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# CONTRIBUTIONS TO METHOD DEVELOPMENT IN CAPILLARY ELECTROPHORESIS AND SUPERCRITICAL FLUID CHROMATOGRAPHY FOR THE ANALYSIS OF PHARMACEUTICAL COMPOUNDS

Thesis submitted in fulfilment of the requirements for PhD studies with joint supervision and awarded degrees of Doctor of Pharmaceutical Analysis (Charles University) and of Doctor of Pharmaceutical Sciences (Vrije Universiteit Brussel).

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2023-2024

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# ACKNOWLEDGEMENTS

I would like to thank my supervisors doc. RNDr. Miroslav Polášek, CSc., Prof. dr. Debby Mangelings, Prof. dr. Yvan Vander Heyden and consultant PharmDr. Pavel Jáč, Ph.D for the chance to enrol into the PhD programs and for their valuable advice, guidance and support throughout the duration of my studies.

I would like to acknowledge prof. RNDr. Petr Solich, Csc. for the opportunity to travel abroad for internships and for a chance to present my results on local and international conferences.

I would like to thank my colleagues at the Department of Analytical Chemistry at CU and at the Department of Analytical Chemistry, Applied Chemometrics and Molecular Modelling at VUB for their friendship, help, collaboration, and enjoyable working environment.

I would like to acknowledge the following funds and grants for financial support:

- Specific research projects SVV 260 548, SVV 260 412, and SVV 260 662
- Erasmus + program
- Mobility Fund of the Charles University

## STATEMENT OF ORIGINALITY

I declare that this thesis is my original work, which I developed independently under the supervision of my supervisors and consultant. All literature and other sources, used during the work processing, are listed in the list of references, and cited properly in the thesis. This work has not been used to obtain another or the same title.

In Nijmegen to the date of 20.3.2024 Petra Riasová

# ABSTRACT

The goal of this thesis is to contribute to the field of separation science by investigating various approaches of method development for the separation of structurally similar pharmaceutical compounds, using capillary electrophoresis (CE) and supercritical fluid chromatography (SFC) as separation techniques. The theoretical part outlines the main principles and aspects of method development in CE and SFC and introduces the relevant analytes studied throughout the thesis. The experimental part consists of four publications and brief introductions to them.

The first publication describes the development of a micellar electrokinetic chromatography method for the separation of indomethacin and its three impurities and includes method validation and application. The method was developed using a multivariate approach, where a quarter-fraction factorial design was used for screening and a face-centred central composite design for optimization. Baseline separation of indomethacin and its three impurities was achieved within 10 min. The method was validated in terms of linearity, precision, and accuracy, and applied to pharmaceutical samples.

The second work focused on the optimization, validation, and subsequent application of a CE method for the determination of the main components of silymarin. In contrast to previous work, univariate optimization was used. The first approach focused on the optimization of a cyclodextrin-modified micellar electrokinetic chromatography method. However, because of the poor repeatability of migration times, the optimization continued with an electrokinetic chromatography method, with heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin as additive in the background electrolyte. Baseline separation was achieved for all analytes, including the diastereomers of silybin and isosilybin. The electrokinetic chromatography method was validated for linearity, precision, and accuracy, and applied to dietary supplements containing Milk Thistle extract.

The third publication describes the evaluation and improvement of retention prediction of analytes on coupled column systems in SFC. A test set consisting of structurally similar compounds, including chiral compounds, diastereomers, and structural isomers, was analysed on a selection of five chiral and four achiral columns. Analyses were performed both on individual and coupled columns. First, an equation providing the best correlation between predicted and experimental retention factors was selected. Secondly, the prediction precision was improved by adjusting the flow rate and backpressure.

The best performing strategy was applied in the fourth paper to select columns for the separation of silymarin flavonolignans by means of SFC. The method was initially optimized in terms of the selection of an organic modifier. In a following step, the content of organic modifier, flow rate, additives concentration, backpressure, and column temperature were varied using a quarter-fraction factorial design. Further optimized parameters were sample solvent and chemistry of mobile phase additives. However, despite the extensive optimization, a baseline separation of all analytes could not be achieved.

This work demonstrates various methods and approaches for the separation of structurally similar compounds. Both multivariate and univariate optimization were successfully used to achieve baseline separation for the developed CE methods. However, even after optimizing

the coupled column selection and applying multivariate optimization, the SFC separation of silymarin components was not achieved.

# ABSTRAKT

Cieľom predloženej dizertačnej práce je skúmanie nových spôsobov vývoja separačných metód pre analýzu štruktúrne podobných farmaceutických látok. Ako analytické separačné metódy boli použité kapilárna elektroforéza (CE) a superkritická fluidná chromatografia (SFC). Teoretická časť tejto dizertačnej práce popisuje hlavné princípy a aspekty vývoja CE a SFC metód a uvádza hlavné študované analyty. V experimentálnej časti je prezentovaný komentovaný súbor štyroch publikácií zaoberajúcich sa praktickou aplikáciou týchto separačných metód vo farmaceutickej analýze.

Prvá publikácia sa zaoberá vývojom, validáciou a aplikáciou CE metódy na separáciu indometacínu a jeho troch nečistôt. Metóda bola vyvinutá za použitia multivariantnej optimalizácie. Frakčný faktoriálny design bol použitý na screening, a centrálne kompozitný design na optimalizáciu metódy. Úplná separácia indometacínu a jeho troch nečistôt bola dosiahnutá v rámci 10 minút za použitia micelárnej elektrokinetickej chromatografie. V rámci validácie bola hodnotená linearita, presnosť a správnosť metódy a následne bola metóda aplikovaná na analýzu farmaceutických prípravkov.

Druhá práca je zameraná na optimalizáciu, validáciu, a aplikáciu CE metódy pre stanovenie hlavných komponentov silymarínu. Na vývoj tejto metódy bola použitá univariantná optimalizácia. Prvý prístup vo vývoji bol zameraný na optimalizáciu cyklodextrínom modifikovanej micelárnej elektrokinetickej chromatografickej metódy. V dôsledku zlej opakovateľnosti migračných časov, optimalizácia metódy pokračovala použitím elektrokinetickej chromatografie s chirálnym selektorom heptakis(2,3,6-tri-O-methyl)-β-cyklodextrínom. Použitím tejto metódy bola dosiahnutá úplná separácia všetkých analytov, vrátane diastereomérov silybínu a isolsilybínu. Počas validácie bola overená linearita, presnosť a správnosť elektrokinetickej chromatografickej metódy, a metóda bola aplikovaná na doplnky stravy s obsahom pestreca mariánskeho.

Tretia publikácia opisuje evaluáciu a zlepšenie predpovedaného retenčného správania analytov v systéme spojených kolón v SFC. Chirálne analyty, diastereoméry a štruktúrne izoméry boli testované na vybraných piatich chirálnych a štyroch achirálnych kolónach, zapojených jednotlivo a v sériovo spojenom systéme. V prvom kroku bola vybratá rovnica, ktorá poskytovala najlepšiu koreláciu medzi predikovanými a experimentálnymi retenčnými faktormi. Predpovedaná presnosť bola zlepšená prispôsobením rýchlosti toku mobilnej fázy a spätného tlaku.

Stratégia, ktorá viedla k najlepším výsledkom, bola využitá v štvrtej práci na selekciu kolón pre separáciu flavonolignanov silymarínu. Ďalší optimalizovaný parameter metódy bol výber organického modifikátora. V nasledujúcom kroku bol použitý frakčný faktoriálny design za účelom skúmania efektov rôznych koncentrácií organického modifikátora, rýchlostí toku, koncentrácií aditív, spätných tlakov, a teplôt kolóny. Ďalšie optimalizované parametre boli typy rozpúšťadiel vo vzorke a typy aditív v mobilnej faze. Napriek rozsiahlej optimalizácii, úplná separácia všetkých analytov nebola dosiahnutá.

V predloženej dizertačnej práci sú demonštrované rôzne metódy a postupy separácie štruktúrne podobných látok. Multivariantná a univariantná optimalizácia boli úspešne použité pre vývoj CE metód, ktorých použitie viedlo k úplnej separácii analytov. Aj napriek použitiu multivariantnej optimalizácie, separácia flavonolignanov silymarínu nebola dosiahnutá v SFC systéme ani za použitia spojených kolón.

# SAMENVATTING

Dit doctoraat beoogt om een bijdrage te leveren aan het onderzoek omtrent het scheiden van structuuranaloge farmaceutische verbindingen met behulp van capillaire elektroforese (CE) en superkritische vloeistofchromatografie (SFC), via verschillende benaderingen voor de methodeontwikkeling. Het theoretisch deel beschrijft de belangrijkste principes en aspecten van methodeontwikkeling in CE en SFC, en introduceert de componenten die in het proefschrift zijn bestudeerd. Het experimentele deel bestaat uit vier studies, voorafgegaan door korte inleidende paragrafen.

De eerste publicatie beschrijft de ontwikkeling van een micellaire elektrokinetische chromatografie methode voor de scheiding van indomethacine en drie onzuiverheden. De methode werd ontwikkeld met behulp van een multivariate benadering, waarbij een vierde-fractie factorieel experimenteel design werd gebruikt voor de screening van de factoren en een face-centred central composite design voor de optimalisatie van de belangrijkste factoren. Een basislijnscheiding van indomethacine en de drie onzuiverheden werd bekomen in 10 minuten. De methode werd gevalideerd op het niveau van lineariteit, precisie en accuraatheid, en werd daarna toegepast op farmaceutische stalen.

De tweede studie richtte zich op de optimalisatie, validatie en daaropvolgende applicatie van een CE methode voor de bepaling van de hoofdbestanddelen van silymarine. In tegenstelling tot de vorige studie werd hier een univariate optimalisatie gebruikt. In een eerste fase werd geprobeerd om een cyclodextrine-gemodificeerde micellaire elektrokinetische chromatografie methode te optimaliseren. Echter, wegens de slechte repeteerbaarheid van migratietijden werd de methode verder ontwikkeld in elektrokinetische chromatografie met heptakis(2,3,6-tri-O-methyl)-β-cyclodextrine als additief in het background electrolyte. Een basislijnscheiding werd bereikt voor alle stoffen, inclusief de diastereomeren van silybine en isosilybine. De elektrokinetische chromatografie methode werd gevalideerd op het niveau van lineariteit, precisie en accuraatheid, en daarna toegepast op voedingssupplementen die Mariadistelextract bevatten.

De derde publicatie beschrijft de evaluatie en verbetering van de voorspelling van retentie van componenten op gekoppelde kolomsystemen in SFC. Een testset bestaande uit structuuranaloge verbindingen, waaronder chirale verbindingen, diastereomeren en structurele isomeren, werd geanalyseerd op een selectie van vijf chirale en vier achirale kolommen. Analyses werden uitgevoerd op zowel individuele kolommen als op gekoppelde kolomsystemen. Eerst werd een vergelijking geselecteerd die de beste correlatie tussen voorspelde en experimentele retentiefactoren gaf. Daarna werd de nauwkeurigheid van de voorspelling verbeterd door het debiet en de tegendruk tijdens de analyses aan te passen.

De beste strategie van de derde studie werd toegepast in de vierde om gekoppelde kolommen te selecteren voor de scheiding van silymarine flavonolignanen door middel van SFC. In eerste instantie werd de selectie van de organische modifier geoptimaliseerd. In een volgende stap werden het percentage van de organische modifier, het debiet, de concentratie additieven, de tegendruk en de kolomtemperatuur gevarieerd met behulp van een vierde-fractie factorieel design. Andere geoptimaliseerde parameters waren het oplosmiddel van het staal

en de mobiele fase additieven. Ondanks de uitgebreide optimalisatie kon echter geen basislijnscheiding van alle geanalyseerde stoffen worden bereikt.

Dit werk toonde aan dat verschillende methoden en aanpakken kunnen gevolgd worden voor de scheiding van structuuranaloge verbindingen. Zowel multivariate als univariate optimalisatie werden succesvol gebruikt om een basislijnscheiding te bereiken met de ontwikkelde CE methoden. Echter, in SFC kon de scheiding van alle silymarine componenten niet bereikt worden na een uitgebreide (o.a. multivariate) optimalisatie van het gekoppeld kolomsysteem.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| ACE | affinity capillary electrophoresis |
| APCI | atmospheric pressure chemical ionization |
| API | atmospheric pressure ionization |
| BBD | Box-Behnken design |
| BGE | background electrolyte |
| BPR | backpressure regulator |
| CAD | charged aerosol detector |
| CCompD | central composite design |
| CD | cyclodextrin |
| CE | capillary electrophoresis |
| CGE | capillary gel electrophoresis |
| CHBA | 4-chlorobenzoic acid |
| CIEF | capillary isoelectric focusing |
| CITP | capillary isotachophoresis |
| CMC | critical micellar concentration |
| CSP | chiral stationary phase |
| CZE | capillary zone electrophoresis |
| DAD | diode array detector |
| DCHIND | 3,4-dichloroindomethacin |
| DOE | design of experiment |
| EKC | electrokinetic chromatography |
| ELSD | evaporative light scattering detector |
| EMA | European Medicines Agency |
| EOF | electroosmotic flow |
| ESI | electrospray ionization |
| FDA | Food and Drug Administration |
| FFD | full factorial design |

| | |
|---|---|
| FrFD | fractional factorial design |
| GAC | green analytical chemistry |
| GC | gas chromatography |
| HPLC | high-performance liquid chromatography |
| i.d. | inner diameter |
| IND | indomethacin |
| IPA | isopropylamine |
| ISB | isosilybin |
| ISBA | isosilybin A |
| ISBB | isosilybin B |
| LA-1 | Lux Amylose-1 |
| LC-2 | Lux Cellulose-2 |
| LC-3 | Lux Cellulose-3 |
| LIF | laser induced fluorescence |
| LOD | limit of detection |
| MEKC | micellar electrokinetic chromatography |
| MMIAA | 5-methoxy-2-methyl-3-indoleacetic acid |
| MS | mass spectrometry |
| NP-HPLC | normal phase high-performance liquid chromatography |
| NSAID | non-steroidal anti-inflammatory drug |
| o.d. | outer diameter |
| PAG | polyacrylamide gel |
| PBD | Plackett-Burman design |
| $P_c$ | critical pressure |
| pI | isoelectric point |
| Q | quadrupole |
| QqQ | triple quadrupole |
| Q-TOF | quadrupole time-of-flight |
| RP-HPLC | reversed phase high-performance liquid chromatography |
| SB | silybin |

| | |
|---|---|
| SBA | silybin A |
| SBB | silybin B |
| SCH | silychristin |
| SD | silydianin |
| SDS | sodium dodecyl sulphate |
| SFC | supercritical fluid chromatography |
| $T_c$ | critical temperature |
| TD | Taguchi design |
| TFA | trifluoroacetic acid |
| TX | taxifolin |
| UHPLC | ultra-high-performance liquid chromatography |
| UV | ultraviolet |
| UV-Vis | ultraviolet – visible |

# 1 Introduction

The need to separate structurally similar compounds has been demonstrated for a variety of applications, such as the separation of degradation products from the main active compound, the separation of chiral compounds (enantiomers) with different pharmacological properties, or the determination of components originating from a given plant. The therapeutic efficacy of herbal preparations is usually attributed to multiple active components of the same origin. The quality of the herbal medicine is determined by the amount of the active components, making separation and quantitation of the plant components an important issue [1]. A successful impurity profiling of pharmaceuticals requires a complete separation and sensitive quantification of all potential impurities, including degradation products, from the active pharmaceutical ingredient [2]. The presence of unwanted chemicals can compromise the quality, efficacy, and safety of pharmaceutical products. This is reflected in the policies of the regulatory agencies, such as the Food and Drug Administration (FDA), European Medicines Agency (EMA), and Pharmaceuticals and Medical Devices Agency (PMDA) which are for example imposing strict guidelines on the development of stereoisomeric drugs following the thalidomide affair in the 1960s [3–7].

Since structurally similar compounds may have similar physicochemical properties, their separation may be difficult. CE has been widely used for the separation of related substances in pharmaceutical analysis. For example, a CE method was developed for separation of omeprazol and its impurities [8] or for simultaneous chiral purity determination and impurity profiling of tamsulosin [9]. SFC has demonstrated its capabilities for determination of pharmaceutical impurities and is becoming increasingly important technique in pharmaceutical quality control [10,11].

