was especially surprising since several studies have been conducted on the effects of the modulation parameters[182,183]. A possible explanation for this is the relatively low resolution of the high-MW oligomers in the RPLC dimension which makes the ASM effect is less noticeable. Similarly, no change was observed in the SFC dimension upon varying the ASM parameters. This, seemingly contradicting the Hagen-Poiseuille law called for forming a hypothesis about the dilution process. Briefly, it is assumed that instead of the dilution factor, it is the splitting itself in the ASM valve that is important since it provides a mixing chamber where the sCO_2-based mobile phase is allowed to vigorously mix with the fraction to be injected. Nevertheless, this is yet to be investigated further and confirmed by more rigorous experimentation.

5 Concluding remarks

This thesis work, while aimed to address particular challenges regarding the analysis of lignophenolic compounds in technical lignin samples, was also intended to yield additional knowledge on the techniques applicable in other fields as well. The major focus was on method development work and systematic evaluation of molecular-level interactions and processes in the studied systems. The developed methods were applied to address current challenges in the lignin field, ranging from quantitative analysis to multidimensional separations.

The studies involving supercritical fluid chromatography of lignophenolics focused on exploiting the superior kinetic efficiency and rapid separation speed of the technique. An SFC separation, combined with a universal charged aerosol detection was specifically useful for quantifying lignin dimers in a complex lignin oil sample. This is a challenge that otherwise would require long gas chromatographic separations after derivatization. The investigation of the uniformity of the detector signal yielded results allowing for the proposal of a single-standard quantification strategy, which directly addressed the problem of scarcely available reference standards for lignin dimers. A main limitation of the method was the selectivity of the SFC method, which proved to be insufficient for such a complex sample. Other one-dimensional chromatographic methods address this by enhancing the selectivity by sample cleanup or selective detectors, but our study inherently excluded these options from the aims. This urged us to combine other separation techniques. Using SFC in a two-dimensional setup combined with gel permeation chromatography was successfully investigated in both offline and online configurations. The offline coupling, featuring a single heart-cut fraction from the GPC transferred to SFC, yielded a chromatogram with much less interference and purer analyte peaks arriving to the CAD, thus contributing to the accuracy of the quantification. From the studies performed in an online fashion, valuable insights into the retention properties of lignophenolics were obtained. Most importantly, it was found that the commonly used polar SFC stationary phases do not exhibit significantly different selectivity from each other and the relative retention is more dependent on the properties of the analytes. For instance, lignophenolics with polar functionalities are typically strongly retained on the investigated stationary phases. Nevertheless, the differences in stationary phase chemistry can be further exploited to alter the absolute retention of phenolics and thus improve the resolution.

Gel permeation chromatography was used throughout the work for size-based separations, however, the trueness of the determined molecular weight had to be critically evaluated. This was carried out by using a complementary, multi-technique approach and allowed us to uncover the error of molecular weight determination not just for complete lignin samples but also for distinct, narrow-MW fractions of kraft lignin. A quantitative model developed for monomers and functional-group-based analysis revealed the significance of hydrogen bonds in aggregation, associated with a positive bias. On the other hand, indirect evidence from pfg-diffusion NMR experiments suggested that reversed-phase-type interactions between analytes and the column packing also occur. At the same time, the positive bias which was observed for monomers gradually decreased towards the higher molecular weight fractions, suggesting that for non-depolymerized technical lignins linear polystyrene calibration yields a reliable molecular weight determination. For monomer fractions of depolymerized lignins however, the error may be unacceptably high. An important, albeit unexpected discovery was made on the stability of lignin solutions, suggesting that repolymerization processes take place in the course of a few weeks.

To benefit from the increased separation power, the coupling of gel permeation and supercritical fluid chromatography in two-dimensional systems was explored. Not reported before, one study focused on a more conventional approach with short modulation and simple instrumentation, to evaluate whether the combination offers benefits compared to the more commonly employed methods based on reversed-phase liquid chromatography. Here, multiple pitfalls were identified, including the low contribution of peak capacity from the GPC system, insufficient time for the development of the SFC chromatogram and inadequate transfer of particular fractions to the second dimension. A valve-based approach was used to address these and other arising challenges. First of all, using peak-parking devices allowed for the decoupling of the modulation time from the ^{2}D cycle time, permitting longer gradients to run, which provided better resolving power in the SFC dimension. Furthermore, using the cutting-edge ASM technology, the detrimental effect of large volumes of ^{1}D eluent were largely resolved, further contributing to the improvements in the ^{2}D run. Lastly, to tackle the transfer of large lignin oligomers to the second dimension, the commercial 2DLC valve was configured to switch from sCO_2-based SFC to water-ACN-based reversed-phase LC. This was crucial to achieve full comprehensiveness, since the mentioned lignin oligomers were not transferred to the column by the SFC solvent due to limited solubility. With this build, we addressed not only the challenges in the lignin field, but also obstacles that have been identified in other research areas. The assembled system was coupled to high resolution mass spectrometry, forming an advanced analysis platform for the comprehensive qualitative analysis of technical lignin samples.

5.1 Future research aspects

While four years is a sufficiently long time to conduct good research and discover scientific novelties, it is inevitable that some of the research questions framed in the project proposal remain unexplored. In addition, new ideas originate during the research itself, thus fueling future endeavors.

Although **Paper I** explored how charged aerosol detection can be utilized to quantify lignin dimers with only a single standard, multiple questions stayed open in that study. First of all, the number of investigated dimers was limited, meaning that extrapolating our results too far may result in serious errors. For this reason, the proposed single-standard strategy should be re-evaluated by including more model compounds with more consistent purity and preferably extending the investigation to trimers and tetramers, which have never been addressed in quantification studies. Furthermore, the influence of chromatographic variables such as flow rates, solvent types and additives on the CAD signal was not appropriately addressed and requires to be uncovered in a separate study.

The complementary multi-technique approach in **Paper II** enabled the exploration of the processes occurring during a GPC separation in the column from various angles, thus giving a more complete picture. However, the study faced several limitations due to the low concentration of the isolated fractions. Additional mass spectrometry experiments could target this shortcoming. Furthermore, more concentrated fractions can be analyzed by pfg-diffusion NMR, thus providing an opportunity to compare the three techniques directly. Additionally, the stability of lignin solutions as an overlooked condition requires a more controlled and extensive study. Another, more explorative study could evaluate size-based separations in pressurized environments with sCO_2 as an eluent component. This requires careful consideration of the employed packing material from a pressure-tolerance viewpoint as well as studies regarding the solubility of lignin in such solvents.

Our online two-dimensional studies have shown that while the combination of GPC and SFC is practically feasible, it cannot compete with more powerful methods. Nevertheless, the difficulties faced during the development in **Paper III** gave rise to the creative and innovative use of valves, addressing limitations that were also recognized in the literature. Building on this, further efforts into the exploration of the switch concept can focus on including more selective modes such as reversed-phase LC in the first dimension. Fraction transfer, being one of the main limitations of the developed method in **Paper IV**, deserves further attention centering around refocusing the analytes in a trapping phase, either in a chip configuration or in a more conventional way.

6 Acknowledgments

The journey of nearly five years during this PhD work was not a lonely one, luckily. I have met countless new colleagues, researchers, engineers and friends while having the constant support of my family and friends from earlier. The contribution of all these people made this thesis possible and I cannot thank enough them for all of it.

First of all, the greatest thanks are due to my wonderful supervisors, Lotta, Maggan and Tommy. I honestly say that I could not have gotten a better team to guide me through the sometimes happy, sometimes rough life of PhD students. While it was definitely a scary experience in the beginning to get literally complete freedom in my research, it has taught me a lot about making decisions, being committed to my ideas and how to become the creative problem-solver I am now. Even though I was not always right, they gave me the necessary support and helped me to get back on the right track. Lotta, it might sound crazy, but I really enjoyed our long discussions over research, both of us being stubborn and eventually, always agreeing on some compromise. I learned a lot from you about how to critically assess my results, even when I had believed that they were sound and solid. Maggan, I would like to say huge thanks for your constantly positive and supportive attitude you showcased whenever I knocked on your door with something to discuss. Our discussions about nerdy chromatography-related questions, instrument building and lab teaching will remain as good memories. Tommy, I wish we had the opportunity to spend more time together and I could get more involved in your research area; sadly, I was too focused on analytical questions. Still, I appreciate that you did not decide to stay away but helped where you could, from giving valuable feedback on my manuscripts to dropping by the office and having a talk for a few minutes.

Massive thanks go to other senior scientists whose ears were always open when I needed their opinion on a particular scientific problem or I just wanted to discuss some minor things. Peter was almost like a fourth supervisor, always asking the right questions which moved my research forward. Sofia, or as we PhD students often said among each other, our cool aunt, helped extremely much with mass spectrometry experiments and even more, she taught me all the little tricks with the instruments which are not found in textbooks, but mean a lot in need. Also thanks for the extra language practice! Fiona deserves a lot of chocolate for her all-around help as well as acting as a second mom to us younger students, even when it included telling us badly off for leaving the office messy.

I owe my gratitude to the support staff at CAS and Kemicentrum for making things go as smoothly as possible. Maria, Sara, Kornelije, Annette, Zoltán, Katarina and Fatima: tusen tack! Hereby, I would like to say thanks to all the fellow students and coworkers at CAS for creating an inspiring atmosphere for research.