Nowadays, around 50% of the drugs used in therapy are chiral, from which only 25 – 30% are used as pure enantiomers, with the rest being marketed in the racemic form [12]. Pharmacological activity is often attributed to one of the enantiomers, while the other one can be less active, have a different pharmacological profile, can cause undesirable side effects, or have toxic effects. Therefore, the chiral compounds are required to undergo an analytical characterization and clinical testing before a chiral drug is commercialized, including the determination of limits for all isomeric components, impurities, and contaminants, as well as in vivo and human testing [4,12,13].

To demonstrate the purity of pharmaceuticals, selective and sensitive analytical separation methods are required. Because of the unpredictability of enantioselectivity, the development of new analytical methods for a chiral separation is a challenge. While liquid chromatography remains the most applied enantioseparation technique, CE and SFC gained in the past years much popularity for chiral separations as well. Both techniques are seen as a green technology, thanks to their short analysis time, and solvent and waste reduction [12,14].

This work will apply univariate and multivariate approaches to the method development for the separation of indomethacin and its impurities, test set of structurally similar compounds, and main active components of the *Silybum marianum* plant using CE and SFC.

For simplicity reasons, the term capillary electrophoresis (CE) will also be used to indicate capillary electromigration (CE) techniques that combine chromatographic principles as well as electromigration, such as electrokinetic chromatography (EKC) and micellar electrokinetic chromatography (MEKC).

# 2 Aims and Objectives

The main aim of this thesis is to explore different approaches of method development for the separation of structurally similar compounds using CE and SFC. The selection of the analytes was focused on addressing gaps in previously reported methods (i.e., developing a separation method for previously unvalidated indomethacin impurity, achieving base-line separation of silymarin components using CE method, creating and applying approach for retention prediction of structurally similar analytes for serially coupled columns in SFC). Throughout the thesis, the analyzed compounds included an active pharmaceutical ingredient and its impurities, components of a herbal extract, or a test set of mixtures of enantiomers, diastereomers, and structural isomers. The developed methods were intended to be more environmentally friendly alternatives to other separation techniques such as high-performance liquid chromatography (HPLC).

The main objectives were:

- The development and validation of a micellar electrokinetic chromatography method for the determination of indomethacin impurities, 4-chlorobenzoic acid, 5-methoxy-2-methyl-3-indoleacetic acid, and 3,4- dichloroindomethacin, for the first time.

- The development and validation of the first CE method for the base-line separation of all main structurally similar flavonolignans and the flavonoid taxifolin in silymarin complex.

- The evaluation and improvement of retention prediction of structurally similar analytes in serially coupled chiral and achiral stationary phases in SFC, based on a limited number of initial analyses.

- Attempt to apply the retention prediction in serially coupled stationary phases in SFC for the development of a novel method for the separation of the most important silymarin components.

# 3 Theoretical part

## 3.1 Capillary electrophoresis

### 3.1.1 History of capillary electrophoresis

Electrophoresis has been defined as the migration of charged particles or ions in an electric field [15]. As a separation technique, electrophoresis was introduced in 1937 by Tiselius, who was studying the electrophoretic mobility of proteins and later developed the first electrophoretic instrument [16].

In moving boundary electrophoresis, migrating components are prone to mixing due to the heat convection and differences in density, therefore failing to provide complete separation of the migrating zones. To increase the resolution, the moving boundary electrophoresis was gradually replaced by different strategies. One of the first was zone electrophoresis, that initially was introduced in a form of paper electrophoresis [17], and later on was routinely used in biomedical analysis [18].

Different gel types can also be used as supporting media in electrophoresis. In 1955 Smithies used starch gels for the separation of serum proteins [19]. Later on, agarose and polyacrylamide gels (PAG) were used [18,20,21]. Electrophoresis in PAG in the presence of sodium dodecyl sulphate (SDS) is up till now a widely used technique in proteomics [18,22].

After the 1960s, narrow tubes or capillaries were used for isotachophoresis or displacement electrophoresis as an alternative to electrophoresis with supporting media [23]. In 1967, Hjérten described an instrument for free zone electrophoresis in revolving open tubes, with an inner diameter (i.d.) of 0.3 cm [24]. Reduction of the i.d. resulted in higher resolution between the zones of the tested proteins and was improved further by using glass and teflon, using capillaries with an even smaller i.d., or applying high voltages [25–27]. Nowadays, capillaries with an internal diameter of 25 - 100 µm are typically used.

In 1973, the first electrophoresis instrument for isotachophoresis became commercially available [28]. Expansion to capillary electrophoresis as we know today happened at the beginning of the 1990s, after the introduction of the first commercially available capillary electrophoresis instrument [18]. In the last decades, many scientific papers were published demonstrating the practical application of capillary electrophoresis in pharmaceutical, clinical, forensic, agrochemical or environmental analysis [29–32].

### 3.1.2 Basic principles of electrophoresis

**Electrophoretic mobility**

The principle of separation in electrophoresis is based on differences of electrophoretic mobilities ($\mu_e$) of analyte ions resulting in different ion velocities ($v$) under the influence of an electric field ($E$):

$$v = \mu_e \mathrm{E} \qquad \text{(Eq. 1)}$$

The strength of an electric field (*E*) is the voltage (*U*) applied over the length (*L*) of a capillary ($Vm^{-1}$).

$$E = \frac{U}{L} \qquad \text{(Eq. 2)}$$

Ion velocities are determined by dividing the effective length (*l*) of the capillary (distance from injection site to the detector) by the migration time of the ion ($ms^{-1}$). The electrophoretic mobility is characteristic for a given ion and a medium. Electrophoretic mobility ($m^2V^{-1}s^{-1}$) can be described in terms of physical parameters as:

$$\mu_e = \frac{q}{6\pi\eta r} \qquad \text{(Eq. 3)}$$

where *q* is the ion charge, *r* the ion radius and $\eta$ the solution viscosity. From this equation it is clear that small, highly charged ions will have higher mobilities, while large, minimally charged species will possess a lower mobility. However, neutral molecules do not show any electrophoretic mobility due to the missing charge [14,33,34]. The effective mobility ($\mu_{eff}$) of weak electrolytes (weak bases and acids) is dependent on the pH of the background electrolyte (BGE), i.e., it is affected by the ionisation of the analyte:

$$\mu_{eff} = \alpha_i \mu_e \qquad \text{(Eq. 4)}$$

where $\alpha_i$ is the degree of ionization or dissociation of a molecule [33].

**Electroosmotic flow (EOF)**

The EOF generated in fused silica capillaries is caused by the presence of negatively charged silanol groups on the interior surface of the capillary wall. The surface density of this charge increases with the pH of the solution. BGE cations, which build up near the negatively charged capillary surface to maintain charge balance, form an electric double layer. An inner layer of cations tightly held by the silanol groups is referred to as stagnant (fixed) layer. Cations further away from the silanol groups are more loosely bound, creating mobile (diffuse) layer fully neutralizing the negative charges on the capillary surface (Figure 1). Potential difference between the mobile and stagnant layers create the so-called electrokinetic potential. The application of an electric field to the capillary causes the migration of the positively charged mobile layer towards the cathode, resulting in the flow of the bulk liquid [33–36]. This flow of a liquid (the BGE) in a capillary is the electroosmotic flow.

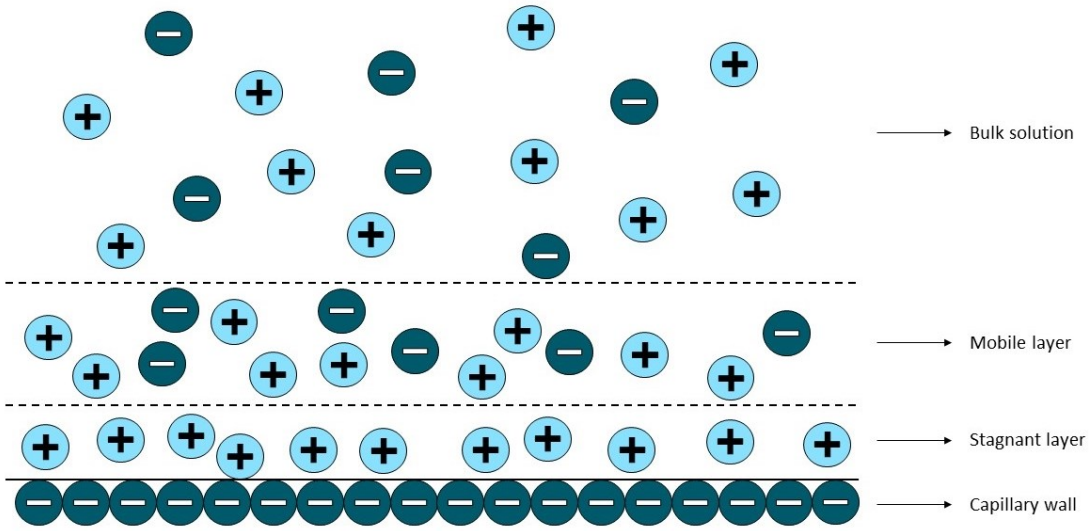


**Figure 1:** Illustration of an electric double layer.

A specific feature of the EOF in the capillary is its nearly flat flow velocity profile. The electro-driven force of the EOF is evenly distributed at the capillary walls, therefore the velocity is nearly uniform throughout the narrow bore capillary with i.d. up to 100 µm. In contrast to the parabolic velocity profile generated by pressure, there is no pressure drop along the length of the capillary. This is beneficial, since a flat velocity profile does not contribute to the broadening of the solute zones during their migration (Fig. 2) [33,35,36].

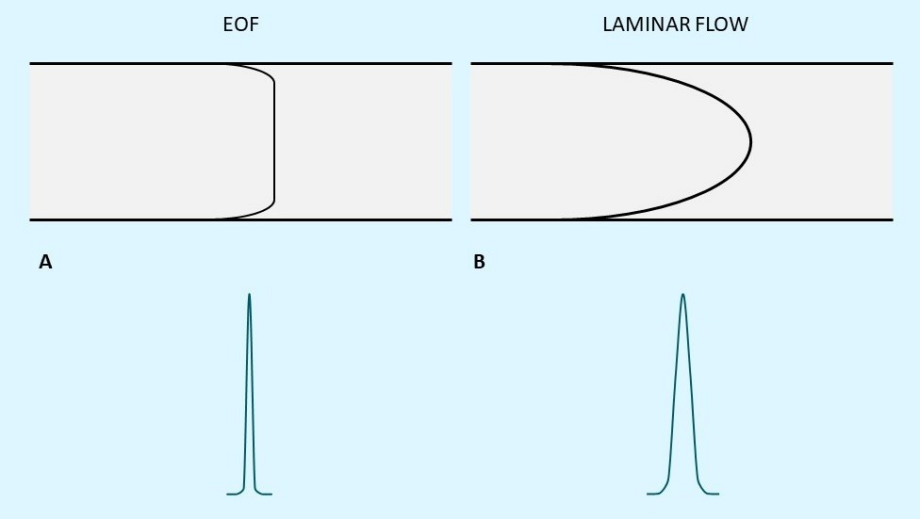


**Figure 2:** Flow velocity profiles with solute zones (top) and corresponding peak shapes (bottom) in electro- (A) and pressure-driven (B) flows. Reproduced with permission from [33].

The magnitude of the EOF may be quite strong, resulting in the migration of all analytes from anode to cathode (under normal conditions). Since all analytes are moving in one direction, anions, neutral compounds, and cations can be detected in one run. Cations, which have the electrophoretic mobility in the same direction as EOF, migrate first. Neutral compounds migrate at the same velocity as the EOF and are not separated. Anions, which are attracted to the anode, may still be dragged by the EOF to the cathode, but will migrate last (Fig. 3). The

EOF affects how long the solutes remain in the capillary, but does not affect the selectivity [33].

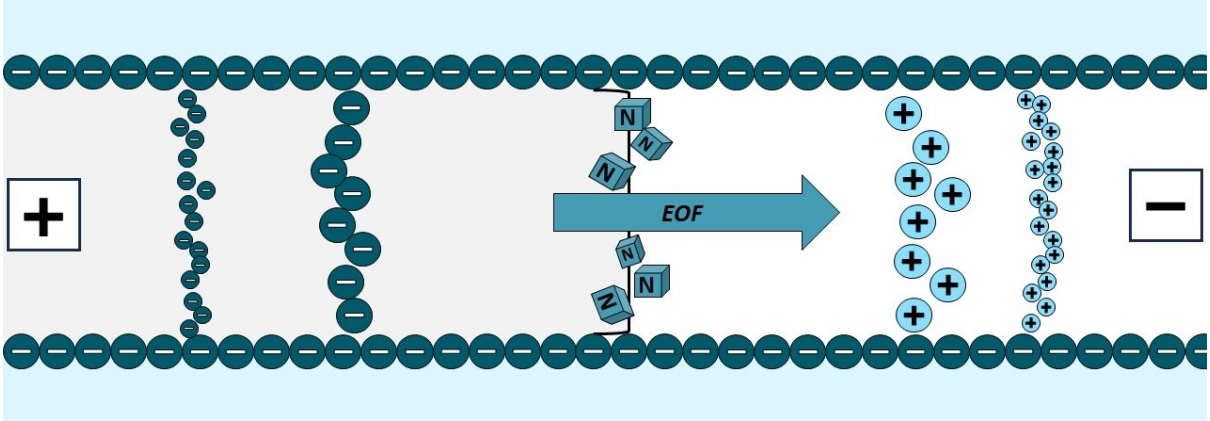


**Figure 3:** Illustration of solute migration in fused silica capillary under the influence of electroosmotic flow under normal polarity. Reproduced with permission from [33].

The magnitude of EOF can be expressed as velocity or mobility:

$$v_{EOF} = (\varepsilon\zeta/\eta)\, E \qquad \text{(Eq. 5)}$$

$$\mu_{EOF} = (\varepsilon\zeta/\eta) \qquad \text{(Eq. 6)}$$

where $v_{EOF}$ is the velocity of EOF, $\mu_{EOF}$ the EOF mobility, $\varepsilon$ the electric permittivity of the solvent, which expresses the capability of the solvent to separate electrolyte into ions (i.e., measure of its polarity), $\zeta$ the electrokinetic potential, which is the potential difference in the double layer (i.e., between fixed and mobile layer), $\eta$ the solution viscosity and $E$ the electric field strength [33,37–39].

The rate of the EOF can be increased by increasing the electric field, resulting in a shorter total analysis time and higher separation efficiencies. An increase in the applied electric field leads to higher currents and increased Joule heating. By decreasing the voltage, the magnitude of EOF will decrease, having a negative effect on analysis time and separation efficiency [33,34].

The magnitude of the EOF can be controlled by changing the BGE properties. Altering the pH of the BGE can have a significant effect on EOF as it changes the electrokinetic potential. By increasing the BGE pH, the electrokinetic potential is increased due to a higher deprotonation of the silanol groups on the inner capillary wall, resulting in a higher EOF. BGEs of low pH will lead to a decreased dissociation of silanol groups on the inner capillary wall, lowering the electrokinetic potential and the EOF magnitude. At pH 2 and lower, there is no EOF anymore because the silanol groups are not dissociated [33,34].

The EOF can also be reduced by increasing the BGE concentration. A higher electrolyte concentration causes a decrease in the electrokinetic potential and an increase in the viscosity of the solution. Typical BGE concentrations are in the range of 10 to 100 mM and are limited by the generated heating within the capillary [33,34,38,39].

Another way of controlling the EOF is an adjustment of the temperature, resulting in the change of viscosity of the BGE. In general, an increase in temperature means a decrease in viscosity, which leads to an increased EOF. The effect of organic modifier is less

straightforward and depends on the effect of the modifier on viscosity, electric permittivity of the solvent, and electrokinetic potential created by the BGE [34].

Modification of the capillary wall charge can either increase, decrease, or reverse the EOF. Dynamic coating is achieved by addition of the coating agent to the buffer, ensuring continuous regeneration of the coating. Modifiers that are able to reverse the EOF, enabling the reversal of migration order of cations and anions, are, for example, cationic polymers (polybrene, poly(ethylene imine)) or quaternary ammonium salts such as cetyltrimethylammonium bromide [33].