Naturally, my groupmates had an enormous role in this ride. Some contributed by just listening to me talking about some of my crazy ideas, probably wishing that I jump out of the window; others by giving useful advice; again others by showcasing good research and inspiring me to improve further. I am endlessly thankful for this. Here comes a definitely not complete list of individuals who were part of the Analytical Chemistry Group in these five years: Linda, Larissa, Bárbara, Daniel, Jens, Oksana, Thamani, Fiona, Federica, Veronika, Nabomo, Mahdi, Hamidreza, Maider, Cristina, Shandilya, Mynta, Josep, Simon, Erika, Azemina and Mona. From these, special thanks go to Thamani for being able to put up with me sitting in the same office, blasting music loudly and disturbing his workflow in the worst possible moments. Often the first one to see my manuscripts, his contribution to my research output is surely more than what one would expect from an officemate. I hope I could give something back when you turned to your left to ask me about chromatography or Matlab coding. I would like to extend my special thanks to Mynta, my sister-in-arms when it came to lignin research. Dubbing ourselves the "new Bob and Jens", we regularly ended up having nerdy discussions about how to tackle the complexity of this huge molecule, continuously pushing and pulling each other to come up with ideas. Besides, going to shows in Köpenhamn was also a lot of fun!

My gratitude goes out to my collaborators, who made my life so much easier with their contributions to my work. Without the help of Joseph, Thanya and Daria, who synthesized the dimer standards for my first-ever paper, probably the whole project would have gone åt skogen. I hope the results we had gotten made up for the efforts they put into the work. Also, I must acknowledge Göran, the Jedi master of NMR, who not only performed the experiments but actively tried to pass all his knowledge to me. It is not his fault that I am still not an NMR expert.

While it is easy to forget about them, I would like to thank my amazing Agilent team, who were always extremely helpful when I reached out with my issues: Kai-Uwe, Jane-Marie, Juho, Diana and Linnea. Furthermore, hereby I acknowledge Vetenskapsrådet for the financial support of the project.

Finally, I would like to say thanks for the never-ending patience, love and support of my family, both from Poland and Hungary. They provided me with the strong background I needed for my work, lifted me up in case of failure and celebrated success with me. Without them, this journey would not have been possible. Bardzo dziękuję za/köszönöm szépen all that I got from you.

Nihil verum, omnia licita

7 References

(1) Glasser, W. G. About Making Lignin Great Again—Some Lessons from the Past. *Front Chem* **2019**, *7* (August), 1–17. https://doi.org/10.3389/fchem.2019.00565.

(2) Boerjan, W.; Ralph, J.; Baucher, M. Lignin Biosynthesis. *Annu Rev Plant Biol* **2003**, *54*, 519–546. https://doi.org/10.1146/annurev.arplant.54.031902.134938.

(3) Vogt, T. Phenylpropanoid Biosynthesis. *Mol Plant* **2010**, *3* (1), 2–20. https://doi.org/10.1093/mp/ssp106.

(4) Lan, W.; Lu, F.; Regner, M.; Zhu, Y.; Rencoret, J.; Ralph, S. A.; Zakai, U. I.; Morreel, K.; Boerjan, W.; Ralph, J. Tricin, a Flavonoid Monomer in Monocot Lignification. *Plant Physiol* **2015**, *167* (4), 1284–1295. https://doi.org/10.1104/pp.114.253757.

(5) del Río, J. C.; Rencoret, J.; Gutiérrez, A.; Kim, H.; John, R. Hydroxystilbenes Are Monomers in Palm Fruit Endocarp Lignins. *Plant Physiol* **2017**, *174* (4), 2072–2082. https://doi.org/10.1104/pp.17.00362.

(6) Zevallos Torres, L. A.; Lorenci Woiciechowski, A.; de Andrade Tanobe, V. O.; Karp, S. G.; Guimarães Lorenci, L. C.; Faulds, C.; Soccol, C. R. Lignin as a Potential Source of High-Added Value Compounds: A Review. *J Clean Prod* **2020**, *263*. https://doi.org/10.1016/j.jclepro.2020.121499.

(7) Windeisen, E.; Wegener, G. Lignin as Building Unit for Polymers. In *Polymer Science: A Comprehensive Reference*; Matyjaszewski, K., Möller, M., Eds.; Elsevier B.V., 2012; Vol. 10, pp 255–265. https://doi.org/10.1016/B978-0-444-53349-4.00263-6.

(8) Chakar, F. S.; Ragauskas, A. J. Review of Current and Future Softwood Kraft Lignin Process Chemistry. *Ind Crops Prod* **2004**, *20* (2), 131–141. https://doi.org/10.1016/j.indcrop.2004.04.016.

(9) Argyropoulos, D. D. S.; Crestini, C.; Dahlstrand, C.; Furusjö, E.; Gioia, C.; Jedvert, K.; Henriksson, G.; Hulteberg, C.; Lawoko, M.; Pierrou, C.; Samec, J. S. M.; Subbotina, E.; Wallmo, H.; Wimby, M. Kraft Lignin: A Valuable, Sustainable Resource, Opportunities and Challenges. *ChemSusChem* **2023**, *16* (23). https://doi.org/10.1002/cssc.202300492.

(10) Wang, H.; Pu, Y.; Ragauskas, A.; Yang, B. From Lignin to Valuable Products–Strategies, Challenges, and Prospects. *Bioresour Technol* **2019**, *271* (4), 449–461. https://doi.org/10.1016/j.biortech.2018.09.072.

(11) Bajwa, D. S.; Pourhashem, G.; Ullah, A. H.; Bajwa, S. G. A Concise Review of Current Lignin Production, Applications, Products and Their Environment Impact. *Ind Crops Prod* **2019**, *139* (February), 111526. https://doi.org/10.1016/j.indcrop.2019.111526.

(12) Berlin, A.; Balakshin, M. Industrial Lignins: Analysis, Properties, and Applications. In *Bioenergy Research: Advances and Applications*; Gupta, V. K., Tuohy, M. G., Kubicek, C. G., Saddler, J., Xu, F., Eds.; Elsevier, 2014; pp 315–336. https://doi.org/10.1016/B978-0-444-59561-4.00018-8.

(13) Abdelaziz, O. Y.; Brink, D. P.; Prothmann, J.; Ravi, K.; Sun, M.; García-Hidalgo, J.; Sandahl, M.; Hulteberg, C. P.; Turner, C.; Lidén, G.; Gorwa-Grauslund, M. F. Biological Valorization of Low Molecular Weight Lignin. *Biotechnol Adv* **2016**, *34* (8), 1318–1346. https://doi.org/10.1016/j.biotechadv.2016.10.001.

(14) Kline, L. M.; Hayes, D. G.; Womac, A. R.; Labbé, N. Simplified Determination of Lignin Content in Hard and Soft Woods via UV-Spectrophotometric Analysis of Biomass Dissolved in Ionic Liquids. *Bioresources* **2010**, *5* (3), 1366–1383. https://doi.org/10.15376/biores.5.3.1366-1383.

(15) Boeriu, C. G.; Bravo, D.; Gosselink, R. J. A.; Van Dam, J. E. G. Characterisation of Structure-Dependent Functional Properties of Lignin with Infrared Spectroscopy. *Ind Crops Prod* **2004**, *20* (2), 205–218. https://doi.org/10.1016/j.indcrop.2004.04.022.

(16) Faix, O. Classification of Lignins from Different Botanical Origins by FT-IR Spectroscopy. *Holzforschung* **1991**, *45* (s1), 21–28. https://doi.org/10.1515/hfsg.1991.45.s1.21.

(17) Rönnols, J.; Danieli, E.; Freichels, H.; Aldaeus, F. Lignin Analysis with Benchtop NMR Spectroscopy. *Holzforschung* **2020**, *74* (2), 226–231. https://doi.org/10.1515/hf-2018-0282.

(18) Lagerquist, L.; Rahkila, J.; Eklund, P. Utilization of 31P PULCON for Quantitative Hydroxyl Group Determination in Lignin by NMR Spectroscopy. *ACS Sustain Chem Eng* **2019**, *7* (9), 9002–9006. https://doi.org/10.1021/acssuschemeng.9b01269.

(19) Zhang, J.; Jiang, Y.; Easterling, L. F.; Anstner, A.; Li, W.; Alzarieni, K. Z.; Dong, X.; Bozell, J.; Kenttämaa, H. I. Compositional Analysis of Organosolv Poplar Lignin by Using High-Performance Liquid Chromatography/High-Resolution Multi-Stage Tandem Mass Spectrometry. *Green Chemistry* **2021**, *23* (2), 983–1000. https://doi.org/10.1039/d0gc03398g.

(20) Constant, S.; Wienk, H. L. J.; Frissen, A. E.; Peinder, P. De; Boelens, R.; Van Es, D. S.; Grisel, R. J. H.; Weckhuysen, B. M.; Huijgen, W. J. J.; Gosselink, R. J. A.; Bruijnincx, P. C. A. New Insights into the Structure and Composition of Technical Lignins: A Comparative Characterisation Study. *Green Chemistry* **2016**, *18* (9), 2651–2665. https://doi.org/10.1039/c5gc03043a.

(21) Fritz, J. O.; Moore, K. J. Separation and Quantification of Lignin-Derived Phenolic Monomers Using High-Resolution Gas Chromatography. *J Agric Food Chem* **1987**, *35* (5), 710–713. https://doi.org/10.1021/jf00077a017.

(22) Sonoda, T.; Ona, T.; Yokoi, H.; Ishida, Y.; Ohtani, H.; Tsuge, S. Quantitative Analysis of Detailed Lignin Monomer Composition by Pyrolysis-Gas Chromatography Combined with Preliminary Acetylation of the Samples. *Anal Chem* **2001**, *73* (22), 5429–5435. https://doi.org/10.1021/ac010557c.

(23) Liu, Q.; Li, P.; Liu, N.; Shen, D. Lignin Depolymerization to Aromatic Monomers and Oligomers in Isopropanol Assisted by Microwave Heating. *Polym Degrad Stab* **2017**, *135*, 54–60. https://doi.org/10.1016/j.polymdegradstab.2016.11.016.

(24) Hafezisefat, P.; Lindstrom, J. K.; Brown, R. C.; Qi, L. Non-Catalytic Oxidative Depolymerization of Lignin in Perfluorodecalin to Produce Phenolic Monomers. *Green Chemistry* **2020**, *22* (19), 6567–6578. https://doi.org/10.1039/d0gc02505d.

(25) Deepa, A. K.; Dhepe, P. L. Lignin Depolymerization into Aromatic Monomers over Solid Acid Catalysts. *ACS Catal* **2015**, *5* (1), 365–379. https://doi.org/10.1021/cs501371q.

(26) Chen, F.; Zhuo, C.; Xiao, X.; Pendergast, T. H.; Devos, K. M. A Rapid Thioacidolysis Method for Biomass Lignin Composition and Tricin Analysis. *Biotechnol Biofuels* **2021**, *14* (1), 1–9. https://doi.org/10.1186/s13068-020-01865-y.

(27) Zhao, J.; Xiuwen, W.; Hu, J.; Liu, Q.; Shen, D.; Xiao, R. Thermal Degradation of Softwood Lignin and Hardwood Lignin by TG-FTIR and Py-GC/MS. *Polym Degrad Stab* **2014**, *108*, 133–138. https://doi.org/10.1016/j.polymdegradstab.2014.06.006.

(28) Kiyota, E.; Mazzafera, P.; Sawaya, A. C. H. F. Analysis of Soluble Lignin in Sugarcane by Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectrometry with a Do-It-Yourself Oligomer Database. *Anal Chem* **2012**, *84* (16), 7015–7020. https://doi.org/10.1021/ac301112y.

(29) Song, W. Y.; Park, H.; Kim, T. Y. Improving Liquid Chromatography-Mass Spectrometry Sensitivity for Characterization of Lignin Oligomers and Phenolic Compounds Using Acetic Acid as a Mobile Phase Additive. *J Chromatogr A* **2022**, *1685*, 463598. https://doi.org/10.1016/j.chroma.2022.463598.

(30) Prothmann, J.; Spégel, P.; Sandahl, M.; Turner, C. Identification of Lignin Oligomers in Kraft Lignin Using Ultra-High-Performance Liquid Chromatography/High-Resolution Multiple-Stage Tandem Mass Spectrometry (UHPLC/HRMSn). *Anal Bioanal Chem* **2018**, *410* (29), 7803–7814. https://doi.org/10.1007/s00216-018-1400-4.

(31) Fischer, K.; Höffler, S. RP-HPLC Analysis of Phenolic Lignin Monomers with DAD and Coulometric Array Detection. *J Chromatogr Sci* **2021**, *59* (4), 312–323. https://doi.org/10.1093/chromsci/bmaa105.

(32) Lobbes, J. M.; Fitznar, H. P.; Kattner, G. High-Performance Liquid Chromatography of Lignin-Derived Phenols in Environmental Samples with Diode Array Detection. *Anal Chem* **1999**, *71* (15), 3008–3012. https://doi.org/10.1021/ac981175i.

(33) Galletti, G. C.; Piccaglia, R.; Concialini, V. Optimization of Electrochemical Detection in the High-Performance Liquid Chromatography of Lignin Phenolics from Lignocellulosic by-Products. *J Chromatogr A* **1990**, *507* (C), 439–450. https://doi.org/10.1016/S0021-9673(01)84223-0.

(34) Sun, M.; Lidén, G.; Sandahl, M.; Turner, C. Ultra-High Performance Supercritical Fluid Chromatography of Lignin-Derived Phenols from Alkaline Cupric Oxide Oxidation. *J Sep Sci* **2016**, *39* (16), 3123–3129. https://doi.org/10.1002/jssc.201600169.

(35) Prothmann, J.; Sun, M.; Spégel, P.; Sandahl, M.; Turner, C. 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. *Anal Bioanal Chem* **2017**, *409* (30), 7049–7061. https://doi.org/10.1007/s00216-017-0663-5.

(36) Prothmann, J.; Palmer, S.; Turner, C.; Sandahl, M. Separation of Monomeric and Dimeric Phenolic Compounds in Lignosulphonate Lignin on Different Stationary Phases Using Ultrahigh-Performance Supercritical Fluid Chromatography. *J Chromatogr A* **2021**, *1653*, 462408. https://doi.org/10.1016/j.chroma.2021.462408.

(37) Prothmann, J.; Li, K.; Hulteberg, C.; Spégel, P.; Sandahl, M.; Turner, C. Nontargeted Analysis Strategy for the Identification of Phenolic Compounds in Complex Technical Lignin Samples. *ChemSusChem* **2020**, *13* (17), 4605–4612. https://doi.org/10.1002/cssc.202000951.

(38) Himmel, M. E.; Oh, K. K.; Sopher, D. W.; Chum, H. L. High-Performance Size Exclusion Chromatography of Low-Molecular-Weight Lignins and Model Compounds. *J Chromatogr A* **1983**, *267* (C), 249–265. https://doi.org/10.1016/S0021-9673(01)90845-3.

(39) Gidh, A. V.; Decker, S. R.; Vinzant, T. B.; Himmel, M. E.; Williford, C. Determination of Lignin by Size Exclusion Chromatography Using Multi Angle Laser Light Scattering. *J Chromatogr A* **2006**, *1114* (1), 102–110. https://doi.org/10.1016/j.chroma.2006.02.044.

(40) Baumberger, S.; Abaecherli, A.; Fasching, M.; Gellerstedt, G.; Gosselink, R.; Hortling, B.; Li, J.; Saake, B.; De Jong, E. Molar Mass Determination of Lignins by Size-Exclusion Chromatography: Towards Standardisation of the Method. *Holzforschung* **2007**, *61* (4), 459–468. https://doi.org/10.1515/HF.2007.074.

(41) Olcese, R.; Carré, V.; Aubriet, F.; Dufour, A. Selectivity of Bio-Oils Catalytic Hydrotreatment Assessed by Petroleomic and GC*GC/MS-FID Analysis. *Energy and Fuels* **2013**, *27* (4), 2135–2145. https://doi.org/10.1021/ef302145g.

(42) Lago, V.; Briens, C.; Berruti, F. Effect of Bed Material, Lignin Content, and Origin on the Processability of Biomass in Fast Pyrolysis Reactors. *Canadian Journal of Chemical Engineering* **2018**, *96* (1), 132–144. https://doi.org/10.1002/cjce.22932.

(43) Wang, L.; Li, J.; Chen, Y.; Yang, H.; Shao, J.; Zhang, X.; Yu, H.; Chen, H. Investigation of the Pyrolysis Characteristics of Guaiacol Lignin Using Combined Py-GC × GC/TOF-MS and In-Situ FTIR. *Fuel* **2019**, *251* (February), 496–505. https://doi.org/10.1016/j.fuel.2019.04.061.

(44) Yin, H.; Lu, J.; Liu, G.; Niu, Z.; Zha, X.; Wu, D.; Feng, A.; Hu, Y. Application of Chemometrics for Coal Pyrolysis Products by Online Py-GC×GC-MS. *ACS Omega* **2021**, *6* (5), 3763–3770. https://doi.org/10.1021/acsomega.0c05359.

(45) Dao Thi, H.; Van Aelst, K.; Van den Bosch, S.; Katahira, R.; Beckham, G. T.; Sels, B. F.; Van Geem, K. M. Identification and Quantification of Lignin Monomers and Oligomers from Reductive Catalytic Fractionation of Pine Wood with GC × GC – FID/MS. *Green Chemistry* **2022**, *24* (1), 191–206. https://doi.org/10.1039/d1gc03822b.

(46) van den Hurk, R. S.; Pursch, M.; Stoll, D. R.; Pirok, B. W. J. Recent Trends in Two-Dimensional Liquid Chromatography. *TrAC - Trends in Analytical Chemistry* **2023**, *166* (May). https://doi.org/10.1016/j.trac.2023.117166.

(47) Tammekivi, E.; Geantet, C.; Lorentz, C.; Faure, K. Two-Dimensional Chromatography for the Analysis of Valorisable Biowaste: A Review. *Anal Chim Acta* **2023**, *1283* (September). https://doi.org/10.1016/j.aca.2023.341855.

(48) Le Masle, A.; Angot, D.; Gouin, C.; D'Attoma, A.; Ponthus, J.; Quignard, A.; Heinisch, S. Development of On-Line Comprehensive Two-Dimensional Liquid Chromatography Method for the Separation of Biomass Compounds. *J Chromatogr A* **2014**, *1340*, 90–98. https://doi.org/10.1016/j.chroma.2014.03.020.