**Migration time, separation efficiency, and resolution**

The apparent mobility ($\mu_{app}$) of a solute is the sum of both the effective electrophoretic mobility and the mobility of the EOF [34,40]:

$$\mu_{app} = \mu_{eff} + \mu_{EOF} \qquad \text{(Eq. 7)}$$

The apparent mobility ($\mu_{app}$) can be calculated from the experimental values. The migration time ($t_m$) is the time that an analyte requires to move from the injection end of a capillary to the point of detection after a voltage was applied over the capillary:

$$\mu_{app} = \frac{l}{t_m E} = \frac{lL}{t_m U} \qquad \text{(Eq. 8)}$$

where $l$ is the effective length of the capillary (from the point of injection to the point of detection) and $L$ represents the total length of the capillary.

The separation efficiency, expressed as the number of theoretical plates ($N$), can be calculated by the following equation:

$$N = 5.54 \left(\frac{t_m}{w_{1/2}}\right)^2 \qquad \text{(Eq. 9)}$$

where $w_{1/2}$ is the peak width at half height.

Separation efficiency can be also described as a height equivalent to a theoretical plate ($H$) [33]:

$$H = l/N \qquad \text{(Eq. 10)}$$

Resolution is used to assess the quality of the separation because the peak widths are taken into account. Numerous equations are used to express resolution in capillary electrophoresis separation. In the European Pharmacopeia [41] the resolution is defined as:

$$R_s = \frac{1.18\,(t_{m(b)} - t_{m(a)})}{w_{1/2(a)} + w_{1/2(b)}} \qquad \text{(Eq. 11)}$$

while the United States Pharmacopoeia [42] is using the following equation:

$$R_s = \frac{2(t_{m(b)} - t_{m(a)})}{w_a + w_b} \qquad \text{(Eq. 12)}$$

where $t_{m(a)}$ is the migration time of the faster migrating analyte (peak $a$), $t_{m(b)}$ the migration time of the slower migrating analyte (peak $b$), $w_{1/2(a)}$ the width at half height for peak $a$, $w_{1/2(b)}$ the width at half height for peak $b$, $w_a$ the baseline width for peak $a$, applying the tangent method, and $w_b$ the baseline width for peak $b$.

**Zone broadening**

Separation quality in CE is strongly dependent on the width of the analyte zones. Any zone broadening is resulting in a reduced separation efficiency. Therefore dispersion effects leading to zone broadening should be controlled [33,39,43]. The basic phenomena influencing the zone broadening in CE are briefly described below.

*Joule heating*

The heat generated by the electric current passing through the length of the capillary is called Joule heating. Because it causes thermal diffusion, it is one of the most important factors that can cause zone broadening in capillary electrophoresis and poor repeatability of migration times. The Joule heating can cause (i) increased dispersion due to temperature gradients with higher temperature in the centre of the capillary than at the capillary walls, and (ii) differences in viscosity, and subsequently increased dispersion. The generated heat depends on the applied voltage, the conductivity of the BGE and the capillary dimensions. Joule heating can be controlled by (i) using narrow-bore capillaries with smaller diameters, where the surface to volume ratio is higher, (ii) lowering the applied voltage, (iii) using BGE with lower conductivities, and (iv) using capillary cooling systems [33,39,43,44].

*Diffusion*

Thanks to the plug-flow velocity profile, radial diffusion (across the capillary) is negligible, however longitudinal diffusion (along the capillary) can contribute to the zone broadening and have a direct effect on the separation efficiency. Longitudinal diffusion occurs during the analyte migration, and it depends on the diffusion coefficient of the solute and on the analysis time. Smaller inorganic ions tend to have higher diffusion coefficients, resulting in higher zone broadening compared to large biomolecules, such as peptides or proteins [33,34].

*Other factors*

In order to reduce zone broadening, the sample injection plug length should be minimized to 1 to 2% of the total capillary length [33]. Sample adsorption caused by interaction of the solute with the capillary wall can negatively affect separation efficiency in CE. High area to volume ratio of the capillary contributes to sample adsorption. This is mainly observed when analysing large peptides or proteins, which possess numerous positive charges and hydrophobic moieties. Differences in conductivities of the sample zone and the running BGE can result in asymmetric peaks. To avoid zone broadening, it is important that conductivities of the BGE and the samples are matched. If the capillary inlet and outlet reservoirs are not placed at the same height, siphoning through the capillary may occur. This will introduce a hydrodynamic flow that can lead to broadening of the zones. Additionally, the detection path length should be kept small relative to the sample zone length [33,43,45].

### 3.1.3 Instrumentation

The overall simple instrumentation is considered as a key feature of CE. Basic components of generic CE system are shown in a schematic diagram in Fig. 4 [33].

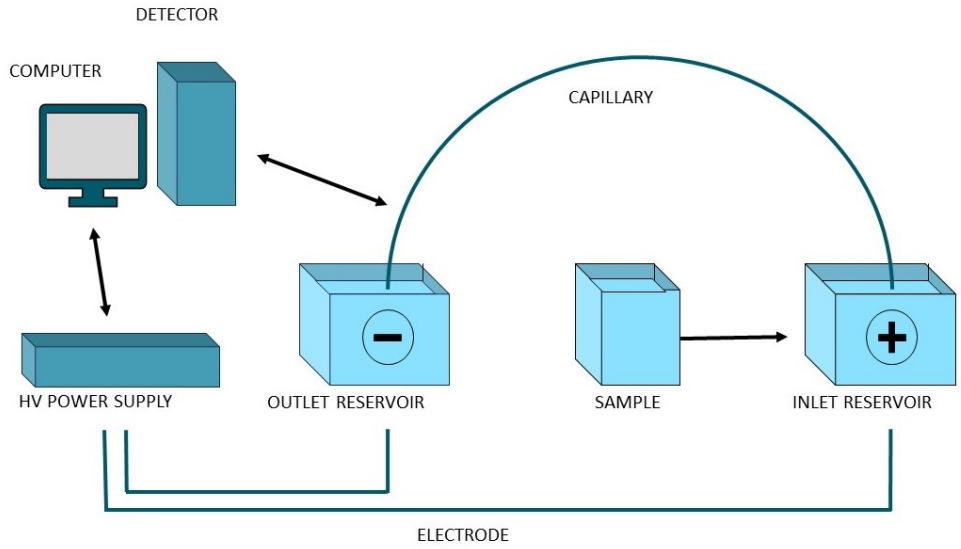


**Figure 4:** Basic components of a CE instrument. Reproduced with permission from [33].

**High voltage power supply**

A dual polarity high voltage power supply is used in CE to deliver up to 30 kV with current levels of about 200 to 300 µA. Because the EOF in bare fused silica capillaries is moving towards the cathode, the polarity of the power supply is commonly set with the anode at the injection end of the capillary and the cathode close to the detection end of the capillary. The power supply should be able to switch to reversed polarity, which can be beneficial under particular experimental conditions, for instance, when short-end analyses are aimed for or for separations under reversed EOF [33,45].

CE analysis is mostly performed at a constant voltage, but it is occasionally beneficial to apply a constant current or power to maintain reproducibility [46]. The use of a constant current or power mode is particularly beneficial for experiments for isoelectric focusing, in isotachophoretic experiments, or to compensate for temperature changes when the capillary temperature is not adequately controlled. To ensure safety of the instrumentation, the high voltage source should be equipped with a ground-leak detector, grounding the voltage source at detector side of the capillary [33,45].

**Sample injection**

Very small volumes of sample are introduced into the capillary, ranging from 1 to 50 nL, restricted by the small volume of the separation capillary. Hydrodynamic and electrokinetic injection are typical injection modes used for sample introduction into the separation capillary [33,47,48].

Hydrodynamic injection is a simple, non-specific way to introduce a sample solution into the separation capillary. The sample is introduced into the capillary by a hydrodynamic flow, achieved by a pressure difference between the inlet and outlet ends of the capillary. The pressure difference can be created by applying pressure at the injection end of the capillary, vacuum at the outlet of the capillary, or by applying a height difference between inlet and outlet reservoir (siphoning), while the injection end of the capillary is dipped in the sample. During the injection, sample, except with siphoning, and BGE reservoirs should be at equal

heights to avoid unwanted sample injection, sample overloading or poor injection repeatability.

The sample injection volume ($V_{inj}$) can be calculated by the Hagen-Poiseuille equation:

$$V_{inj} = \frac{\Delta P d^4 \pi t_{inj}}{128 \eta L} \qquad \text{(Eq. 13)}$$

where $\Delta P$ is the pressure difference along the capillary, $d$ is the capillary inside diameter, and $t_{inj}$ the injection time, $\eta$ is the buffer viscosity, $L$ is total capillary length.

The length of the sample zone depends on the viscosity of the liquid inside of the capillary, which is maintained constant by thermostating. The viscosity of the sample has a negligible effect on the length of the injected zone, because of the very small volume of the sample compared to the total liquid volume in the capillary [33,44,47].

With an electrokinetic injection, a voltage 3 to 5 times smaller than that used for separation is applied over the capillary. As a consequence, a narrow band of the sample migrates into the capillary tube. The sample is injected by a combination of electromigration and electroosmotic flow. Consequently, the amount of the analyte ion injected is dependent on its mobility. Electrokinetic injection is a simple and effective method, causing a minimal band broadening by the lack of a parabolic flow as opposed to hydrodynamic injection. However, the repeatability of the method is lower compared to hydrodynamic injection, as it depends on factors which are more difficult to control [33,44,47].

Stacking methods are used to increase the sensitivity of the CE analysis by concentrating dilute sample mixtures during or just after sample introduction. They are based on differences in field strength between sample zone and running BGE [33,49].

**Separation capillaries**

Separation capillaries are the parts of the instrumentation where separation and detection take place. Capillaries used in electrophoresis can be made of different materials, such as fused silica, glass or teflon. The length of the capillary typically ranges from 25 to 75 cm, with i.d. of 25 to 100 µm and outer diameters (o.d.) of 350 to 400 µm. General requirements for capillaries used in CE are: (i) chemical and physical resistance, (ii) ultraviolet (UV) transparency, (iii) flexibility, (iv) robustness, and (v) low price. Fused silica meets most of the abovementioned requirements and is therefore the most popular capillary material [33,44,45].

Fused silica capillaries are normally protected by a polyimide coating, making them flexible, more robust and easier to handle. For optical detection, the coating is burned off, creating a UV transparent detection window. However, this window with removed protecting coating is also much more fragile [33,35].

To achieve the best performance in terms of repeatability, it is important to condition the inner walls of the capillary. New capillaries are conditioned with a strong basic solution (typically with 0.1 - 1.0 M NaOH solution), dissolving a film of silica on the inner capillary walls. Ionization of the acidic silanol groups on fused silica by a NaOH solution is also done during daily capillary conditioning prior to analysis, and between the runs to regenerate the capillary surface. In the subsequent steps, the capillary is rinsed with water and then BGE to maintain a stable EOF [33,44].

The major drawback of fused silica capillaries is that they are not chemically inert and have a tendency to adsorb analytes, resulting in band broadening and loss of efficiency, which was observed, for example, during the separation of proteins by CE [45]. To prevent unwanted interactions of analytes with the inner capillary wall, two approaches can be applied: permanent coating of the capillary walls by polymers, or dynamic coating using BGE additives. Wall-coated capillaries, with shielded silanol groups, may also be used to change the magnitude or direction of EOF [33,45].

**Temperature control**

Temperature control in CE is important for reducing Joule heating, isolating the capillary from ambient temperature changes, and consequently ensuring repeatability of the separation. To maintain capillary temperature, two approaches are commonly used: thermostating by forced air stream or by a liquid coolant. The liquid thermostating is considered more efficient. However, forced air thermostating is sufficient for the amount of heat generated in CE, while it is instrumentally simpler and easier to handle. The temperature, which changes viscosity, EOF, and analysis time, can be also altered as optimization parameter [33,44].

**Detection**

Ideally, the CE detector is universal and highly sensitive at the same time, with detection cells preventing additional zone broadening of already separated analytes. The most commonly utilized detection systems in CE are on-capillary ultraviolet – visible (UV-Vis), fluorescence and conductivity detectors, and off-capillary mass-spectrometric detection. Table 1 compares the limits of detection (LOD) of these systems [50].

The most commonly used detection mode in CE is UV-VIS absorption. Here the intensity of the light beam passing the capillary is measured. The detection is performed in a short section of the separation capillary, from which the external coating was removed. Limited sensitivity of UV-Vis detection is caused by the short detection pathway in normal capillaries. To improve the sensitivity of the UV-Vis detection, capillaries with an extended optical path length are available. Examples of such capillaries are bubble cell capillaries, with a wider i.d. of the capillary at the detection window, or high sensitivity cells with Z-shaped optical cells with axial illumination detection. The use of bubble cells shows no deterioration of resolution or separation efficiency. Z-shaped cells have a longer optical path length than bubble cells, making the detection more sensitive. The selectivity of absorbance detection can be improved by employing a diode array detector (DAD), that provides information about the peak spectra and their purity. For analytes not exhibiting UV-Vis absorption, UV-Vis detection can be applied indirectly by the addition of an absorbing co-ion to the BGE that produces a high background signal [33,49–51].

Fluorescence is a very sensitive and selective detection method, measuring the light intensity emitted by molecules transitioning from an excited to the ground electron state after the previous excitation by absorption of radiation. The sensitivity of the technique can be increased further by replacing lamp-based fluorescence with laser induced fluorescence (LIF), which is providing a higher excitation energy. The applicability is limited, as the technique is linked to very specific molecular structures, usually containing several aromatic rings and/or an extended conjugated system of double bonds. Since not so many compounds are naturally fluorescent, fluorescence detection can be accomplished by derivatization of the monitored analytes. However, the derivatization processes are often complex and time-consuming, and

are therefore avoided. Similarly to absorbance detection, indirect fluorescence detection can be applied with a fluorescent BGE [44,45,49,50].

Contactless conductivity detection is accomplished by conductivity electrodes, mounted around the outer wall of the capillary, which are measuring electrical conductivity of the solution in the separation capillary. It is a universal, instrumentally simple detection technique suitable for compounds without UV absorbance or fluorescence, such as inorganic ions. The detectors do not interfere with the flow in the separation capillary and do not contribute to the zone broadening [44,49,50].

CE can be advantageously coupled with the sensitive and universal mass spectrometric (MS) detection, which separates ionized particles based on their different charge to mass ratios and determines the molecular mass of the ions [52]. The complementarity of the CE-MS coupling is mainly shown when (i) compounds co-eluting in CE can be successfully distinguished by MS, or (ii) analytes of the same molecular mass, normally undistinguishable by MS, are easily separated with CE. Electrospray ionization (ESI) is the most suitable ionization technique used in the online coupling of liquid phase CE separation with MS. The very low flow rates generated in a CE separation increase the sensitivity of the ESI. Additionally, ESI is particularly well suited to ionize polar and charged compounds usually separated by CE. The challenge in CE-ESI coupling is that both CE and ESI are based on electric fields with different CE and ESI currents. The requirements in the magnitude and direction of the electric fields applied may also differ for CE and ESI. A sheath-liquid interface is the most robust technique for coupling CE with MS, where the sheath liquid is providing the electrical contact and hydrodynamic flow necessary to establish a stable electrospray. The disadvantage is that the analytes leaving the capillary are diluted. On the other hand, the sensitivity can be tuned by the chemistry of the sheath liquid to enhance the ionization of analytes [33,45,51].

**Table 1:** Comparison of LODs of the most commonly used CE detectors [50].

| **Detection technique** | **LOD (mol)** |
|---|---|
| UV-Vis Absorption | $10^{-12} – 10^{-15}$ |
| Fluorescence | $10^{-15} – 10^{-20}$ |
| Conductivity | $10^{-16}$ |
| Mass spectrometry | $10^{-17}$ |

### 3.1.4 Capillary electromigration methods

Various separation modes can be employed with little to no changes to the CE instrumentation [33,35].

**Capillary zone electrophoresis**

Capillary zone electrophoresis (CZE) is the most direct and simple application of the CE principles described above. After a sample is introduced into a separation capillary filled with BGE, a voltage is applied. The separation of analytes is based on a combination of the different mobilities of the analytes and the EOF. The analysis time is determined by the magnitude of the electric field applied. Cations and anions are separated based on their different charge-to-size ratios. Neutral compounds migrate unseparated with the EOF [33,34,49,53].