(49) Sarrut, M.; Corgier, A.; Crétier, G.; Le Masle, A.; Dubant, S.; Heinisch, S. Potential and Limitations of On-Line Comprehensive Reversed Phase Liquid Chromatography×Supercritical Fluid Chromatography for the Separation of Neutral Compounds: An Approach to Separate an Aqueous Extract of Bio-Oil. *J Chromatogr A* **2015**, *1402*, 124–133. https://doi.org/10.1016/j.chroma.2015.05.005.

(50) Sun, M.; Sandahl, M.; Turner, C. Comprehensive On-Line Two-Dimensional Liquid Chromatography × Supercritical Fluid Chromatography with Trapping Column-Assisted Modulation for Depolymerised Lignin Analysis. *J Chromatogr A* **2018**, *1541*, 21–30. https://doi.org/10.1016/j.chroma.2018.02.008.

(51) Supercritical Fluid. In *IUPAC Compendium of Terminology*; International Union of Pure and Applied Chemistry, 2019. https://doi.org/10.1351/goldbook.S06138 WV - 3.0.1.

(52) Knez; Markočič, E.; Leitgeb, M.; Primožič, M.; Knez Hrnčič, M.; Škerget, M. Industrial Applications of Supercritical Fluids: A Review. *Energy* **2014**, *77*, 235–243. https://doi.org/10.1016/j.energy.2014.07.044.

(53) Clifford, A. A.; Williams, J. R. Introduction to Supercritical Fluids and Their Applications. In *Supercritical Fluid Methods and Protocols*; Clifford, A., Williams, J., Eds.; Humana Totowa, 2000; Vol. 13.

(54) Nordbotten, J. M.; Celia, M. A.; Bachu, S. Injection and Storage of CO2 in Deep Saline Aquifers: Analytical Solution for CO2 Plume Evolution during Injection. *Transp Porous Media* **2005**, *58* (3), 339–360. https://doi.org/10.1007/s11242-004-0670-9.

(55) Nováková, L.; Grand-Guillaume Perrenoud, A.; Francois, I.; West, C.; Lesellier, E.; Guillarme, D. Modern Analytical Supercritical Fluid Chromatography Using Columns Packed with Sub-2μm Particles: A Tutorial. *Anal Chim Acta* **2014**, *824*, 18–35. https://doi.org/10.1016/j.aca.2014.03.034.

(56) Kirkwood, J. G. The Dielectric Polarization of Polar Liquids. *J Chem Phys* **1939**, *7* (10), 911–919. https://doi.org/10.1063/1.1750343.

(57) Moriyoshi, T.; Kita, T.; Uosaki, Y. Static Relative Permittivity of Carbon Dioxide and Nitrous Oxide. *Berichte der Bunsengesellschaft für physikalische Chemie* **1993**, *97*, 589–596.

(58) Jumaah, F.; Jędrkiewicz, R.; Gromadzka, J.; Namieśnik, J.; Essén, S.; Turner, C.; Sandahl, M. Rapid and Green Separation of Mono- and Diesters of Monochloropropanediols by Ultrahigh Performance Supercritical Fluid Chromatography-Mass Spectrometry Using Neat Carbon Dioxide as a Mobile Phase. *J Agric Food Chem* **2017**, *65* (37), 8220–8228. https://doi.org/10.1021/acs.jafc.7b02857.

(59) Jin, W.; Yang, J.; Liu, D.; Zhong, Q.; Zhou, T. Determination of Inflammation-Related Lipids in Depressive Rats by on-Line Supercritical Fluid Extraction-Supercritical Fluid Chromatography-Tandem Mass Spectrometry. *J Pharm Biomed Anal* **2021**, *203*, 114210. https://doi.org/10.1016/j.jpba.2021.114210.

(60) Lavison, G.; Bertoncini, F.; Thiébaut, D.; Beziau, J. F.; Carrazé, B.; Valette, P.; Duteurtre, X. Supercritical Fluid Chromatography and Two-Dimensional Supercritical Fluid Chromatography of Polar Car Lubricant Additives with Neat CO2 as Mobile Phase. *J Chromatogr A* **2007**, *1161* (1–2), 300–307. https://doi.org/10.1016/j.chroma.2007.05.068.

(61) Ibanez, E.; Lopez-Sebastian, S.; Tabera, J.; Reglero, G. Separation of Carotenoids by Subcritical Fluid Chromatography with Coated, Packed Capillary Columns and Neat Carbon Dioxide. *J Chromatogr A* **1998**, *823*, 313–319.

(62) Cunico, L. P.; Turner, C. Supercritical Fluids and Gas-Expanded Liquids. In *The Application of Green Solvents in Separation Processes*; Pena-Pereira, F., Tobiszewski, M., Eds.; Elsevier Inc., 2017; pp 155–214. https://doi.org/10.1016/B978-0-12-805297-6.00007-3.

(63) Souvignet, I.; Olesik, S. V. Solvent-Solvent and Solute-Solvent Interactions in Liquid Methanol/Carbon Dioxide Mixtures. *J. Phys. Chem* **1995**, *99*, 16800–16803.

(64) Lesellier, E.; West, C. The Many Faces of Packed Column Supercritical Fluid Chromatography - A Critical Review. *J Chromatogr A* **2015**, *1382*, 2–46. https://doi.org/10.1016/j.chroma.2014.12.083.

(65) West, C.; Lesellier, E. Characterization of Stationary Phases in Subcritical Fluid Chromatography by the Solvation Parameter Model: I. Alkylsiloxane-Bonded Stationary Phases. *J Chromatogr A* **2006**, *1110*, 181–190. https://doi.org/10.1016/j.chroma.2006.01.125.

(66) West, C.; Lesellier, E. Characterisation of Stationary Phases in Subcritical Fluid Chromatography with the Solvation Parameter Model IV. Aromatic Stationary Phases. *J Chromatogr A* **2006**, *1115*, 233–245. https://doi.org/10.1016/j.chroma.2006.02.050.

(67) West, C.; Khater, S.; Lesellier, E. Characterization and Use of Hydrophilic Interaction Liquid Chromatography Type Stationary Phases in Supercritical Fluid Chromatography. *J Chromatogr A* **2012**, *1250*, 182–195. https://doi.org/10.1016/j.chroma.2012.05.008.

(68) West, C.; Lesellier, E. Characterisation of Stationary Phases in Subcritical Fluid Chromatography with the Solvation Parameter Model: III. Polar Stationary Phases. *J Chromatogr A* **2006**, *1110*, 200–213. https://doi.org/10.1016/j.chroma.2006.01.109.

(69) Gros, Q.; Molineau, J.; Noireau, A.; Duval, J.; Bamba, T.; Lesellier, E.; West, C. Characterization of Stationary Phases in Supercritical Fluid Chromatography Including Exploration of Shape Selectivity. *J Chromatogr A* **2021**, *1639*, 461923. https://doi.org/10.1016/j.chroma.2021.461923.

(70) Tammekivi, E.; Batteau, M.; Laurenti, D.; Lilti, H.; Faure, K. A Powerful Two-Dimensional Chromatography Method for the Non-Target Analysis of Depolymerised Lignin. *Anal Chim Acta* **2024**, *1288* (September). https://doi.org/10.1016/j.aca.2023.342157.

(71) West, C.; Lesellier, E. Effects of Mobile Phase Composition on Retention and Selectivity in Achiral Supercritical Fluid Chromatography. *J Chromatogr A* **2013**, *1302*, 152–162. https://doi.org/10.1016/j.chroma.2013.06.003.

(72) Brunelli, C.; Zhao, Y.; Brown, M. H.; Sandra, P. Pharmaceutical Analysis by Supercritical Fluid Chromatography: Optimization of the Mobile Phase Composition on a 2-Ethylpyridine Column. *J Sep Sci* **2008**, *31* (8), 1299–1306. https://doi.org/10.1002/jssc.200700555.

(73) den Uijl, M. J.; Schoenmakers, P. J.; Pirok, B. W. J.; van Bommel, M. R. Recent Applications of Retention Modelling in Liquid Chromatography. *J Sep Sci* **2021**, *44* (1), 88–114. https://doi.org/10.1002/jssc.202000905.

(74) Jayaraman, K.; Alexander, A. J.; Hu, Y.; Tomasella, F. P. A Stepwise Strategy Employing Automated Screening and DryLab Modeling for the Development of Robust Methods for Challenging High Performance Liquid Chromatography Separations: A Case Study. *Anal Chim Acta* **2011**, *696* (1–2), 116–124. https://doi.org/10.1016/j.aca.2011.04.010.

(75) Ovchinnikov, D. V.; Ul'yanovskii, N. V.; Kosyakov, D. S.; Pokrovskiy, O. I. Some Aspects of Additives Effects on Retention in Supercritical Fluid Chromatography Studied by Linear Free Energy Relationships Method. *J Chromatogr A* **2022**, *1665*, 33–35. https://doi.org/10.1016/j.chroma.2022.462820.

(76) Glenne, E.; Samuelsson, J.; Leek, H.; Forssén, P.; Klarqvist, M.; Fornstedt, T. Systematic Investigations of Peak Distortions Due to Additives in Supercritical Fluid Chromatography. *J Chromatogr A* **2020**, *1621*. https://doi.org/10.1016/j.chroma.2020.461048.

(77) Chollet, C.; Boutet-Mercey, S.; Laboureur, L.; Rincon, C.; Méjean, M.; Jouhet, J.; Fenaille, F.; Colsch, B.; Touboul, D. Supercritical Fluid Chromatography Coupled to Mass Spectrometry for Lipidomics. *Journal of Mass Spectrometry* **2019**, *54* (10), 791–801. https://doi.org/10.1002/jms.4445.

(78) Soukup, J.; Jandera, P. Adsorption of Water from Aqueous Acetonitrile on Silica-Based Stationary Phases in Aqueous Normal-Phase Liquid Chromatography. *J Chromatogr A* **2014**, *1374*, 102–111. https://doi.org/10.1016/j.chroma.2014.11.028.

(79) Brunelli, C.; Zhao, Y.; Brown, M. H.; Sandra, P. Development of a Supercritical Fluid Chromatography High-Resolution Separation Method Suitable for Pharmaceuticals Using Cyanopropyl Silica. *J Chromatogr A* **2008**, *1185* (2), 263–272. https://doi.org/10.1016/j.chroma.2008.01.050.

(80) Khalikova, M. A.; Šatínský, D.; Solich, P.; Nováková, L. Development and Validation of Ultra-High Performance Supercritical Fluid Chromatography Method for Determination of Illegal Dyes and Comparison to Ultra-High Performance Liquid Chromatography Method. *Anal Chim Acta* **2015**, *874*, 84–96. https://doi.org/10.1016/j.aca.2015.03.003.

(81) Perfetti, T. A.; Ashraf-Khorassani, M.; Coleman, W. M.; Dube, M. F. Qualitative and Quantitative Analyses of the Enantiomers of Nicotine and Related Alkaloids Employing Chiral Supercritical Fluid Chromatography in Commercial Nicotine Samples and in E-Cigarette Products. *Contributions to Tobacco & Nicotine Research* **2023**, *32* (3), 77–89. https://doi.org/10.2478/cttr-2023-0010.

(82) Dai, J.; Zhang, Y.; Wang-Iverson, D. B.; Tymiak, A. A. Supercritical Fluid Chromatography. In *ADME-Enabling Technologies in Drug Design and Development*; Zhang, D., Surapaneni, S., Eds.; John Wiley & Sons, Ltd, 2016; pp 363–376. https://doi.org/10.1201/9780203494240-17.

(83) Toribio, L.; Bernal, J.; Martín, M. T.; Ares, A. M. Supercritical Fluid Chromatography Coupled to Mass Spectrometry: A Valuable Tool in Food Analysis. *TrAC - Trends in Analytical Chemistry* **2021**, *143*. https://doi.org/10.1016/j.trac.2021.116350.

(84) Andrianova, A. A.; DiProspero, T.; Geib, C.; Smoliakova, I. P.; Kozliak, E. I.; Kubátová, A. Electrospray Ionization with High-Resolution Mass Spectrometry as a Tool for Lignomics: Lignin Mass Spectrum Deconvolution. *J Am Soc Mass Spectrom* **2018**, *29* (5), 1044–1059. https://doi.org/10.1007/s13361-018-1916-z.

(85) Brunelli, C.; Górecki, T.; Zhao, Y.; Sandra, P. Corona-Charged Aerosol Detection in Supercritical Fluid Chromatography for Pharmaceutical Analysis. *Anal Chem* **2007**, *79* (6), 2472–2482. https://doi.org/10.1021/ac061854q.

(86) Scholz, K.; Lipphardt, A.; Wienken, C. M.; Tiso, T.; Hayen, H. Hyphenation of Supercritical Fluid Chromatography with Different Detection Methods for Identification and Quantification of Liamocin Biosurfactants. *J Chromatogr A* **2020**, *1631*, 461584. https://doi.org/10.1016/j.chroma.2020.461584.

(87) Takeda, H.; Takahashi, M.; Hara, T.; Izumi, Y.; Bamba, T. Improved Quantitation of Lipid Classes Using Supercritical Fluid Chromatography with a Charged Aerosol Detector. *J Lipid Res* **2019**, *60* (8), 1465–1474. https://doi.org/10.1194/jlr.D094516.

(88) Vaccher, C.; Decaudin, B.; Sautou, V.; Lecoeur, M. Analysis of Non-Phthalates Plasticizers on Porous Graphitic Carbon by Supercritical Fluid Chromatography Using Evaporative Light Scattering Detection. *J Chromatogr A* **2014**, *1359*, 277–286. https://doi.org/10.1016/j.chroma.2014.07.036.

(89) Vehovec, T.; Obreza, A. Review of Operating Principle and Applications of the Charged Aerosol Detector. *J Chromatogr A* **2010**, *1217* (10), 1549–1556. https://doi.org/10.1016/j.chroma.2010.01.007.

(90) Mourey, T. H.; Oppenheimer, L. E. Principles of Operation of an Evaporative Light-Scattering Detector for Liquid Chromatography. *Anal Chem* **1984**, *56* (13), 2427–2434. https://doi.org/10.1021/ac00277a039.

(91) Robinson, M. W.; Hill, A. P.; Readshaw, S. A.; Hollerton, J. C.; Upton, R. J.; Lynn, S. M.; Besley, S. C.; Boughtflower, B. J. Use of Calculated Physicochemical Properties to Enhance Quantitative Response When Using Charged Aerosol Detection. *Anal Chem* **2017**, *89* (3), 1772–1777. https://doi.org/10.1021/acs.analchem.6b04060.

(92) Wahab, M. F.; Gritti, F.; O'Haver, T. C.; Hellinghausen, G.; Armstrong, D. W. Power Law Approach as a Convenient Protocol for Improving Peak Shapes and Recovering Areas from Partially Resolved Peaks. *Chromatographia* **2019**, *82* (1), 211–220. https://doi.org/10.1007/s10337-018-3607-0.

(93) Wahab, M. F.; O'Haver, T. C.; Gritti, F.; Hellinghausen, G.; Armstrong, D. W. Increasing Chromatographic Resolution of Analytical Signals Using Derivative Enhancement Approach. *Talanta* **2019**, *192* (2018), 492–499. https://doi.org/10.1016/j.talanta.2018.09.048.

(94) Hellinghausen, G.; Wahab, M. F.; Armstrong, D. W. Improving Peak Capacities over 100 in Less than 60 Seconds: Operating above Normal Peak Capacity Limits with Signal Processing. *Anal Bioanal Chem* **2020**, *412* (8), 1925–1932. https://doi.org/10.1007/s00216-020-02444-8.

(95) Andrianova, A. A.; Yeudakimenka, N. A.; Lilak, S. L.; Kozliak, E. I.; Ugrinov, A.; Sibi, M. P.; Kubátová, A. Size Exclusion Chromatography of Lignin: The Mechanistic Aspects and Elimination of Undesired Secondary Interactions. *J Chromatogr A* **2018**, *1534*, 101–110. https://doi.org/10.1016/j.chroma.2017.12.051.

(96) Lubomirsky, E.; Khodabandeh, A.; Preis, J.; Susewind, M.; Hofe, T.; Hilder, E. F.; Arrua, R. D. Polymeric Stationary Phases for Size Exclusion Chromatography: A Review. *Anal Chim Acta* **2021**, *1151*, 338244. https://doi.org/10.1016/j.aca.2021.338244.

(97) Striegel, A. M. Viscometric Detection in Size-Exclusion Chromatography: Principles and Select Applications. *Chromatographia* **2016**, *79* (15–16), 945–960. https://doi.org/10.1007/s10337-016-3078-0.

(98) Liu, Y.; Bo, S. Hydrodynamic Radius Characterization of a Vinyl-Type Polynorbornene by Size-Exclusion Chromatography with a Static and Dynamic Laser Light Scattering Detector. *Chromatographia* **2004**, *59* (5–6), 299–303. https://doi.org/10.1365/s10337-003-0160-1.

(99) Waldeck, A. R.; Kuchel, P. W.; Lennon, A. J.; Chapman, B. E. NMR Diffusion Measurements to Characterise Membrane Transport and Solute Binding. *Prog Nucl Magn Reson Spectrosc* **1997**, *30*, 39–68.

(100) Zhang, X.; Zhang, J.; Yang, H.; He, C.; Ke, Y.; Singh, S.; Cheng, G. Determination of the Structures of Lignin Subunits and Nanoparticles in Solution by Small-Angle Neutron Scattering: Towards Improving Lignin Valorization. *ChemSusChem* **2022**, *15* (19). https://doi.org/10.1002/cssc.202201230.

(101) Zhao, W.; Xiao, L. P.; Song, G.; Sun, R. C.; He, L.; Singh, S.; Simmons, B. A.; Cheng, G. From Lignin Subunits to Aggregates: Insights into Lignin Solubilization. *Green Chemistry* **2017**, *19* (14), 3272–3281. https://doi.org/10.1039/c7gc00944e.

(102) Vainio, U.; Maximova, N.; Hortling, B.; Laine, J.; Stenius, P.; Simola, L. K.; Gravitis, J.; Serimaa, R. Morphology of Dry Lignins and Size and Shape of Dissolved Kraft Lignin Particles by X-Ray Scattering. *Langmuir* **2004**, *20* (22), 9736–9744. https://doi.org/10.1021/la048407v.

(103) Chakraborty, S.; Ding, J.; Thies, M. C.; Kitchens, C. L. More Elongated Solution Structures in Lignin Precursors Improve Properties of Resultant Carbon Fibers. *ACS Appl Polym Mater* **2019**, *1* (10), 2561–2565. https://doi.org/10.1021/acsapm.9b00530.