**Micellar electrokinetic chromatography**

MEKC can be used for the separation of both charged and neutral compounds. A pseudostationary phase is created by the addition of surfactant into the BGE solution at a concentration above its critical micellar concentration (CMC). At this concentration, surfactants form micelles, with their hydrophobic chains oriented towards the centre and the charged hydrophilic heads towards the BGE. Micelles are usually spherically shaped and charged and migrate with or against the EOF direction. The separation is based on a different partitioning of the analytes between the hydrophilic BGE and the lipophilic core of the micelles. The most frequently used surfactants are SDS, bile salts, and long alkyl chain quaternary ammonium salts.
The neutral analytes are separated based on their different partitioning between the micelles and the running buffer. The separation mechanism of neutral analytes is in general chromatographic, with the difference that the pseudostationary phase (micelles), have their own migration velocity ($t_{mc}$). The migration velocity of micelles is measured by a water-insoluble dye completely retained by the micelles (e.g., Sudan III, Sudan IV). More hydrophobic analytes will interact more with the micelles, making their migration longer compared to polar analytes. The moles of the analyte in the micellar phase and in the aqueous phase is expressed by the retention factor ($k$):

$$k = \frac{(t_r - t_0)}{t_0\left(1 - \frac{t_r}{t_{mc}}\right)} = K\left(\frac{V_S}{V_M}\right) \qquad \text{(Eq. 14)}$$

where $t_r$ is the migration time of the solute, $t_0$ the migration time of unretained solute moving at the EOF rate, $t_{mc}$ the micelle migration time, $K$ the partition coefficient of micellar and mobile phase, $V_S$ the volume of the micellar phase, and $V_M$ the volume of the aqueous phase.

The interaction between the analytes and micelles can be manipulated by the addition of organic modifiers [33,34,53,54].

**Electrokinetic chromatography**

EKC is another technique that includes partitioning between the BGE and a pseudostationary phase, which may be also charged. EKC is typically used for enantioseparations and chiral selectors (e.g., cyclodextrins) act as a pseudostationary phase. From a physicochemical perspective, the formation of enantioselective complexes is a chromatographic process. On the other hand, the mobility of the analytes is an electrokinetic parameter [53,55].

**Capillary gel electrophoresis**

In capillary gel electrophoresis (CGE) the capillary is filled with a gel or a polymeric solution, usually polyacrylamide, dextran, polyethylenoxide, or agarose, which is acting as a sieving medium. The separation is based on size of the analytes. CGE is used for the separation of large biomolecules with a similar charge, but a different mass [34,54].

**Capillary isoelectric focusing**

Capillary isoelectric focusing (CIEF) is providing high resolution separation of amphoteric analytes, such as peptides and proteins, based on their isoelectric point (pI). In CIEF, the capillary is filled with a mixture of carrier ampholytes and sample. When an electric field is applied, a pH gradient is established. The migration of the analytes stops in the place where

pH in the separation capillary corresponds to the pI value of the amphoteric analyte. To ensure that the migration of the solutes is solely based on differences in their pI, the EOF should be supressed. Upon the completion of focusing step that is accompanied by the decrease of the current to the constant value, the analytes are transported to the detector by hydrodynamic flow or by electrophoretic mobilization [34,49,54,56].

**Capillary isotachophoresis**

Capillary isotachophoresis (CITP) is a „moving boundary" technique for the separation of either cations or anions. This principle is commonly used for the preconcentration of diluted samples by transient isotachophoresis before their CZE separation [34,49].

**Affinity capillary electrophoresis**

The separation in affinity capillary electrophoresis (ACE) is based on modification of the charge-to-size ratio through interactions of analytes with specific ligands. By successively increasing the amount of ligand, the electrophoretic mobility of analytes changes. ACE is widely used for investigation of various biomolecules, mainly proteins, polysaccharides, and hormones [57,58], studying the complexation between metal ions and organic ligands [57] as well as for the determination of binding constants between chiral selectors and enantiomers of a chiral analyte [57,59].

### 3.1.5 Important parameters in CE method development

The initial in CE method development is the selection of the CE separation mode, based on the properties of the analysed compounds. The described parameters below influence the quality and speed of the CE separation and need to be considered during method development.

**Type and concentration of the BGE**

A variety of BGE systems can be used in CE, and their selection has an important influence on the separation. Ideally, the selected BGE should have a high buffer capacity. However, the buffer capacity of most buffer systems is only sufficient in a limited pH range. Additionally, the conductivity of the BGE should be low, in order to keep the current and heat generation low as well. Moreover, the BGE co-ion mobility should be close to the mobility of analytes to minimize electromigration dispersion [44].

The BGE concentration has an effect on the EOF, selectivity, and resolution. The BGE concentrations typically range from 10 to 100 mM. With higher BGE concentrations, the EOF is decreased, the migration times of the analytes are longer. The use of a BGE with higher concentration can limit coulombic interaction of the solute with the capillary wall and therefore decrease the effective charge at the capillary wall. More concentrated BGEs can lead to Joule heating as a result of higher conductivity. The created temperature gradient can result in peak broadening, having a negative effect on the separation [33,44,53].

Decreasing the BGE concentration will increase the EOF. However, lower BGE concentrations may also decrease the buffering capacity of the BGE, creating unstable conditions [33,44,53].

**BGE pH**

The pH of the electrolyte has a major effect on the separation selectivity, and even small changes of the pH can have a significant effect on the resolution of analytes. The pH of the BGE has a strong influence on the effective mobility (see equation 4) of analytes, particularly weak acids or bases, as the degree of ionization or dissociation of these compounds is pH-dependent. [49,53].

The EOF velocity is also affected by the pH and increases with higher pH, with a maximum at pH 7-8. The effect of the pH on EOF is greatly reduced with a BGE pH below 3 as most silanol groups are then not dissociated [33,34,44,49,53].

**Type and content of organic modifier**

Organic modifiers can be used to alter the viscosity and the polarity of the electrolyte and affect both selectivity and separation efficiency. Organic solvents, such as methanol, ethanol, isopropyl alcohol, and acetonitrile, are typically used up to 30% (v/v). The permittivity of the aqueous solution decreases by the addition of an organic solvent. Consequently, EOF and electrophoretic mobility of analytes are affected by the addition of an organic modifier to the system. In general, the addition of an organic modifier reduces the electroosmotic mobility. Additionally, the organic solvents are used for solubilization of analytes that are poorly soluble in aqueous solutions [43,49,53]. The use of non-aqueous capillary electrophoresis is advantageous for the separation of compounds that are insoluble in water [60].

**Electric field strength**

The field strength applied along the capillary is the cause of the ion migration in CE. A voltage applied over the length of the capillary affects the speed and the separation efficiency. With an increase of the field strength, the analysis time is reduced. However, application of a high voltage can lead to the occurrence of excessive heating, resulting in a loss of resolution and lowered peak efficiency [38,39].

**Temperature and other parameters**

Changes in temperature cause changes in viscosity, pKa and pH values, which consequently affect mobilities. Due to the increase in EOF at higher temperatures, the analysis time is generally shorter. With increasing temperatures, the zone broadening also increases. Because of the typically negative effects of temperature on the separation, most separations are performed thermostated at 25°C [38,39,47].

Other aspects that need to be considered during the CE method development are the type of capillary, the way of sample injection, and separation mode. These parameters have been described in sections 3.1.3 and 3.1.4.

## 3.2 Supercritical fluid chromatography

### 3.2.1 Supercritical fluids

In SFC, the mobile phase is a supercritical fluid, i.e., a substance with its temperature and pressure above the critical temperature ($T_c$) and critical pressure ($P_c$) (Fig. 5). At the critical point, the density of a gas and a liquid phase becomes equal and a supercritical fluid is formed. The density of a supercritical fluid is higher than that of a gas, while its viscosity is lower, and its diffusivity is higher than that of the liquid state. These characteristics allow to operate at higher velocities in SFC, while generating lower pressures along a column [61–63].

Several substances were studied as supercritical mobile phases, however $CO_2$, with a relatively low critical temperature (31°C) and critical pressure (74 bar), became the eluent of choice (Fig. 5). There are many additional reasons to favour $CO_2$ as mobile phase, including its safety, non-flammability, low cost, availability in adequate purity, inertness, and miscibility with a variety of organic solvents. $CO_2$ is easy to remove after analysis, which is reducing waste generation and is the reason why SFC is seen as a green separation technique [3,64–66].

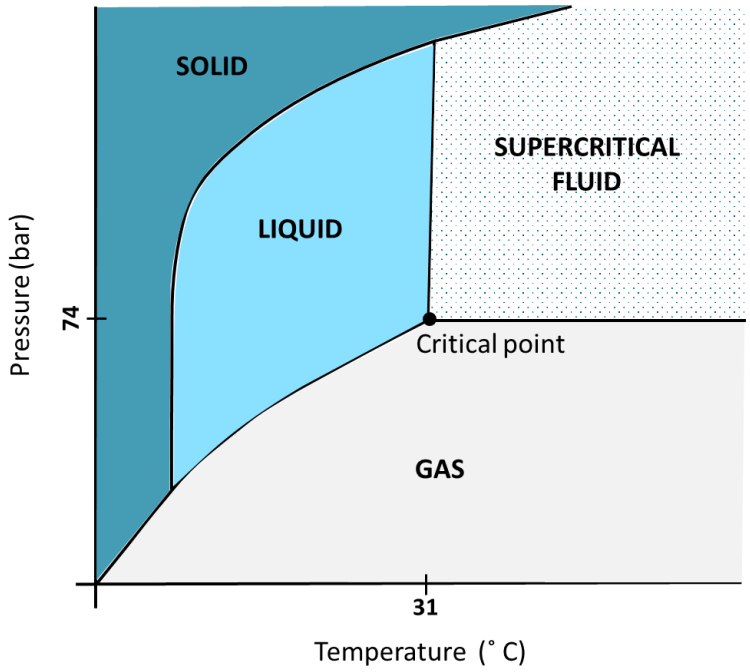


**Figure 5:** Phase diagram for $CO_2$. Reproduced with permission from [63].

The density is considered as the most influential feature of SFC, because it controls the solvating power of the supercritical fluid, especially when using pure $CO_2$. Neat $CO_2$ is a non-polar fluid, with properties similar to hexane or heptane, limiting the application range to the separation of non-polar analytes. The miscibility of $CO_2$ enables the addition of a variety of organic modifiers and the analysis of more polar analytes, which makes SFC a very versatile technique. However, mixing the primary eluent ($CO_2$) with modifiers increases the critical values of pressure and temperature. As a consequence, SFC is often executed in the subcritical region, using typical SFC conditions such as 100 – 120 bar and 40°C. Under subcritical conditions, the density, and consequently the solvating power of the mobile phase, does not change much with pressure, unlike under supercritical conditions. Under subcritical

conditions, the mobile phase is a binary mixture of liquified gas and organic modifier [64,67,68].

### 3.2.2 History of SFC

The first systematic study of the gas-liquid critical region of $CO_2$ was done in 1869 [69]. It took nearly a century until the use of a supercritical fluid as mobile phase was demonstrated by Klesper et al. in 1962. The technique was called high pressure gas chromatography (GC) at that time [70]. The experiment demonstrated that enhanced mobility can be achieved on a chromatographic column by operating pressures above the critical pressure, while the operating temperature remains lower than in traditional GC.

$CO_2$ was used as mobile phase by Sie et al. [71] to study the effect of mobile phase pressure on the partition coefficient of analytes. They used both gas-liquid and gas-solid separation modes and employed the term supercritical fluid chromatography in 1967 for this new separation technique [72–74]. In 1968, an SFC system with a mechanical backpressure regulator (BPR) was reported, controlling the pressure independent of the flow rate [75].

In the 1970s, an SFC system with pressure programmer was reported, creating an SFC pressure gradient [76]. Effects of temperature, pressure, eluent composition, flow rate, and stationary phase type on the chromatographic performance were studied by Novotny et al. [77] and it was stated that due to the pressure drop along the column, packed columns could not give high efficiency. The rapid growth of SFC occurred in the 1980s, leading to the commercialization of SFC instruments [69].

In 1981, open tubular capillary column SFC, with a small pressure drop along the capillary, was introduced [78]. Instrumentation for capillary column SFC was based on a GC system [79]. Independently of capillary column SFC, packed-column SFC with an electronically controlled backpressure regulator was developed by modification of an HPLC system [80,81]. The packed columns had a wider application range than open tubular columns, including chiral separations. Combined with instrumental restrictions, this contributed to the reduction in utilization of capillary column SFC in the early 1990s [82,83]. The application range of SFC expanded further by studying the influence of additives on the peak shapes of analytes [84,85].

Recent advances in SFC instrumentation allowed to achieve method robustness and accuracy comparable to HPLC or ultra-high-performance liquid chromatography (UHPLC) systems. Development of stationary phases, such as the 2-ethylpyridine phase, tailored for SFC and the use of selective detectors continue to broaden the application scope of SFC [86–88]. Currently, SFC is used as a complementary technique to reversed phase HPLC and it is a preferred technique for chiral separations [63].

### 3.2.3 Instrumentation

A schematic diagram of the typical components of an SFC system is shown in Fig. 6.

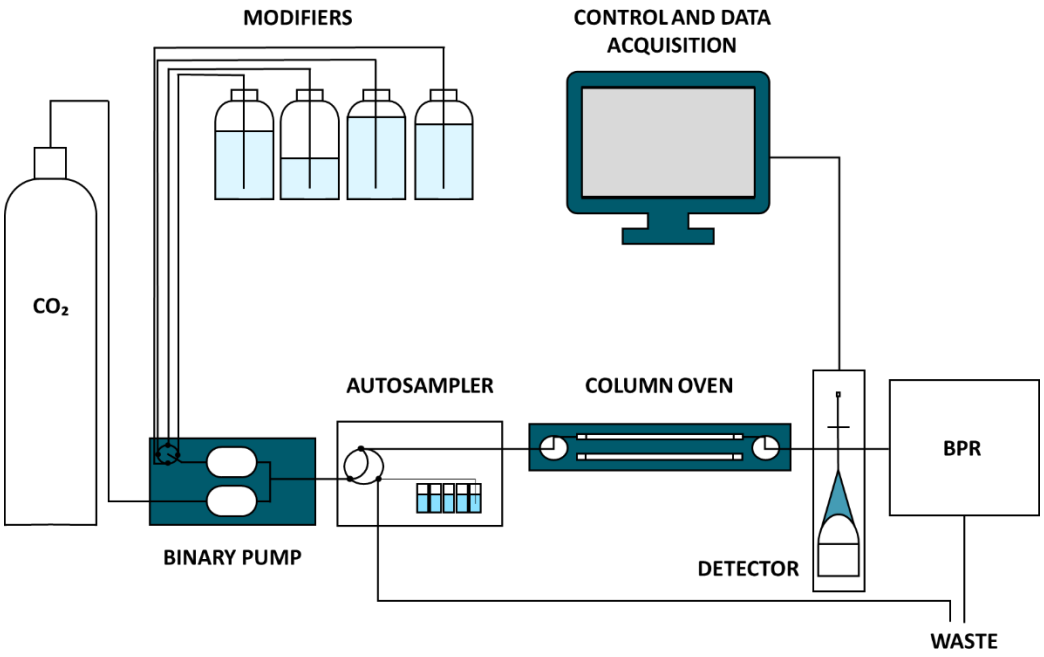


**Figure 6:** Basic components of SFC instrumentation. Reproduced with permission from [63].

**SFC pumps**

In SFC, high pressure pumps are required. Because of the difference in pressure and compressibility of $CO_2$ and the modifier, a binary pumping system is used, where one pump is used for $CO_2$, and the other for the modifier [89,90]. To avoid gaseous bubbles in the liquid flow, $CO_2$ is pumped in the liquid state. This is achieved by placing a sub-cooler between the $CO_2$ source and the pump's suction valve, and having the $CO_2$ and $CO_2$ pump head cooled to 4 to 5°C [90,91].

**Autosamplers**

Because of the nature of the mobile phase, SFC is incompatible with autosamplers commonly used in HPLC. In SFC, autosamplers use injection valves with internal or external loops. Only the loops are exposed to high pressures. During the sample loading, the sample in a vial is drawn into the needle and loop. The needle with the sample is pushed into a needle seat under high pressure and the valve is switched to the injection position. When the valve is switched to the injection position, the highly compressed mobile phase fills the syringe, tubing, and the needle of the autosampler. After switching the valve back to the load position, the remaining mobile phase is emptied to the waste. An empty syringe cannot aspire liquid samples. Therefore, a separate low pressure wash pump is used to flush the system to prevent carry-over, and to fill the injection system with the wash solvent [63,89,90].

**Column oven**

The density and selectivity of the $CO_2$-based mobile phases are considerably affected by the temperature. Therefore, the column compartment of the SFC system is thermostated to

control the column temperature. The temperature typically ranges between 10 and 90°C [89,92].

**Backpressure regulator**

The backpressure regulator controls the pressure in the SFC system. A reliable BPR limits pressure changes and consequently variations in the mobile phase density. This makes the system more reliable and prevents the creation of noise in UV-Vis detectors [2,92]. The minimal pressure required to reach liquid-like densities for $CO_2$ is 80 ± 20 bar (depending on the temperature). The most commonly used pressures in SFC are 100 – 150 bar, with BPRs capable of pressures up to 400 bar [92]. When binary or tertiary mixtures are used, pressure has in general only a small influence on retention and selectivity, but for a pure $CO_2$ mobile phase, pressure and density are the main means of changing solvent strength for relatively non-polar solutes [89,92].

**Detection**

SFC systems currently use UV, DAD, evaporative light scattering (ELSD), charged aerosol (CAD) and MS detectors [67].

*Ultraviolet detectors*

The UV detectors used in SFC measure light absorbed by analytes eluted off the column and must be capable of withstanding high pressures. Typically, the flow cell is able to withstand the maximal pressure delivered by the pump on the BPR. The wavelength range covered by the modern detectors is from 190 to 750 nm or higher, which covers also the visible light range [89,92].

*Evaporative light scattering and charged aerosol detectors*

The evaporation-based detectors transform mobile phase into droplets. The droplets are then nebulized, the eluent is evaporated, and a cloud of aerosol is formed. The aerosol goes through a light beam (ELSD) and the light scattered by the particles is detected, or the aerosol is electrically charged (CAD) and charged particles are detected [93,94]. These evaporation-based detectors are often referred to as universal detectors, because the response they provide is independent on the molecular structure of the analytes [2,95]. The prerequisite of using evaporation-based detectors is that the solute should be considerably less volatile than the mobile phase [93]. For non-volatile analytes, these detectors provide a nearly universal mass dependent response at a constant mobile-phase composition [95]. On the other hand, the drawback of these detectors is the non-linear response in gradient conditions, meaning that identical injected amounts result in different peak areas. The resulting peak areas depend on the composition of the mobile phase entering the detector [94].

*Mass spectrometry detectors*

Nowadays, commercial SFC-MS coupling is performed using atmospheric pressure ionization (API) sources [96]. The mainly used ionization source is ESI, which is also considered as the most versatile ionization source for analytes of moderate to high polarity. In SFC-ESI-MS a high voltage is applied on the tip of a capillary, creating charged droplets. Ions are formed by evaporation and Coulomb fission. The second most-often used ionization technique is atmospheric pressure chemical ionization (APCI), where the solvent molecules are first

ionized by corona discharge, followed by ionization of sample molecules with solvent ions during molecular interactions. APCI is considered to be an ionization in the gas phase, as opposed to ESI, where the ionization occurs in the liquid phase. The APCI is the most suitable ionization for lipophilic to intermediately polar molecules [97].

The most used MS analyser in SFC is the triple quadrupole (QqQ), thanks to its high sensitivity, large dynamic range, and medium to high scanning speed. Other commonly used analysers are single quadrupole (Q), utilized mainly for the analysis of less complex matrices, and quadrupole time-of-flight (Q-TOF) utilized for its high resolution, high scanning speed and mass accuracy in both qualitative and quantitative measurements, especially in *omics* analyses [97].

### 3.2.4 Important parameters in SFC method development

**Stationary phase**

In the recent past, packed HPLC columns have been used as columns of choice for SFC [98]. The dominant column technology uses porous silica particles with siloxane-bonded surface modification [99]. Thanks to the increased interest in SFC, columns specifically designed for SFC have also been manufactured [98]. The first stationary phase dedicated to SFC was 2-ethylpyridine bonded on porous silica particles, aimed to reduce tailing of basic compounds [100]. The advantages of these columns are better repeatability, robustness, and compatibility with a range of modifiers, additives and higher operating temperatures. As the mobile phase does not restrict the type of stationary phase used in SFC, both reversed-phase and normal-phase columns have been used. The use of non-polar stationary phases (e.g., phenylhexyl-, C8-, C18 bonded silica), mostly retaining hydrophobic compounds is similar to reversed phase HPLC (RP-HPLC), while the use of polar stationary phases (e.g., silica bonded with aminopropyl, propanediol, bare silica), where polar analytes are the most retained, is comparable to normal phase HPLC (NP-HPLC) [101,102]. A unique retention pattern is achieved by using stationary phases with mixed polarity (e.g., C18-bonded silica with polar endcapping, silica with aromatic ligands) [64].

Typically, the column is the main determinant of the separation. The selectivity may be further improved by changing the modifier type or the modifier gradient, and enhanced by using additives, changing the mobile phase pressure, or varying the column temperature [98]. Additionally, thanks to the relatively low viscosity of the mobile phase and the compatibility of the same mobile phase with a variety of stationary phases, it is possible to couple different columns to create a system behaving like a new column with a different selectivity.

In SFC, 5 µm fully porous particle packed columns are mostly employed. More recently there is a growing trend in the use of sub-2 micron fully porous particles and core shell particles [2].

Retention in chromatography is generally expressed by the retention time ($t_r$), or retention factor ($k$):

$$k = \frac{t_r - t_0}{t_0} \qquad \text{(Eq. 15)}$$

where $t_0$ is the column void time [103].

**Mobile phase**

Carbon dioxide is the main eluent in SFC. Properties and advantages of $CO_2$ are introduced in section 3.2.1. $CO_2$ is too apolar to elute polar compounds [2]. To increase the mobile phase polarity and facilitate the elution of polar compounds, modifiers are added to carbon dioxide. As a result, analyte solubility in the mobile phase is increased, while analyte interactions with the stationary phase are decreased [2]. Addition of organic modifiers with H-bond acceptor properties can also have a positive effect on peak shapes [2]. Short chain alcohols, i.e., methanol, ethanol, and isopropyl alcohol, are the most universal organic modifiers in SFC [104]. Because of its high eluotropic strength, high polarity, low viscosity, low UV cut-off value, availability, relatively low toxicity and inexpensiveness, methanol is the most widely used modifier [90,104]. Acetonitrile, or a mixture of acetonitrile and methanol, can improve selectivity [104].

In general, even small concentrations of polar modifiers can increase the solvent strength. A decrease in retention is usually observed with an increasing amount of organic modifier, with a stronger decrease up to 10% of the modifier. In practice, modifier concentrations are mostly kept below 50% [64,105]. An exception to this is enhanced-fluidity liquid chromatography, where an amount of $CO_2$ up to 50% is added to a methanol/water mobile phase. The purpose of adding $CO_2$ in this mode is to reduce the viscosity of the mobile phase, improving separation efficiency, and reducing analysis time by increasing flow rate and changing solvent strength [64].

When analysing strong acids and bases, or amphoteric compounds, carbon dioxide with organic modifiers may still lack sufficient elution strength or provide poor peak shapes. For these analytes, the addition of a highly polar additive promotes the peak elution and symmetry [90,98]. The most used acidic additives are acetic acid, formic acid, citric acid, and trifluoroacetic acid (TFA), while commonly used basic additives are isopropylamine (IPA), diethylamine, and trimethylamine. In general, acidic additives are beneficial for the analysis of organic acids, while basic additives are preferred for the analysis of bases, resulting in a suppressed ionization of analytes and improved interaction with certain stationary phases, such as for example polysaccharide-based chiral stationary phases (CSP) [2,3]. For the simultaneous analysis of acids and bases, both an acidic and a basic additive may be mixed with the modifier. Volatile additives, such as ammonium formate, ammonium hydroxide, and ammonium acetate, can also improve peak shapes of both acidic and basic analytes, and are compatible with MS detection. Depending on the nature of the analytes and the stationary phase, the concentration of the additives is usually in the range of 0.1 to 2% [2]. In addition to acids and bases, water can also be used as an additive in SFC mobile phases, and serves to improve peak shapes and the elution of polar analytes [2].

**Extra-column dispersion**

A parameter that can negatively affect resolution and efficiency of the SFC separation is extra-column dispersion. It is a band broadening occurring outside of the chromatographic column. The properties of supercritical fluids, such as low viscosity and high diffusivity, contribute to increased extra-column band broadening effects. The extra-column dispersion can be reduced by optimizing sample solvent and injection volume, minimizing volume of the detection cell and extra column volume, and by using efficient columns with appropriate dimensions [106,107].

**Backpressure and temperature**

The mobile phase density, which partially determines the solvent strength, depends on the temperature, pressure, and composition of the mobile phase [3,62]. The effect of pressure on retention is quite straightforward. An increase in pressure results in higher density, which leads to lower analyte retention. Mobile phases with organic modifiers have a lower compressibility, and increased density compared to pure $CO_2$, therefore a pressure variation will have a limited impact on density and retention [98,104].

Temperature has a more complex effect on SFC separation. An increase in temperature will reduce the mobile phase density, resulting in higher retention factors [3]. However, an increase in temperature will cause increase in vapor pressure of the fluid resulting in lower retention factors [2]. Pressure and temperature are considered secondary parameters for fine tuning a method [62]. Generally, changes in temperature result in smaller effects on retention, but may have a significant effect on selectivity, as the temperature variations have an effect on the chemical properties of the mobile phase and the analytes [2,62,90]. It is therefore recommended to start SFC method development at intermediate values of pressure and temperature, which are for instance 150 bar and 25-30°C, respectively [64].

**Flow rate**

Generally, higher flow rates tend to shorten the run time of an analysis. However, in SFC the increase in the flow rate also increases the pressure drop in the column and in the system. Because the backpressure regulator is keeping the outlet pressure constant, an increase in pump pressure is observed, which results in a mild increase in density and decrease in retention [90].

**Sample solvent**

In SFC, the samples are often diluted in pure modifier, e.g., methanol. The modifiers have a considerably higher elution strength than the SFC mobile phase, which can cause peak distortion and affect the separation efficiency. This can be mitigated by reducing the injected volumes. Non-polar solvents with a polarity closer to $CO_2$, such as hexane and heptane, are considered to be better solvent option in SFC compared to pure methanol or other modifiers [108,109]. Aprotic solvents, such as methyl *t*-butyl ether, dichloromethane, acetonitrile, and cyclopentyl methyl ether, are well suited for injections of large volumes [110].

## 3.3 Application of CE and SFC for the separation of structurally similar compounds

CE was successfully utilized in separation of various structurally similar compounds, including analysis of phytochemicals [1], impurity profiling [14], or separation of structural isomers [111–114]. Additionally, the separation of enantiomers has proven to be one of the most prominent applications of CE. The first CE separation of enantiomers was achieved by Zare's group and published in 1985 [115]. However, the potential of this technique for enantiomer separation was recognized later [116,117]. The major advantage of this technique is its flexibility, because a large variety of chiral selectors, that may possess their own mobility in the BGE, can be used individually or in a combination. The concentration of the chiral selectors in a BGE can also be easily varied [40,118]. Chiral CE has been successfully applied in food, environmental, agrochemical, clinical, and pharmaceutical analysis [31,118].

For the separation of enantiomers, two strategies can be employed, i.e., direct or indirect. The basis of a direct chiral separation is the formation of transient diastereomeric complexes between a chiral selector that is part of the BGE, and the chiral analytes. In an indirect chiral separation, both enantiomers undergo a derivatization procedure with a chiral reagent, forming diastereomeric derivatives of the target analytes, which possess different physico-chemical properties and thus can be separated in an achiral environment [119,120].

The most widely used chiral selectors in CE are cyclodextrins (CD). CDs are cyclic oligosaccharides, produced during starch digestion by the enzyme cyclodextrin glycosyltransferase. Those applied in CE contain 6 (α-CD), 7 (β-CD), or 8 (γ-CD) glucopyranose units. The units form a hollow torus, with a lipophilic cavity and a hydrophilic outside surface. The wider rim contains the secondary 2- and 3-hydroxyl groups and the narrower rim the primary 6-hydroxyl groups. These hydroxyl groups can be derivatized with charged or uncharged substituents, creating a large variety of CDs [40,121]. The chiral separation is based on a difference in affinity of the inclusion of the hydrophobic group of the analyte into the hydrophobic cavity and lateral interactions of the hydroxyl groups with the analyte [121,122]. Benefits of CDs are their UV-transparency, structural variety and commercial availability; they can be used either in aqueous and nonaqueous BGEs [40,121]. Other important chiral selectors in CE are crown ethers, macrocyclic antibiotics, proteins, and chiral surfactants [122].

Similar to CE, SFC found its use in impurity profiling or in analysis of structural isomers [123–125]. However, one of the key applications of SFC are chiral separations, with the first chiral SFC separation reported also in 1985 [126]. Enantioselectivity in SFC was found superior when compared to that of HPLC. Additionally, the analysis time in chiral SFC is often shorter, resulting in faster chiral screening and method development when compared to HPLC. Chiral SFC separations are important especially in the field of pharmaceutical analysis, bioanalysis, and in preparative separations (for preparative purposes) [127,128].

In chromatography, most chiral separations are conducted by using a CSPs. The selection of a stationary phase is a crucial step in chiral method development. CSPs contain chiral selectors (polysaccharides, CDs, proteins, and macrocyclic antibiotics). The chiral separation is enabled

through the formation of transient diastereoisomeric complexes of different stability between the stationary phase and the solutes. Polysaccharide-based CSPs are the most widely used. To enhance the enantioselective interactions, chlorinated and methylated polysaccharide derivatives were developed. Nowadays, a variety of amylose and cellulose-based chiral stationary phases selection is available, such as amylose tris(3,5-dimethylphenylcarbamate), cellulose tris(3,5-dimethylphenylcarbamate), cellulose tris(methylbenzoate), cellulose tris(3-chloro-4-methylphenylcarbamate), cellulose tris(4-chloro-3-methylphenylcarbamate), or amylose tris(3-chloro-4-methylphenylcarbamate). There are several reasons for their popularity, namely the wide application window, high loading capability, long-term stability when used under proper conditions, and the availability of numerous different chemistries. The selectors in the CSPs are available in coated form, showing a broad enantioselectivity, or in immobilized form, allowing moreover the use of stronger solvents, without the risk of stationary phase bleeding [2,3,129].

Although the majority of chiral SFC separations is done using polysaccharide-based phases, other CSPs, containing CDs, macrocyclic antibiotics, proteins, synthetic polymer, or Pirkle-type selectors are also used [3].

## 3.4 Experimental designs

Two strategies can be employed for the optimization of an analytical method, i.e. univariate and multivariate optimization. When using univariate optimization, the influence of only one factor at a time is monitored, while interactions between factors are not taken into consideration. As only one factor at a time is varied, this approach leads to an increase in the number of experiments to develop the method, when the number of factors increases. Consequently, this strategy is more time consuming, results in an increased consumption of materials and reagents, while global optimal conditions may not be found [130,131].

On the other hand, multivariate optimization allows the simultaneous evaluation of multiple factors at several levels. In general, this strategy takes less effort, time, and resources than univariate optimization, while it allows the collection of a large amount of information from a limited number of experiments [130,131].

When using the multivariate approach by means of a design of experiments (DOE), two design categories can be identified based on the objectives of the experiments: screening designs and optimization designs (response surface designs) [130,132]. The aim of the screening designs is to identify the most dominating factors and occasionally of factor interactions by calculating their effects. The purpose of the optimization designs is to identify the conditions with the best possible performance. The selection of the design depends mainly on number of factors, but also on goals of the study, feasibility, time consumption, and cost-effectiveness of the experiments [132,133].

Full Factorial designs (FFD), Fractional Factorial designs (FrFD), and Plackett-Burman designs (PBD) are the most frequently used screening designs [132,133]. FrFD and PBD are used when large number of factors need to be evaluated, while when using FFD, the number of factors should not exceed four, as this would require too many experiments [131].

The optimization is usually carried out after the screening step, where the most significant factors were identified. Three-level FFD, Central Composite designs (CCompD), Box-Behnken designs (BBD), Taguchi designs (TD), and Doehlert designs or D-optimal designs are typically used for optimization. BBD is used for modelling of three or more factors, all studied at three equally spaced levels. Doehlert designs do not have the same number of levels for all studied factors, which is beneficial when factors are subject to instrumental limitations. Doehlert designs require smaller number of experiments and are considered as a more practical alternative to BBD [130,132].