(104) Cheng, G.; Kent, M. S.; He, L.; Varanasi, P.; Dibble, D.; Arora, R.; Deng, K.; Hong, K.; Melnichenko, Y. B.; Simmons, B. A.; Singh, S. Effect of Ionic Liquid Treatment on the Structures of Lignins in Solutions: Molecular Subunits Released from Lignin. *Langmuir* **2012**, *28* (32), 11850–11857. https://doi.org/10.1021/la300938b.

(105) Cathala, B.; Saake, B.; Faix, O.; Monties, B. Association Behaviour of Lignins and Lignin Model Compounds Studied by Multidetector Size-Exclusion Chromatography. *J Chromatogr A* **2003**, *1020* (2), 229–239. https://doi.org/10.1016/j.chroma.2003.08.046.

(106) Contreras, S.; Gaspar, A. R.; Guerra, A.; Lucia, L. A.; Argyropoulos, D. S. Propensity of Lignin to Associate: Light Scattering Photometry Study with Native Lignins. *Biomacromolecules* **2008**, *9* (12), 3362–3369. https://doi.org/10.1021/bm800673a.

(107) Guerra, A.; Gaspar, A. R.; Contreras, S.; Lucia, L. A.; Crestini, C.; Argyropoulos, D. S. On the Propensity of Lignin to Associate: A Size Exclusion Chromatography Study with Lignin Derivatives Isolated from Different Plant Species. *Phytochemistry* **2007**, *68* (20), 2570–2583. https://doi.org/10.1016/j.phytochem.2007.05.026.

(108) Deng, Y.; Feng, X.; Zhou, M.; Qian, Y.; Yu, H.; Qiu, X. Investigation of Aggregation and Assembly of Alkali Lignin Using Iodine as a Probe. *Biomacromolecules* **2011**, *12* (4), 1116–1125. https://doi.org/10.1021/bm101449b.

(109) Lindström, T. The Colloidal Behaviour of Kraft Lignin Part I.: Association and Gelation of Kraft Lignin in Aqueous Solutions. *Colloid Polym Sci* **1979**, *257*, 277–285.

(110) Lee, J. H.; Kim, T. M.; Choi, I. G.; Choi, J. W. Phenolic Hydroxyl Groups in the Lignin Polymer Affect the Formation of Lignin Nanoparticles. *Nanomaterials* **2021**, *11* (7). https://doi.org/10.3390/nano11071790.

(111) Bylin, S.; Wells, T.; Sun, Q.; Ragauskas, A.; Theliander, H. Lignin Structure and Aggregation Behavior in a Two-Component Ionic Liquid Solvent System. *Bioresources* **2014**, *9* (4), 6002–6018.

(112) Ringena, O.; Lebioda, S.; Lehnen, R.; Saake, B. Size-Exclusion Chromatography of Technical Lignins in Dimethyl Sulfoxide/Water and Dimethylacetamide. *J Chromatogr A* **2006**, *1102* (1–2), 154–163. https://doi.org/10.1016/j.chroma.2005.10.037.

(113) Asikkala, J.; Tamminen, T.; Argyropoulos, D. S. Accurate and Reproducible Determination of Lignin Molar Mass by Acetobromination. *J Agric Food Chem* **2012**, *60* (36), 8968–8973. https://doi.org/10.1021/jf303003d.

(114) Sameni, J.; Krigstin, S.; Sain, M. Solubility of Lignin and Acetylated Lignin in Organic Solvents. *Bioresources* **2017**, *12* (1), 1548–1565. https://doi.org/10.15376/biores.12.1.1548-1565.

(115) Xue, Z.; Zhao, X.; Sun, R. C.; Mu, T. Biomass-Derived γ-Valerolactone-Based Solvent Systems for Highly Efficient Dissolution of Various Lignins: Dissolution Behavior and Mechanism Study. *ACS Sustain Chem Eng* **2016**, *4* (7), 3864–3870. https://doi.org/10.1021/acssuschemeng.6b00639.

(116) Sun, J.; Dutta, T.; Parthasarathi, R.; Kim, K. H.; Tolic, N.; Chu, R. K.; Isern, N. G.; Cort, J. R.; Simmons, B. A.; Singh, S. Rapid Room Temperature Solubilization and Depolymerization of Polymeric Lignin at High Loadings. *Green Chemistry* **2016**, *18* (22), 6012–6020. https://doi.org/10.1039/c6gc02258h.

(117) Evstigneev, E. I. Specific Features of Lignin Dissolution in Aqueous and Aqueous-Organic Media. *Russian Journal of Applied Chemistry* **2010**, *83* (3), 509–513. https://doi.org/10.1134/S1070427210030250.

(118) Boeriu, C. G.; Fiţigău, F. I.; Gosselink, R. J. A.; Frissen, A. E.; Stoutjesdijk, J.; Peter, F. Fractionation of Five Technical Lignins by Selective Extraction in Green Solvents and Characterisation of Isolated Fractions. *Ind Crops Prod* **2014**, *62*, 481–490. https://doi.org/10.1016/j.indcrop.2014.09.019.

(119) Duval, A.; Vilaplana, F.; Crestini, C.; Lawoko, M. Solvent Screening for the Fractionation of Industrial Kraft Lignin. *Holzforschung* **2016**, *70* (1), 11–20. https://doi.org/10.1515/hf-2014-0346.

(120) Mu, L.; Shi, Y.; Wang, H.; Zhu, J. Lignin in Ethylene Glycol and Poly(Ethylene Glycol): Fortified Lubricants with Internal Hydrogen Bonding. *ACS Sustain Chem Eng* **2016**, *4* (3), 1840–1849. https://doi.org/10.1021/acssuschemeng.6b00049.

(121) Wang, Y.; Wei, L.; Li, K.; Ma, Y.; Ma, N.; Ding, S.; Wang, L.; Zhao, D.; Yan, B.; Wan, W.; Zhang, Q.; Wang, X.; Wang, J.; Li, H. Lignin Dissolution in Dialkylimidazolium-Based Ionic Liquid-Water Mixtures. *Bioresour Technol* **2014**, *170*, 499–505. https://doi.org/10.1016/j.biortech.2014.08.020.

(122) Glas, D.; Van Doorslaer, C.; Depuydt, D.; Liebner, F.; Rosenau, T.; Binnemans, K.; De Vos, D. E. Lignin Solubility in Non-Imidazolium Ionic Liquids. *Journal of Chemical Technology and Biotechnology* **2015**, *90* (10), 1821–1826. https://doi.org/10.1002/jctb.4492.

(123) Pu, Y.; Jiang, N.; Ragauskas, A. J. Ionic Liquid as a Green Solvent for Lignin. *Journal of Wood Chemistry and Technology* **2007**, *27* (1), 23–33. https://doi.org/10.1080/02773810701282330.

(124) Malaeke, H.; Housaindokht, M. R.; Monhemi, H.; Izadyar, M. Deep Eutectic Solvent as an Efficient Molecular Liquid for Lignin Solubilization and Wood Delignification. *J Mol Liq* **2018**, *263*, 193–199. https://doi.org/10.1016/j.molliq.2018.05.001.

(125) Sosa, F. H. B.; Abranches, D. O.; Da Costa Lopes, A. M.; Coutinho, J. A. P.; Da Costa, M. C. Kraft Lignin Solubility and Its Chemical Modification in Deep Eutectic Solvents. *ACS Sustain Chem Eng* **2020**, *8* (50), 18577–18589. https://doi.org/10.1021/acssuschemeng.0c06655.

(126) Ma, Q.; Yu, C.; Zhou, Y.; Hu, D.; Chen, J.; Zhang, X. A Review on the Calculation and Application of Lignin Hansen Solubility Parameters. *Int J Biol Macromol* **2024**, *256*. https://doi.org/10.1016/j.ijbiomac.2023.128506.

(127) Gaugler, E. C.; Radke, W.; Vogt, A. P.; Smith, D. A. Molar Mass Determination of Lignins and Characterization of Their Polymeric Structures by Multi-Detector Gel Permeation Chromatography. *J Anal Sci Technol* **2021**, *12* (1). https://doi.org/10.1186/s40543-021-00283-5.

(128) Lange, H.; Rulli, F.; Crestini, C. Gel Permeation Chromatography in Determining Molecular Weights of Lignins: Critical Aspects Revisited for Improved Utility in the Development of Novel Materials. *ACS Sustain Chem Eng* **2016**, *4* (10), 5167–5180. https://doi.org/10.1021/acssuschemeng.6b00929.

(129) Gellerstedt, G. Gel Permation Chromatography. In *Methods in Lignin Chemistry*; Lin, S. Y., Dence, C. W., Eds.; Springer Berlin: Heidelberg, 1992; pp 487–497.

(130) Hu, Z.; Du, X.; Liu, J.; Chang, H. M.; Jameel, H. Structural Characterization of Pine Kraft Lignin: BioChoice Lignin vs Indulin AT. *Journal of Wood Chemistry and Technology* **2016**, *36* (6), 432–446. https://doi.org/10.1080/02773813.2016.1214732.

(131) Jacobs, A.; Dahlman, O. Absolute Molar Mass of Lignins by Size Exclusion Chromatography and MALDI-TOF Mass Spectroscopy. *Nord Pulp Paper Res J* **2000**, *15* (2), 120–127. https://doi.org/10.3183/npprj-2000-15-02-p120-127.