A fractional factorial design used for screening is a two-level design. Using a FrFD, a relatively large number of factors can be evaluated at two levels in a rather limited number of experiments (table 2). In FrFD, some effects are estimated together. Because of these confounded estimations, not all major factor effects and interaction effects are estimated separately. The smaller the fractional of the full factorial the less experiments are required but the more effects are estimated confounded. However, confounding also causes loss of some, usually considered not significant, information [130,133]. For a fraction factorial design the number of experiments (*N*) is:

$$N = L^{k-p} \qquad \text{(Eq. 16)}$$

where *L* is the number of examined levels, *k* the number of factors, and *p* is the number of independent design generators, with $1/2^p$ representing the fraction of the full factorial (*p* = 1, 2, 3, etc.) [130,131].

**Table 2:** Experimental matrix for a sixteenth-fraction factorial two-level screening design for seven variables [131].

| **Experiment** | **Factors** | | | | | | |
|---|---|---|---|---|---|---|---|
| | **A** | **B** | **C** | **D** | **E** | **F** | **G** |
| **1** | -1 | -1 | -1 | 1 | 1 | 1 | -1 |
| **2** | 1 | -1 | -1 | -1 | -1 | 1 | 1 |
| **3** | -1 | 1 | -1 | -1 | 1 | -1 | 1 |
| **4** | 1 | 1 | -1 | 1 | -1 | -1 | -1 |
| **5** | -1 | -1 | 1 | 1 | -1 | -1 | 1 |
| **6** | 1 | -1 | 1 | -1 | 1 | -1 | -1 |
| **7** | -1 | 1 | 1 | -1 | -1 | 1 | -1 |
| **8** | 1 | 1 | 1 | 1 | 1 | 1 | 1 |

The central composite designs are the most frequently applied response surface designs, used for modelling responses as a function of two or more factors. These designs combine a two-level full factorial design ($2^k$ experiments), a star design (2k experiments), and a centre point. The factors are usually studied at five levels (Table 3, Fig. 7). The number of experiments (*N*) to examine a given number of factors in a CCompD is defined as follows:

$$N = 2^k + 2k + Cp \qquad \text{(Eq. 17)}$$

where *k* is the number of factors and *Cp* the number of centre point replicates [130,131].

**Table 3:** Experimental matrices for central composite design of two variables [131,134].

| | $k_1$ | $k_2$ |
|---|---|---|
| Factorial design | -1 | -1 |
| | 1 | -1 |
| | -1 | 1 |
| | 1 | 1 |
| Axial points | -α | 0 |
| | α | 0 |
| | 0 | -α |
| | 0 | α |
| Central point | 0 | 0 |

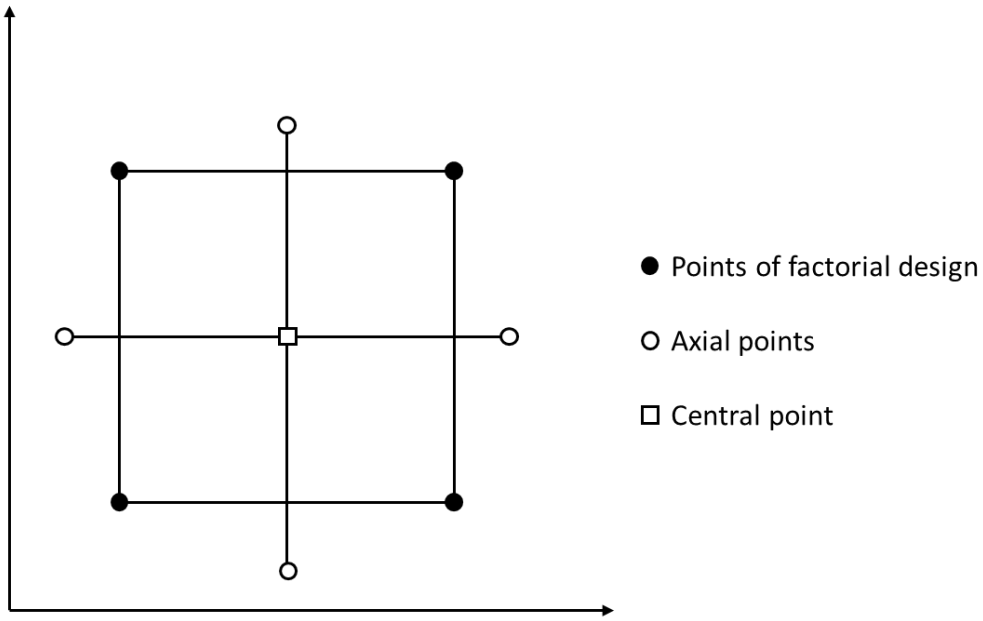


**Figure 7:** Representation of central composite design for the optimization of two variables. Reproduced with permission from [134].

## 3.5 Indomethacin

### 3.5.1 Indomethacin chemistry and impurities

Indomethacin (IND), [1-(4-chlorobenzoyl)-5-methoxy-2-methylindol-3-yl]acetic acid, is a non-steroidal anti-inflammatory drug (NSAID). IND was patented in 1961 and subsequently approved for medical use in 1963 [135,136].

The main mechanism of action of IND is non-selective cyclooxygenase inhibition. Cyclooxygenase is an enzyme that catalyses the biosynthesis of prostaglandins. Prostaglandins have a main role in the generation of an inflammatory response, with symptoms including inflammation, fever, and promotion of pain [137,138].

IND has analgesic and antipyretic properties. It is effectively used for the treatment of rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, and Alzheimer's disease. It can also be used to heal conjunctival inflammation and to accelerate closure of patent ductus arteriosus. IND is widely available in different dosage forms, such as gels, sprays or suppositories [139–141].

IND can have serious adverse effects, such as gastric intolerance and toxicity, renal and pulmonary toxicity, or headache. Other, less common, side effects are light-headedness, confusion, or hallucinations [142].

IND is a white or yellow crystalline powder, which is almost insoluble in water and sparingly soluble in alcohol. It shows polymorphism and can exist as several crystalline forms. The log P of IND is 4.27 and the pKa value of its carboxylic group is 4.5 [41,143].

Ten structurally related compounds are listed in the European Pharmacopoeia as IND impurities, including 5-methoxy-2-methyl-3-indoleacetic acid (MMIAA), 4-chlorobenzoic acid (CHBA), 3,4- dichloroindomethacin (DCHIND) (Fig. 8). The content for each unspecified impurity is limited to 0.10 %, with a total maximum of 0.3% and a reporting threshold of 0.05% [41]. The United States Pharmacopeia defines two related compounds, 5-methoxy-2-methyl-3-indoleacetic acid and 4-chlorobenzoic acid. The acceptance criteria are set to 0.1 and 0.5%, respectively. The limit for unspecified impurities is 0.1% and the total amount of impurities must not exceed 1.0% [42].

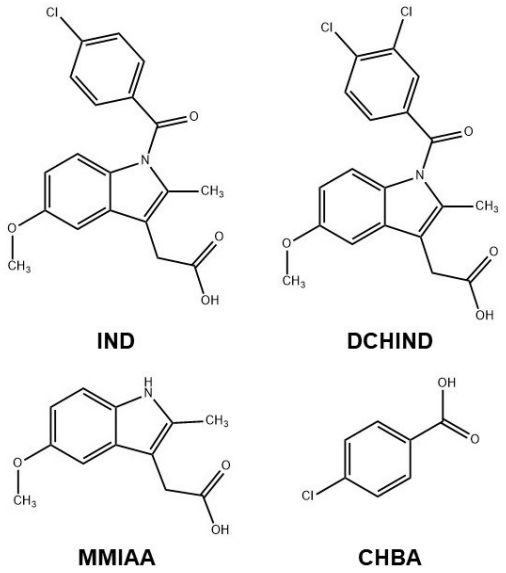


**Figure 8:** Structures of indomethacin (IND) and its three defined impurities 5-methoxy-2-methyl-3-indoleacetic acid (MMIAA), 4-chlorobenzoic acid (CHBA), and 3,4-dichloroindomethacin (DCHIND).

### 3.5.2 Analysis methods for indomethacin and its impurities

Because of their good analytical performance, chromatographic methods are the most commonly used for the determination of IND and its impurities [136]. In the European Pharmacopoeia, the IND assay is carried out using an RP-HPLC method with a C18 stationary phase and mobile phase consisting of acetonitrile, water, and acetic acid. For purity testing, an HPLC method with phenylhexylsilyl-modified silica as the stationary phase is used with detection at 254 nm. The mobile phase contained acetonitrile, water, and formic acid [41].

Several HPLC methods have been developed for the determination of IND and its degradation products, using phenyl stationary phase [144], particle-based RP-phenyl stationary phases of different particle sizes, and monolithic C18 columns with both isocratic [145,146] and gradient elution chromatography [147]. The determination of IND and other NSAIDs was achieved using a cyano- column in an LC-MS method [148].

A capillary electrophoresis, using nonaqueous electrolytes, was applied for the separation of IND from other NSAIDs [149]. Lin et al. [150] developed a method for the determination of IND in premature infants with patent ductus arteriosus, using a BGE consisting of Tris buffer and an anionic surfactant sodium octanesulfonate. A CE method for the determination of traces of NSAIDs in saliva was developed using a combination of solid-phase extraction and large volume stacking with polarity switching in MEKC [151]. The CZE and MEKC methods for the determination of NSAIDs in biological samples were reviewed by Makino et al. [152]. MEKC was efficiently used for determination of NSAIDs in environmental samples [153].

## 3.6 Silymarin

### 3.6.1 Silymarin chemistry and pharmacognosy

Milk thistle (*Silybum marianum*) is a wild herbaceous plant from the Asteraceae family, typical of the Mediterranean regions of Europe, but also growing for centuries in parts of Africa, America, Asia, and Australia. The plant is made of thorny stems, red-purple flowers, and dazzling green leaves with milky white veins. The fruits are black, oblong-shaped achenes [154,155].

The milk thistle fruits have been used for thousands of years to treat respiratory, liver, biliary tract and digestive disorders, such as cough, hepatitis, cirrhosis and fibrosis of the liver, or as an antidote to mushroom poisoning, snake bites, and insect stings [155–157].

The main active component of milk thistle is silymarin [154]. Silymarin is a crude extract from milk thistle fruits. It contains 70-80 % silymarin flavonolignans, namely diastereomers silybin A (SBA) and B (SBB), diastereomers isosilybin A (ISBA) and B (ISBB), silychristin (SCH), and silydianin (SD), along with 20-30% fatty acids and other polyphenolic components, including the flavonoid taxifolin (TX) (Fig. 9) [154,157,158]. Silymarin flavonolignans generally contain 2 to 4 asymmetric centres. Therefore, they usually occur in nature as various stereoisomers [156,158]. The first identified component of silymarin was silybin (SB), followed by SCH. To date, over 20 compounds of the flavonolignan complex were specified in milk thistle [156].

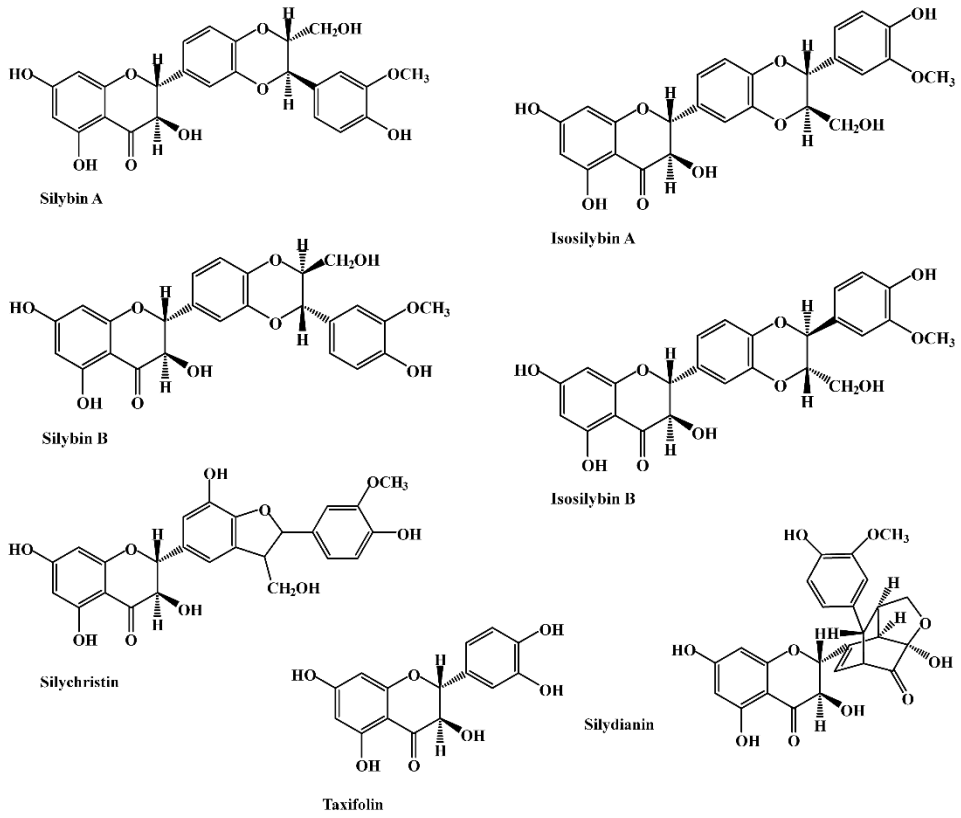


**Figure 9:** Structures of flavonolignans and taxifolin.

Following oral administration, silymarin is distributed to the stomach, intestine, liver, and pancreas. Silymarin reaches its peak plasma concentration after 6-8 hours and is excreted mainly in the form of bile metabolites. Silymarin has a poor aqueous solubility, which contributes to its low bioavailability [154,156].

According to the European Pharmacopoeia and USP, the silymarin content should be in the range of 30-65% in milk thistle dry extract [41,42]. Silymarin should consist of SCH and SD (20–45%), SBA and SBB (40–65%), and ISBA and ISBB (10–20%) [41,42]. Silymarin preparations should contain 90-110% of its labelled amount, as defined in the USP [42]. Dietary supplements are not included in the European and the Czech Pharmacopoeias [41,159]. Therefore the requirements for their quality are significantly lower, i.e., dietary supplements in the Czech Republic are assessed only for their safety, while their efficacy is not evaluated [160].

Silymarin shows a diverse biological and pharmacological potential by interacting directly or indirectly with several molecular targets, including inflammatory mediators, transcription factors, protein kinases, enzymes, and receptors [157]. Recent clinical and preclinical research revealed that silymarin has hepatoprotective, antioxidant, anti-diabetic, anti-cancer, neuroprotective, anti-viral, and photoprotective activities. Additionally, milk thistle strengthens the capillaries, regulates blood pressure, and promotes intestinal regularity [155,157].

Silymarin is available in the form of a suspension, tablets, capsules, granules, powders, tinctures, syrups, and even as a dried ripe fruit [154,155,157]. The milk thistle is a safe, well-tolerated herbal medicine without contra-indications or serious side effects, except mild gastrointestinal or allergic reactions [155,156].

### 3.6.2 Analysis methods for flavonolignans in silymarin

HPLC has been most widely applied for the analysis of silymarin complex [156]. Because of the moderate hydrophobicity (log P = 2.59) of the flavonolignans, reversed-phase stationary phases, such as C-18, have been used for the separation. Because the flavonolignans are weak acids, in general, an acidic mobile phase containing methanol is used for the separation [161–167]. An example of such a method is the LC method described in European Pharmacopoeia. The method is based on a gradient elution on an endcapped octadecylsilyl silica gel stationary phase with a mixture of phosphoric acid, methanol and water as mobile phase [41].

A sensitive LC-ESI/MS method was developed for the simultaneous determination of SBA, SBB, ISBA, ISBB, SCH, and SD, achieving complete separation of the diastereomers using a C18 column and a gradient mobile phase consisting of 5 mM ammonium acetate adjusted to pH 4.0 with formic acid as component A and methanol, water and formic acid in component B. The method was used for the identification and quantification of the silymarin flavonolignans in commercial products [164]. Another method achieving baseline separation of six main active constituents of silymarin used isocratic elution on a C18 column and acidified methanol and water as mobile phase. This LC-ESI/MS method was applied for the determination of flavonolignans in human plasma [168]. A core-shell column with a pentafluorophenyl stationary phase and UV detection was utilized in another method with methanol and phosphate buffer as mobile phase. Baseline separation was achieved for SBA, SBB, ISBA, ISBB, SCH, SD, and TX [169].

Limitations of DAD and UV detection, namely the sensitivity, were solved by developing LC-MS methods for silymarin separation and determination. In LC-ESI-MS method, six flavonolignans were separated with gradient elution on a C18 column with a mobile phase consisting of ammonium acetate, MeOH, water, and formic acid [170]. In a UHPLC-QTOF-MS method, the separation of 11 silymarin compounds was performed on a High Strength Silica-based reversed-phase analytical column with T3 bonding using a mobile phase consisting of water, MeOH, ammonium acetate and acetic acid [171].

As an alternative to the HPLC methods, two CE methods were developed. In the first, silymarin was analysed in an uncoated fused-silica capillary with a BGE of pH 9.0 containing borate buffer, 2,6-dimethyl-β-cyclodextrin, and MeOH [172]. For the second method, a fluorinated ethylene-propylene copolymer capillary was utilized. The BGE consisted of an aqueous solution of ε-aminocaproic acid, ammonium hydroxide, polyvinylpyrrolidone, and hydroxyethyl cellulose [173]. Baseline separation of the flavonolignans was not achieved by neither of the two CE methods.

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# 4 Results and discussion

## 4.1 Development of a micellar electrokinetic chromatography method for the determination of three defined impurities in indomethacin

### 4.1.1 Introduction and summary of the paper "Development of micellar electrokinetic chromatography method for the determination of three defined impurities in indomethacin"

IND is a NSAID used in various forms, mainly for the treatment of rheumatoid arthritis and associated disorders. Ten structurally related impurities of IND are listed in the European Pharmacopoeia [1]. Their amounts in bulk drug as well as in formulations are limited. Mainly RP-HPLC methods were previously used for the determination of IND and its impurities. Although capillary electrophoresis methods for the separation of IND from other NSAIDs or for the determination of IND in biological fluids were developed, no CE method that could be applied for purity testing of IND was reported.

In this study, a micellar electrokinetic chromatography method was developed for determination of three IND impurities, i.e., MMIAA, CHBA, and DCHIND in the parent drug; 1-naphthylacetic acid was used as internal standard. This study resulted in a paper P. Riasová, D. Doubková, L. Pincová, O. Jung, M. Polášek, P. Jáč; Development of micellar electrokinetic chromatography method for the determination of three defined impurities in indomethacin Electrophoresis 39 (20) (2018) 2550-2557.

The method development was carried out by a design of experiments. During the screening phase, a quarter-fraction factorial design was applied to identify the most influential separation parameters on the method performance. During the CZE analysis, i.e., with BGE containing phosphate buffer and organic modifier, the baseline resolution of DCHIND and IND was not achieved. Addition of SDS contributed to a longer analysis time, but also resulted in a different migration order of the analytes. Under MEKC conditions, the resolution of DCHIND and IND was no longer critical, and the average resolution was always higher than in the CZE experiments. Therefore, MEKC was used for further optimization.

Following the screening, a central composite face-centred design was used for the optimization of the BGE composition. The factors SDS concentration, content of methanol, concentration of phosphate buffer, and pH of the buffer were studied at three levels. The selected responses for optimization were migration time of the last analyte, resolution between CHBA and IND, and average resolution.

Based on the data from the optimization, it was concluded that concentration of SDS and content of MeOH had the most significant effect on the analysis time and resolution between IND and CHBA. The average resolution was affected mostly by the SDS concentration, phosphate buffer concentration and its pH. To find the optimal BGE composition, the following response settings were considered: (i) response migration time of the last analyte was minimized, with 10 min as target value and 15 min as maximum value, (ii) response resolution between CHBA and IND was maximized, with a minimum value of 7.5 and target value of 10, (iii) response average resolution was maximized, with a minimum value of 5 and

a target value of 7.5. The three responses were handled with the same weight. Optimal separation conditions were calculated based on the effects of the tested factors on the selected responses.

The optimized BGE consisted of 20 mM phosphate buffer pH 7.57 containing 58 mM SDS and no MeOH. The separation was carried out in a 64.5/56 cm fused silica 50 µm i.d. capillary with an extended light path (150 µm i.d.) detection window, at 30 kV with DAD detection at 224 nm. Sufficient resolution of all compounds with Rs ≥ 3.5 was achieved within 10 min. Correlation between the predicted and observed values for responses migration time of the last analyte and resolution between IND and CHBA were excellent, with deviations of 0.99 and 2.34 %, respectively. The agreement between predicted and observed values of average resolution was acceptable, with a deviation of 17.89 %.

Thanks to the multivariate approach in the method development, the number of experiments required was kept relatively low compared to a univariate approach. In total, 38 experiments were required for screening and optimization. More importantly, the use of a design of experiments for the method development enabled the simultaneous study of several factors including their interactions on the method performance.

The method was validated in a range of 1.25–80.00 µg/mL of each impurity in terms of linearity, precision, and accuracy. The calibration curves were rectilinear with coefficient of determination ($r^2$) exceeding 0.9994. RSD of intermediate precision were in the ranges 3.02 – 6.65% and 1.30 – 9.49% (n = 9) for variations of migration times and peak area ratios, respectively. The accuracy of the method was tested on IND gel with average recoveries (n = 3) of 102.0 ± 1.6, 97.7 ± 2.5, and 92.5 ± 2.3 % for MMIAA, CHBA, and DCHIND, respectively. The method was applied for purity testing of the IND bulk drug and IND gel. In both cases, the concentration of IND was 2.50 mg/mL. MMIAA found in the bulk drug was below the limit of quantification, while no DCHIND or CHBA was detected in the sample. Similarly, no DCHIND was found in the topical gel. However, the contents of MMIAA and CHBA detected in the topical gel sample corresponded to 0.64% ± 0.07% and 1.06 % ± 0.07% relative to the IND content.

The novel MEKC method exhibits worse validation characteristics regarding the precision and accuracy than conventionally applied HPLC methods. However, the method developed in this study was validated for DCHIND for the first time and was applied for the determination of impurities in IND gel.

**References**

[1] European Pharmacopoeia, 9th Edition, Council of Europe, Strassbourg 2014.

### 4.1.2 Full manuscript “Development of micellar electrokinetic chromatography method for the determination of three defined impurities in indomethacin”

*This full text is not included in the online version of the thesis. This full text can be found either in the full printed version of the thesis (pages 49-64) or online under this doi: 10.1002/elps.201800080.*

## 4.2 Development of a capillary electrophoresis method for the separation of flavonolignans in silymarin complex

### 4.2.1 Introduction and summary of the paper "Development of a capillary electrophoresis method for the separation of flavonolignans in silymarin complex"

Silymarin is an extract derived from milk thistle, which consists mainly of structurally similar flavonolignans. It has been historically used for the treatment of liver disorders. Many other beneficial health effects have also been reported. Both the European Pharmacopoeia and United States Pharmacopeia define the contents of the main flavonolignans, SBA, SBB, ISBA, ISBB, SCH, and SDA. Silymarin should consist of 20 - 45% of SCH and SDA, 40 – 65% of SB A and SB B, and 10 – 20% of ISB A and ISB B. According to the United States Pharmacopeia, dietary supplements with silymarin should contain 90 – 110% of its claimed amount.

RP-HPLC based methods have been typically used for the analysis of flavonolignans in silymarin, using a C18 stationary phase and a mixture of MeOH and acidified aqueous solution as mobile phase. The two CE methods described in the literature did not achieve baseline separation of all flavonolignans.

In this work, a CE method for the separation of six main active flavonolignans of the silymarin complex and the flavonoid taxifolin was developed. The suitability of different separation modes for the separation of structurally similar compounds, including two pairs of diastereomers, SB A/B and ISB A/B was tested. This study resulted in a paper P. Riasová, J. Jenčo, D. Moreno-González, Y. Vander Heyden, D. Mangelings, M. Polášek, P. Jáč, Development of a capillary electrophoresis method for the separation of flavonolignans in silymarin complex, Electrophoresis 43 (2022) 930-938.

At a first stage, the method development was focussed on the separation of the SB diastereomers. Baseline separation of SBA and B was achieved by addition of SDS to the BGE. However, SDS had no influence on the separation of ISBA/B. In a second stage, various CDs were tested for the separation of the ISB diastereomers. The best results were achieved with 2-hydroxypropyl-β-cyclodextrin, but the addition of this CD had no effect on the separation of SBA and B. In a next step, a combination of these two separation approaches, i.e., a cyclodextrin-modified MEKC (CD-MEKC) method, was optimized for the separation of two pairs of diastereomers and subsequently transferred to the separation of all seven analytes. During the optimization, significant changes in migration times that caused unrepeatable and unpredictable migration order were observed. Therefore, the CD-MEKC method was not further developed.

In a second approach, an EKC method was developed. The effect of the heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin (TM-β-CD) concentration on separation was studied in the range 1.0-15.0 mM. Partial separation of both diastereomer pairs was achieved using this CD. Therefore, the other separation parameters including capillary length, pH of the borate buffer, and methanol content were optimized further.

Because of the variations in migration times and similar UV spectra of analytes observed in the first optimization approach, univariate optimization was used for both approaches. This means that only the effect of one factor at a time on the method performance was examined, which showed to be a time-consuming strategy. After a relatively large number of experiments for univariate approach (around 50 experiments) compared to the multivariate approach (27 experiments), a baseline separation of all seven analytes was achieved. The optimized background electrolyte consisted of 100 mM boric acid (pH 9.0) containing 5 mM TM-β-CD, and 10% (v/v) of methanol. The separation was carried out in an 80.5/72 cm (50 µm id) fused silica capillary at + 25 kV with UV detection at 200 nm. The resolution between the diastereomers of SBA/B and ISBA/B was 1.73 and 2.59, respectively. Genistein was used as internal standard.

The optimized method was validated for all 7 analytes in a concentration range of 2.5-50 µg $mL^{-1}$ in terms of linearity, precision, and accuracy. The calibration curves were rectilinear with correlation coefficients ≥ 0.9972. The method was applied for the assay of two dietary supplements containing milk thistle extract. The accuracy of the method was evaluated by comparing the results of the novel EKC method with the Unites States Pharmacopeia HPLC assay. A t-test showed no statistically significant difference between both methods. The presented EKC method confirmed that both preparations meet the requirements of the European Pharmacopoeia and United States Pharmacopeia.

The novel CE method is less sensitive compared to LC-UV methods, or other chromatographic methods coupled to a very sensitive MS detector. Although the separation takes almost 25 minutes, the analysis time is shorter than that of the chromatographic methods used in the European Pharmacopoeia and the United States Pharmacopeia. The CE method fulfils two principles of green analytical chemistry (GAC). The 12 GAC principles, as described in proposal of Gałuszka et al [1], are providing guidelines for making analytical laboratories greener. The key components of GAC principles are elimination or reduction of the use of hazardous or toxic chemicals, minimization of energy consumption, analytical waste management and increased safety. The CE method has a lower reagent consumption, smaller sample volumes, and therefore a reduced environmental impact.

For simplicity reasons, the term capillary electrophoresis (CE) will also be used to indicate capillary electromigration (CE) techniques that combine chromatographic principles as well as electromigration, such as electrokinetic chromatography (EKC), micellar electrokinetic chromatography (MEKC).

**References**

[1] Gałuszka, A., Migaszewski, Z., Namieśnik, J., The 12 principles of green analytical chemistry and the SIGNIFICANCE mnemonic of green analytical practices, Trends Anal. Chem. 50 (2013), 78–84. https://doi.org/10.1016/j.trac.2013.04.010.

### 4.2.2 Full manuscript “Development of a capillary electrophoresis method for the separation of flavonolignans in silymarin complex”

*This full text is not included in the online version of the thesis. This full text can be found either in the full printed version of the thesis (pages 67-88) or online under this doi: 10.1002/elps.202100212.*

## 4.3 Coupling of chiral and achiral stationary phases in supercritical fluid chromatography: evaluating and improving retention prediction

### 4.3.1 Introduction and summary of the paper "Coupling of chiral and achiral stationary phases in supercritical fluid chromatography: evaluating and improving retention prediction"

The benefits of SFC in enantioseparation are already well established. The most crucial step in chiral method development is the selection of a suitable stationary phase. Since this is mainly based on a trial-and-error approach, this process is typically time consuming and labour intensive.

Because of the relatively low viscosity of supercritical fluids, it is possible to couple several columns in series to create improved chromatographic selectivity. To study the retention behaviour of analytes on coupled column systems, a test set, consisting of the chiral compounds atenolol, ephedrine, propranolol, mianserin, labetalol, and nadolol, the diastereomers quinine and quinidine, and the structural isomers of aminophenol and aminocresol was analysed. The isomers were screened on the individual chiral columns Lux Cellulose-1, Lux Cellulose-2 (LC-2), Lux Cellulose-3 (LC-3), Lux Cellulose-4, Lux Amylose-2 and the achiral columns Luna NH2, Luna Silica, Synergi RP, and FluoroSep RP and on 60 coupled-column systems. The screening was performed using generic chromatographic conditions that employed a mobile phase composed of 20% MeOH, containing 0.1% (v/v) IPA and 0.1% (v/v) TFA, combined with 80% $CO_2$. The flow rate was 3.0 mL/min, the back pressure was set to 150 bar and the column temperature was maintained at 30°C. This study resulted in a paper P. Riasová, P. Jáč, M. Polášek, Y. Vander Heyden, D. Mangelings, Coupling of chiral and achiral stationary phases in supercritical fluid chromatography: evaluating and improving retention prediction, Journal of Chromatograpy A 1667 (2022) 462883.

Three equations were used to estimate the retention behaviour of the analytes on coupled systems. Their prediction precision was evaluated by comparing the predicted retention factors to the experimentally obtained. The following equation provided the best correlation between predicted and experimental retention factors:

$$k_{A,B} = \frac{\Phi_A k_A + \Phi_B k_B}{\Phi_A + \Phi_B}$$

where $k_{A,B}$ is the predicted retention factor of an analyte in a coupled column system, $k_A$ and $k_B$ are the experimental retention factors on the individual stationary phases, and $\Phi_A$ and $\Phi_B$ represent the column lengths of the stationary phases. Relative deviations of predicted and experimental retention factors ranged from 0.0% to 51.9% using this equation with a median value between 4.7 and 58.2%. In this work, reversal of enantiomer elution order was not studied.

To improve prediction precision of the equations, flow rate and backpressure of the screening conditions were adjusted to generate the same average pressure in the individual column and the coupled system. The average relative deviations of retention factors were reduced to 2.8% - 6.6% by adjusting the flow rate, and to 2.3% - 8.6% by adjusting the back pressure.

While adjusting backpressure provided faster analysis, the adjustment of flow rate provided a higher correlation between the experimental and predicted results. Additionally, when adjusting the backpressure, the prediction of the elution sequence of the closely eluting compounds was unreliable.

The applicability of the flow rate adjustment approach was demonstrated by means of the selection of a column combination for the separation of main silymarin flavonolignans and the flavonoid taxifolin. The same column set was screened, with Lux Cellulose-2 and Lux Cellulose-3 providing the best results. Co-elution of two compounds was observed during the screening when using the two chiral columns separately. However, in the coupled column system, at least a partial resolution of all seven analytes was observed.

This study proposes a strategy for the reliable prediction of the retention behaviour of analytes on a coupled column system in SFC. The limited number of initial analyses needed for the column selection, in addition to the SFC characteristics, also present a reduced impact on the environment.

### 4.3.2 Full manuscript “Coupling of chiral and achiral stationary phases in supercritical fluid chromatography: evaluating and improving retention prediction”

*This full text is not included in the online version of the thesis. This full text can be found either in the full printed version of the thesis (pages 91-114) or online under this doi: 10.1016/j.chroma.2022.462883.*

## 4.4 Chromatographic behaviour of selected flavonolignans from silymarin in supercritical fluid chromatography with coupled chiral columns

### 4.4.1 Introduction and summary of the paper "Chromatographic behaviour of selected flavonolignans from silymarin in supercritical fluid chromatography with coupled chiral columns"

As previously described, silymarin consists of flavonolignans SBA, SBB, ISBA, ISBB, SCH, SD, and flavonoid TX. Due to the structural similarity of the flavonolignans, their separation might be challenging. Thanks to the characteristics of supercritical fluids, when coupling columns SFC may provide a unique selectivity, compared to HPLC. Because the stationary phase is the main determinant of selectivity, the selection of the stationary phase is considered to be the most important factor in method optimization. To select the best performing column combination, the findings from the previous work were employed (Section 4.3).