(132) Gosselink, R. J. A.; Abächerli, A.; Semke, H.; Malherbe, R.; Käuper, P.; Nadif, A.; Van Dam, J. E. G. Analytical Protocols for Characterisation of Sulphur-Free Lignin. *Ind Crops Prod* **2004**, *19* (3), 271–281. https://doi.org/10.1016/j.indcrop.2003.10.008.

(133) Önnerud, H.; Palmblad, M.; Gellerstedt, G. Investigation of Lignin Oligomers Using Electrospray Ionisation Mass Spectrometry. *Holzforschung* **2003**, *57* (1), 37–43. https://doi.org/10.1515/HF.2003.006.

(134) Norgren, M.; Lindström, B. Physico-Chemical Characterization of a Fractionated Kraft Lignin. *Holzforschung* **2000**, *54*, 528–534.

(135) Rönnols, J.; Jacobs, A.; Aldaeus, F. Consecutive Determination of Softwood Kraft Lignin Structure and Molar Mass from NMR Measurements. *Holzforschung* **2017**, *71*, 563–570. https://doi.org/10.1515/hf-2016-0182.

(136) Montgomery, J. R. D.; Bazley, P.; Lebl, T.; Westwood, N. J. Using Fractionation and Diffusion Ordered Spectroscopy to Study Lignin Molecular Weight. *ChemistryOpen* **2019**, *8* (5), 601–605. https://doi.org/10.1002/open.201900129.

(137) Duarte, R. M. B. O.; Brandão, P. F.; Duarte, A. C. Multidimensional Chromatography in Environmental Analysis: Comprehensive Two-Dimensional Liquid versus Gas Chromatography. *J Chromatogr A* **2023**, *1706* (March). https://doi.org/10.1016/j.chroma.2023.464288.

(138) Montero, L.; Herrero, M.; Ibáñez, E.; Cifuentes, A. Profiling of Phenolic Compounds from Different Apple Varieties Using Comprehensive Two-Dimensional Liquid Chromatography. *J Chromatogr A* **2013**, *1313*, 275–283. https://doi.org/10.1016/j.chroma.2013.06.015.

(139) François, I.; Sandra, P. Comprehensive Supercritical Fluid Chromatography × Reversed Phase Liquid Chromatography for the Analysis of the Fatty Acids in Fish Oil. *J Chromatogr A* **2009**, *1216* (18), 4005–4012. https://doi.org/10.1016/j.chroma.2009.02.078.

(140) Aly, A. A.; Górecki, T. Green Comprehensive Two-Dimensional Liquid Chromatography (LC × LC) for the Analysis of Phenolic Compounds in Grape Juices and Wine. *Anal Bioanal Chem* **2022**, *415*, 2383–2398. https://doi.org/10.1007/s00216-022-04241-x.

(141) Zhou, Y.; Zhang, H.; Wang, X.; Qi, D.; Gu, W.; Wu, D.; Liu, B. Development of a Heart-Cutting Supercritical Fluid Chromatography-High-Performance Liquid Chromatography Coupled to Tandem Mass Spectrometry for the Determination of Four Tobacco-Specific Nitrosamines in Mainstream Smoke. *Anal Bioanal Chem* **2019**, 2961–2969. https://doi.org/10.1007/s00216-019-01746-w.

(142) Dolan, J. W.; Snyder, L. R.; Djordjevic, N. M.; Hill, D. W.; Waeghe, T. J. Reversed-Phase Liquid Chromatographic Separation of Complex Samples by Optimizing Temperature and Gradient TimeI. Peak Capacity Limitations. *J Chromatogr A* **1999**, *857*, 1–20. https://doi.org/10.1016/S0021-9673(99)00765-7.

(143) Giddings, J. C. Two-Dimensional Separations: Concept and Promise. *Anal Chem* **1984**, *56* (12), 1258–1270.

(144) Davis, J. M.; Stoll, D. R.; Carr, P. W. Dependence of Effective Peak Capacity in Comprehensive Two-Dimensional Separations on the Distribution of Peak Capacity between the Two Dimensions. *Anal Chem* **2008**, *80* (21), 8122–8134. https://doi.org/10.1021/ac800933z.

(145) Davis, J. M.; Glddings, J. C. *Statistical Theory of Component Overlap in Multicomponent Chromatograms*; 1983; Vol. 55. https://pubs.acs.org/sharingguidelines.

(146) Si, W.; Liu, Y.; Xiao, Y.; Guo, Z.; Jin, G.; Yan, J.; Shen, A.; Zhou, H.; Yang, F.; Liang, X. An Offline Two-Dimensional Supercritical Fluid Chromatography × Reversed Phase Liquid Chromatography Tandem Quadrupole Time-of-Flight Mass Spectrometry System for Comprehensive Gangliosides Profiling in Swine Brain Extract. *Talanta* **2020**, *208*. https://doi.org/10.1016/j.talanta.2019.120366.

(147) Li, Y.; Sun, S. W.; Zhang, X. Y.; Liu, Y.; Liu, X. H.; Zhang, S.; Wang, W.; Wang, J.; Wang, W. Separation of New Coumarin Glycosides from Toddalia Asiatica Using Offline Two-Dimensional High-Performance Liquid Chromatography. *Plants* **2020**, *9* (4). https://doi.org/10.3390/plants9040428.

(148) Wang, W.; Zhu, Y.; Jiang, L.; Mei, L.; Tao, Y.; Liu, Z. Enrichment and Separation of High-Polar Compounds from Saussurea Obvallata Using Solid-Phase Extraction Combining with Offline Two-Dimensional Liquid Chromatography. *J Sep Sci* **2021**, *44* (21), 3967–3975. https://doi.org/10.1002/jssc.202100546.

(149) Stoll, D. R.; Carr, P. W. *Two-Dimensional Liquid Chromatography: Principles, Practical Implementation and Applications*; 2015.

(150) Stoll, D. R.; Carr, P. W. Two-Dimensional Liquid Chromatography: A State of the Art Tutorial. *Anal Chem* **2017**, *89* (1), 519–531. https://doi.org/10.1021/acs.analchem.6b03506.

(151) Bedani, F.; Schoenmakers, P. J.; Janssen, H. G. Theories to Support Method Development in Comprehensive Two-Dimensional Liquid Chromatography - A Review. *J Sep Sci* **2012**, *35* (14), 1697–1711. https://doi.org/10.1002/jssc.201200070.

(152) Stoll, D. R.; Shoykhet, K.; Petersson, P.; Buckenmaier, S. Active Solvent Modulation: A Valve-Based Approach to Improve Separation Compatibility in Two-Dimensional Liquid Chromatography. *Anal Chem* **2017**, *89* (17), 9260–9267. https://doi.org/10.1021/acs.analchem.7b02046.

(153) Sarrut, M.; D'Attoma, A.; Heinisch, S. Optimization of Conditions in On-Line Comprehensive Two-Dimensional Reversed Phase Liquid Chromatography: Experimental Comparison with One-Dimensional Reversed Phase Liquid Chromatography for the Separation of Peptides. *J Chromatogr A* **2015**, *1421*, 48–59. https://doi.org/10.1016/j.chroma.2015.08.052.

(154) Pirok, B. W. J.; Knip, J.; van Bommel, M. R.; Schoenmakers, P. J. Characterization of Synthetic Dyes by Comprehensive Two-Dimensional Liquid Chromatography Combining Ion-Exchange Chromatography and Fast Ion-Pair Reversed-Phase Chromatography. *J Chromatogr A* **2016**, *1436*, 141–146. https://doi.org/10.1016/j.chroma.2016.01.070.

(155) Brudin, S. S.; Shellie, R. A.; Haddad, P. R.; Schoenmakers, P. J. Comprehensive Two-Dimensional Liquid Chromatography: Ion Chromatography×reversed-Phase Liquid Chromatography for Separation of Low-Molar-Mass Organic Acids. *J Chromatogr A* **2010**, *1217* (43), 6742–6746. https://doi.org/10.1016/j.chroma.2010.05.064.

(156) Ding, K.; Xu, Y.; Wang, H.; Duan, C.; Guan, Y. A Vacuum Assisted Dynamic Evaporation Interface for Two-Dimensional Normal Phase/Reverse Phase Liquid Chromatography. *J Chromatogr A* **2010**, *1217* (34), 5477–5483. https://doi.org/10.1016/j.chroma.2010.06.053.

(157) Lazaridi, E.; Janssen, H. G.; Vincken, J. P.; Pirok, B.; Hennebelle, M. A Comprehensive Two-Dimensional Liquid Chromatography Method for the Simultaneous Separation of Lipid Species and Their Oxidation Products. *J Chromatogr A* **2021**, *1644*. https://doi.org/10.1016/j.chroma.2021.462106.

(158) Tian, H.; Xu, J.; Xu, Y.; Guan, Y. Multidimensional Liquid Chromatography System with an Innovative Solvent Evaporation Interface. *J Chromatogr A* **2006**, *1137* (1), 42–48. https://doi.org/10.1016/j.chroma.2006.10.005.

(159) Yang, L.; Nie, H.; Zhao, F.; Song, S.; Meng, Y.; Bai, Y.; Liu, H. A Novel Online Two-Dimensional Supercritical Fluid Chromatography/Reversed Phase Liquid Chromatography–Mass Spectrometry Method for Lipid Profiling. *Anal Bioanal Chem* **2020**, *412* (10), 2225–2235. https://doi.org/10.1007/s00216-019-02242-x.

(160) Pepaj, M.; Wilson, S. R.; Novotna, K.; Lundanes, E.; Greibrokk, T. Two-Dimensional Capillary Liquid Chromatography: PH Gradient Ion Exchange and Reversed Phase Chromatography for Rapid Separation of Proteins. *J Chromatogr A* **2006**, *1120* (1–2), 132–141. https://doi.org/10.1016/j.chroma.2006.02.031.

(161) Li, Y.; Gu, C.; Gruenhagen, J.; Zhang, K.; Yehl, P.; Chetwyn, N. P.; Medley, C. D. A Size Exclusion-Reversed Phase Two Dimensional-Liquid Chromatography Methodology for Stability and Small Molecule Related Species in Antibody Drug Conjugates. *J Chromatogr A* **2015**, *1393*, 81–88. https://doi.org/10.1016/j.chroma.2015.03.027.

(162) Pretorius, N. O.; Willemse, C. M.; de Villiers, A.; Pasch, H. Combined Size Exclusion Chromatography, Supercritical Fluid Chromatography and Electrospray Ionization Mass Spectrometry for the Analysis of Complex Aliphatic Polyesters. *J Chromatogr A* **2014**, *1330*, 74–81. https://doi.org/10.1016/j.chroma.2014.01.018.

(163) Dubuis, A.; Le Masle, A.; Chahen, L.; Destandau, E.; Charon, N. Off-Line Comprehensive Size Exclusion Chromatography × reversed-Phase Liquid Chromatography Coupled to High Resolution Mass Spectrometry for the Analysis of Lignocellulosic Biomass Products. *J Chromatogr A* **2020**, *1609*. https://doi.org/10.1016/j.chroma.2019.460505.

(164) De Saegher, T.; Vercammen, J.; Atanasova, B.; Van Geem, K. M.; De Clercq, J.; Verberckmoes, A.; Lauwaert, J. Efficient Mapping of Lignin Depolymerization Product Pools and Quantification of Specific Monomers through Rapid GPC-HPLC-UV/VIS Analysis. *Anal Chim Acta* **2023**, *1278* (August), 341738. https://doi.org/10.1016/j.aca.2023.341738.

(165) De Saegher, T.; Lauwaert, J.; Vercammen, J.; Van Geem, K. M.; De Clercq, J.; Verberckmoes, A. Fast Screening of Depolymerized Lignin Samples Through 2D-Liquid Chromatography Mapping. *ChemistryOpen* **2021**, *10* (8), 740–747. https://doi.org/10.1002/open.202100088.

(166) Mommers, J.; van der Wal, S. Two Metrics for Measuring Orthogonality for Two-Dimensional Chromatography. *J Chromatogr A* **2019**, *1586*, 101–105. https://doi.org/10.1016/j.chroma.2018.11.081.

(167) Grübner, M.; Dunkel, A.; Steiner, F.; Hofmann, T. Systematic Evaluation of Liquid Chromatography (LC) Column Combinations for Application in Two-Dimensional LC Metabolomic Studies. *Anal Chem* **2021**, *93* (37), 12565–12573. https://doi.org/10.1021/acs.analchem.1c01857.

(168) Groeneveld, G.; Dunkle, M. N.; Pursch, M.; Mes, E. P. C.; Schoenmakers, P. J.; Gargano, A. F. G. Investigation of the Effects of Solvent-Mismatch and Immiscibility in Normal-Phase × Aqueous Reversed-Phase Liquid Chromatography. *J Chromatogr A* **2022**, *1665*. https://doi.org/10.1016/j.chroma.2022.462818.

(169) Montero, L.; Ayala-Cabrera, J. F.; Bristy, F. F.; Schmitz, O. J. Multi-2D LC × LC as a Novel and Powerful Implement for the Maximum Separation of Complex Samples. *Anal Chem* **2023**, *95* (6), 3398–3405. https://doi.org/10.1021/acs.analchem.2c04870.

(170) Van Der Horst, A.; Schoenmakers, P. J. Comprehensive Two-Dimensional Liquid Chromatography of Polymers. *J Chromatogr A* **2003**, *1000*, 693–709. https://doi.org/10.1016/S0021-9673(03)00495-3.

(171) Pursch, M.; Buckenmaier, S. Loop-Based Multiple Heart-Cutting Two-Dimensional Liquid Chromatography for Target Analysis in Complex Matrices. *Anal Chem* **2015**, *87* (10), 5310–5317. https://doi.org/10.1021/acs.analchem.5b00492.

(172) Chapel, S.; Heinisch, S. Strategies to Circumvent the Solvent Strength Mismatch Problem in Online Comprehensive Two-Dimensional Liquid Chromatography. *J Sep Sci* **2022**, *45* (1), 7–26. https://doi.org/10.1002/jssc.202100534.

(173) Stoll, D. R.; Sajulga, R. W.; Voigt, B. N.; Larson, E. J.; Jeong, L. N.; Rutan, S. C. Simulation of Elution Profiles in Liquid Chromatography − II: Investigation of Injection Volume Overload under Gradient Elution Conditions Applied to Second Dimension Separations in Two-Dimensional Liquid Chromatography. *J Chromatogr A* **2017**, *1523*, 162–172. https://doi.org/10.1016/j.chroma.2017.07.041.

(174) Vonk, R. J.; Gargano, A. F. G.; Davydova, E.; Dekker, H. L.; Eeltink, S.; De Koning, L. J.; Schoenmakers, P. J. Comprehensive Two-Dimensional Liquid Chromatography with Stationary-Phase-Assisted Modulation Coupled to High-Resolution Mass Spectrometry Applied to Proteome Analysis of Saccharomyces Cerevisiae. *Anal Chem* **2015**, *87* (10), 5387–5394. https://doi.org/10.1021/acs.analchem.5b00708.

(175) den Uijl, M. J.; Roeland, T.; Bos, T. S.; Schoenmakers, P. J.; van Bommel, M. R.; Pirok, B. W. J. Assessing the Feasibility of Stationary-Phase-Assisted Modulation for Two-Dimensional Liquid-Chromatography Separations. *J Chromatogr A* **2022**, *1679*. https://doi.org/10.1016/j.chroma.2022.463388.

(176) Cacciola, F.; Jandera, P.; Hajdú, Z.; Česla, P.; Mondello, L. Comprehensive Two-Dimensional Liquid Chromatography with Parallel Gradients for Separation of Phenolic and Flavone Antioxidants. *J Chromatogr A* **2007**, *1149* (1), 73–87. https://doi.org/10.1016/j.chroma.2007.01.119.

(177) Sweeney, A. P.; Shalliker, R. A. Development of a Two-Dimensional Liquid Chromatography System with Trapping and Sample Enrichment Capabilities. *J Chromatogr A* **2002**, *968*, 41–52. https://doi.org/https://doi.org/10.1016/S0021-9673(02)00782-3.

(178) Niezen, L. E.; Staal, B. B. P.; Lang, C.; Pirok, B. W. J.; Schoenmakers, P. J. Thermal Modulation to Enhance Two-Dimensional Liquid Chromatography Separations of Polymers. *J Chromatogr A* **2021**, *1653*. https://doi.org/10.1016/j.chroma.2021.462429.

(179) Creese, M. E.; Creese, M. J.; Foley, J. P.; Cortes, H. J.; Hilder, E. F.; Shellie, R. A.; Breadmore, M. C. Longitudinal On-Column Thermal Modulation for Comprehensive Two-Dimensional Liquid Chromatography. *Anal Chem* **2017**, *89* (2), 1123–1130. https://doi.org/10.1021/acs.analchem.6b03279.

(180) Stevenson, P. G.; Bassanese, D. N.; Conlan, X. A.; Barnett, N. W. Improving Peak Shapes with Counter Gradients in Two-Dimensional High Performance Liquid Chromatography. *J Chromatogr A* **2014**, *1337*, 147–154. https://doi.org/10.1016/j.chroma.2014.02.051.

(181) Stephan, S.; Jakob, C.; Hippler, J.; Schmitz, O. J. A Novel Four-Dimensional Analytical Approach for Analysis of Complex Samples. *Anal Bioanal Chem* **2016**, *408* (14), 3751–3759. https://doi.org/10.1007/s00216-016-9460-9.

(182) Stoll, D. R.; Harmes, D. C.; Staples, G. O.; Potter, O. G.; Dammann, C. T.; Guillarme, D.; Beck, A. Development of Comprehensive Online Two-Dimensional Liquid Chromatography/Mass Spectrometry Using Hydrophilic Interaction and Reversed-Phase Separations for Rapid and Deep Profiling of Therapeutic Antibodies. *Anal Chem* **2018**, *90* (9), 5923–5929. https://doi.org/10.1021/acs.analchem.8b00776.

(183) Pardon, M.; Chapel, S.; de Witte, P.; Cabooter, D. Optimizing Transfer and Dilution Processes When Using Active Solvent Modulation in On-Line Two-Dimensional Liquid Chromatography. *Anal Chim Acta* **2023**, *1252*. https://doi.org/10.1016/j.aca.2023.341040.

Printed by Media-Tryck, Lund 2024 NORDIC SWAN ECOLABEL 3041 0903

ISBN 978-91-8096-044-1

Centre for Analysis and Synthesis
Department of Chemistry
Faculty of Science
Lund University