A selection of chiral (Lux Cellulose-1, LC-2, LC-3, Lux Cellulose-4, Lux Amylose-1 (LA-1), Lux Amylose-2) and achiral (Luna NH2, Luna Silica, Synergi Polar RP, FluoroSep-RP Phenyl) columns was screened. The screening was done with a 40% MeOH containing 0.1% IPA and 0.1% TFA and 60% $CO_2$ as mobile phase, with a backpressure of 150 bar and a column temperature of 30°C. The flow rate was adjusted for each column to generate the same system pressure. The retention factors in coupled columns were calculated from the screening results using the equation that was established as providing the best correlation between calculated and experimental values from the previous research. This resulted in a manuscript that is still in preparation.

The most promising column combinations showed to be those of the LC-2 and LC-3 columns, and of the LC-3 and LA-1 columns. Different sequences of the columns in coupled systems were tested. The better performing, in terms of resolution, were LA-1 + LC-3 and LC-3 + LC-2. In the next stage, a selection of organic modifiers was tested: ethanol, isopropyl alcohol, acetonitrile, and methanol. A partial separation of all seven analytes was only observed when methanol was used as organic modifier.

In the next step of method optimization, for both coupled column systems, the effects of the content of MeOH, flow rate, concentration of additives, backpressure, and column temperature on the method performance were studied. A quarter-fraction factorial design was used to examine effects of the five factors at three levels on the resolution of critical peak pairs and on the analysis time.

The significance of the effects of the five studied factors was evaluated using Dong's algorithm. The content of MeOH and the column temperature had a significant effect on the separation in both systems. Based on the effect plots from the screening and the results from additional experiments determining the most suitable column temperature, the best separation conditions were determined. For the LA-1 + LC-3 coupled system, the chromatographic conditions were 40% of MeOH, 2.8 mL/min, 0.1% IPA and TFA, 125 bar and 30°C. Resolution for both SCH and SD, and ISBB and ISBA was 0.62. The best separation conditions on the LC-3 + LC-2 coupled system were 40% MeOH, 2.3 mL/min, 0.1% IPA and

TFA, 140 bar and 40°C. The Rs between SCH and ISBB, and ISBB and SBA was 0.64 and 0.38, respectively.

The method development further focused on the optimization of the sample solution and mobile phase additives. However, the only improvement was seen when only 0.1% TFA was used separately as an additive in the mobile phase. The resolution between SCH and SD, and ISBB and ISBA increased on the LA-1 + LC-3 system to 0.85 and 0.70, respectively. The resolution between SCH and ISBB, and ISBB and SBA remained somewhat constant, with values of 0.70 and 0.42, respectively.

The study demonstrated the successful selection of columns to be used in coupled systems, based on a limited number of experiments. Additionally, this research confirms that the selection of the stationary phase is the most important factor in SFC method development, while illustrating the optimization of other factors.

However, even after extensive optimization, baseline separation of the analytes was not achieved on a coupled column system in SFC. The previously described CE method (section 4.2), where a baseline separation was achieved for all 7 analytes, and which was successfully validated and applied for the determination of silymarin in pharmaceutical products, is therefore a better alternative for the separation of silymarin components.

## 4.4.2 Full manuscript "Chromatographic behaviour of selected flavonolignans from silymarin in supercritical fluid chromatography with coupled chiral columns"

**Chromatographic behaviour of selected flavonolignans from silymarin in supercritical fluid chromatography with coupled chiral columns**

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*Paper in preparation*

**Abstract**

Milk thistle is a medicinal plant that has been used from ancient times as a remedy for a variety of ailments. The active constituent of milk thistle is silymarin, a mixture of flavonolignans and the flavonoid taxifolin. A supercritical fluid chromatographic (SFC) method was optimized for the simultaneous separation of seven silymarin components: diastereomers of silybin (silybin A and silybin B), diastereomers of isosilybin (isosilybin A and isosilybin B), silychristin, silydianin, and taxifolin. The SFC method was optimized on two coupled column systems. A quarter-fraction factorial design was used to study the effects of modifier content, flow rate, additives concentration, backpressure and column temperature on the resulting separation. Other optimized parameters were the type of organic modifier, sample solvent and type of additives. Baseline separation for all analysed compounds could not be achieved. The best performing method was obtained on a Lux Amylose-1 + Lux Cellulose-3 coupled column system with the following analysis conditions: a mobile phase with 40% MeOH containing 0.1% isopropylamine and 0.1% trifluoracetic acid in CO2, a flow rate of 1.23 mL/min, a backpressure of 150 bar and a column temperature of 30°C. The resolutions between the critical peak pairs silychristin and silydianin, and isosilybin A and B were 1.81 and 0.82, respectively.

**Keywords:** silymarin, stereoisomers, method optimization, supercritical fluid chromatography, column coupling

**Abbreviations**

SBA - silybin A, SBB - silybin B, ISBA - isosilybin A, ISBB - isosilybin B, SCH - silychristin, SD - silydianin, TX – taxifolin, MeOH - methanol, EtOH – ethanol, 2-PrOH – isopropyl alcohol, ACN – acetonitrile, Hep - heptane, LC-2 - Lux Cellulose-2, LC-3 - Lux Cellulose-3, LA-1 - Lux Amylose-1, Rs - resolution

## 1. Introduction

Milk thistle (Silybum marianum L. Gaernt.) is an annual to biannual plant from the Asteraceae family, with native habitats in southern Europe, southern Russia, Asia Minor and northern Africa [1]. Medicinal properties of milk thistle have been known for thousands of years [2]. A mixture of flavonolignans isolated from milk thistle, collectively known as silymarin, is responsible for its pharmacological activity, [1]. Nowadays, silymarin is widely used in medicinal preparations and dietary supplements for the treatment of liver disorders, such as cirrhosis, hepatitis, or alcoholic liver diseases [3]. It was reported to have antioxidant, anti-inflammatory, immunomodulating and anti-fibrotic properties [4–7]. Silybin was classified by WHO in the 1970s as official medicine with hepatoprotective properties [8].

Silymarin consists mainly of flavonolignan isomers, including the diastereomers silybin A (SBA) and silybin B (SBB), isosilybin A (ISBA) and isosilybin B (ISBB), silychristin (SCH), silydianin (SD) and the flavonoid taxifolin (TX) (Fig. S-1) [9,10]. The major component of silymarin, representing 50 – 70%, is silybin [1]. Silybin has properties of a weak acid in neutral aqueous solutions. It has a rather hydrophobic character and its solubility in water is poor despite having hydrophilic groups. Silybin is insoluble in non-polar solvents (chloroform, petroleum ether), only poorly soluble in polar protic solvents (ethanol, methanol), but soluble in polar aprotic solvents (acetone, dimethylformamide or tetrahydrofuran)[10].

An LC method for the separation and quantification of the silymarin flavonolignans is described in the European Pharmacopoeia. The method is based on gradient elution on an endcapped octadecylsilyl silica gel stationary phase with a mixture of aqueous phosphoric acid solution and methanol as mobile phase [11]. Analytical methods developed to separate the silymarin flavonolignans are summarized in the review of Csupor et al. [12], showing a prevalence of HPLC methods using C-18 and other types of reversed phase (RP) stationary phases combined with acidic mobile phases, containing methanol as organic modifier.

The first baseline separation of the silymarin flavonolignans SBA, SBB, ISBA, ISBB, SCH and SD, was achieved using isocratic elution on an RP stationary phase and using a mixture of methanol and water acidified with glacial acetic acid as mobile phase. The method was used to determine the flavonolignans in human plasma [13]. The disadvantage of the HPLC methods are their relatively long analysis times (usually > 30 min) [14–17]. The analysis time was reduced with the development of a UHPLC methods [18]. Capillary electrophoresis methods were also employed in the analysis of silymarin [9,19,20].

Gros et al. [21] developed an on-line SFE – SFC approach for extraction and analysis of nonpolar and polar compounds from milk thistle seeds. Polar compounds were analysed on the Chiralpak IJ-3 column, using MeOH:H2O 95:5 (v/v) + 0.1% of formic acid as a co-solvent. The compounds were analysed under the following gradient: 30% of co-solvent for 1 min, increase to 50% in 6 min, 50% of co-solvent for 2 min, reduced to 30% in 0.1 min, 30% of co-solvent until the end of analysis. Separation of 6 polar compounds was reported, with 4 peaks identified as taxofolin, silibyn A, silibyn B, and isosilybin.

Supercritical fluid chromatography (SFC) is a separation technique, known from the 1960s [22], that uses sub- or supercritical CO2 as mobile phase. CO2 has a critical pressure of 73.8 bar and a critical temperature of 31.1°C [23], so its supercritical state can easily be reached. Thanks to the lower viscosity of the mobile phase, higher flow rates can be used on the separation columns, resulting in shorter analysis times and less solvent consumption, compared to classical HPLC [24,25].

SFC gained popularity as a preferred technique for the separation of chiral analytes [26]. Because of the unpredictability of enantioselectivity, chiral method development is a labour-intensive process, often heavily based on trial-and-error approaches [27]. The choice of the column is the most important

step in the method development, as the properties of the stationary phase are the main determinant of the selectivity for the analytes [28].

A low density of the mobile phase, responsible for the low pressure drop along the column, and the compatibility of the mobile phase with a variety of stationary phases are the main characteristics of SFC, allowing column coupling. By coupling stationary phases with diverse selectivities, a new separation medium is created with unique separation properties, potentially providing a possible increase in resolution and efficiency.

Several methods have been published taking advantage of column coupling for the separation of structurally similar compounds, when adequate separation was not achieved using single columns. Phinney et al. [29] used a combination of chiral and achiral stationary phases for the separation of structurally related β-blockers and 1,4-benzodiazepines. Barnhart et al. [30] demonstrated the use of serially coupled Chiralpak AD-H and Chiralcel OD-H columns for the separation of a mixture of four stereoisomers. Benefits of serially coupled achiral and chiral columns for the separation of enantiomers and diastereomers of cinnamonitrile and hydrocinnamonitrile were demonstrated by Alexander end Staab [31]. Tandem coupling of achiral columns was also used for impurity profiling of 25 individual drug substances with various numbers and amounts of impurities [32].

In this paper, the development of an SFC method for the separation of seven components of silymarin, SBA, SBB, ISBA, ISBB, SCH, SD and TX, was described. In-house available chiral and achiral stationary phases with dissimilar properties were screened to separate the silymarin compounds. Two combinations of two serially coupled chiral columns were selected for method optimization based on a methodology developed in [33]. An experimental design approach was applied to further optimize the contents of organic modifier and additives in the mobile phase, flow rate, backpressure and column temperature. In addition, organic modifier type, type of mobile phase additives and sample solvent were varied in an attempt to obtain the best possible separation.

## 2. Material and methods

### 2.1. Chemicals and reagents

The standards of SBB (purity ≥ 95%), ISBA (≥ 95%), SCH (≥ 95%), SD (≥ 95%), TX (≥ 85%) and silymarin were purchased from Sigma-Aldrich (St. Louis, MO, USA). SB (≥ 92.53%) was obtained from HWI Analytik (Rülzheim, Germany). ISBB and SD were provided by PhytoLab (Vestenbergsgreuth, Germany).

Methanol (MeOH), ethanol (EtOH), isopropyl alcohol (2-PrOH), acetonitrile (ACN), heptane (Hep), all HPLC grade, and glacial acetic acid (> 99.9%) were obtained from VWR Chemicals (Fontenay-sous-Bois, France). Trifluoroacetic acid (TFA) (99%), isopropylamine (IPA) (≥ 99.5%) and ammonium acetate (≥ 98%) were purchased from Aldrich (St. Louis), ammonium formate (≥ 99.0%) from Fluka Analytical (Bucharest, Romania) and formic acid (99%) from Biosolve (Dieuze, France). CO2 quality 4.5 (purity > 99.995 %) was provided by Messer (Berchem, Belgium) and ultrapure water was made in-house by a Sartorius Stedim ultra-water purification system (Göttingen, Germany).

### 2.2. Stationary phases

Achiral columns Luna NH2, Luna Silica, both 100 mm x 4.6 mm, 3 µm, and Synergi Polar RP, 100 mm x 4.6 mm, 4 µm, were obtained from Phenomenex (Utrecht, The Netherlands). A FluoroSep-RP Phenyl achiral column with dimensions 150 mm x 4.6 mm, 3 µm was acquired from ES Industries (West Berlin, Germany). The chiral columns Lux Cellulose-1, Lux Cellulose-2 (LC-2), Lux Cellulose-3 (LC-3), Lux Cellulose-4, Lux Amylose-1 (LA-1) and Lux Amylose-2 were purchased from Phenomenex. Dimensions of the chiral columns were 250 mm x 4.6 mm, with 5 µm particle size for the LA-1 column and 3 µm for all other. The columns were coupled in series with PEEK tubing, assembled with a ferrule and a nut at both ends.

### 2.3. Instrument

The experiments were performed on an Acquity Ultra Performance Convergence Chromatography (UPC²) system from Waters (Milford, MA, USA). The system is equipped with a binary pump, a convergence manager, an autosampler with a fixed loop of 10 µL, a backpressure regulator, a photo diode array detector (PDA), and an external column oven. The Empower software (3 V7.10, 2010, Waters) was used for data collection and processing.

### 2.4. Preparation of standard and test solutions

The stock solutions of individual silymarin compounds, SBA, SBB, ISBA, ISBB, SCH, SD, and TX, were prepared by dissolving appropriate amounts of the standards in MeOH to achieve 1 mg/mL concentration. For SB, which contains SBA and SBB, the concentration of the stock solution was 2 mg/mL. Test solutions were prepared by diluting the stock solution with MeOH to achieve a concentration of 100 µg/mL (200 µg/mL for SB). The mixture was prepared by adding 100 µL of each stock solution (for SB, ISBA, ISBB, SCH, SD, and TX)to 400 µL MeOH to achieve a concentration of 100 µg/mL for each compound. The test solutions of individual compounds were injected to determine the elution sequence of the analytes.

To optimize of the sample solvent, silymarin was dissolved in MeOH, EtOH, 2-PrOH, ACN and in mixtures Hep:2-PrOH:EtOH (2:1:2 v/v/v) and Hep:2-PrOH:MeOH (2:1:2 v/v/v) in a concentration 1 mg/mL. The solutions were sonicated 60 min for solvent mixtures containing Hep and 30 min for the other solvents.

### 2.5. Experimental design

A reflected quarter-fraction factorial design (Table 1) was applied to study the effects of SFC related factors on the separation of the silymarin compounds mixture. For both coupled column systems, 16 experiments were performed to examine the effect of five factors: content of organic modifier, flow rate, additives content, backpressure, and column temperature. Each factor was studied at three levels.

The considered responses were resolution (Rs) of critical peak pairs, calculated by the Empower software according to the USP equation [34], and analysis time, determined as the retention time of the last eluting compound. To visualise the effects of the different factors on the responses, effect plots were created by plotting for each factor the average response at a given level as a function of the level.

**Table 1:** Reflected quarter-fraction factorial screening design for five factors at three levels.

| Exp. No. | Factors | | | | |
|---|---|---|---|---|---|
| | **Modifier** | **Flow rate** | **Additives** | **Backpressure** | **Temperature** |
| 1 | 0 | 0 | 0 | +1 | 0 |
| 2 | +1 | 0 | 0 | 0 | +1 |
| 3 | 0 | +1 | 0 | 0 | +1 |
| 4 | +1 | +1 | 0 | +1 | 0 |
| 5 | 0 | 0 | +1 | +1 | +1 |
| 6 | +1 | 0 | +1 | 0 | 0 |
| 7 | 0 | +1 | +1 | 0 | 0 |
| 8 | +1 | +1 | +1 | +1 | +1 |
| 9 | -1 | -1 | -1 | 0 | -1 |
| 10 | 0 | -1 | -1 | -1 | 0 |
| 11 | -1 | 0 | -1 | -1 | 0 |
| 12 | 0 | 0 | -1 | 0 | -1 |
| 13 | -1 | -1 | 0 | 0 | 0 |
| 14 | 0 | -1 | 0 | -1 | -1 |
| 15 | -1 | 0 | 0 | -1 | -1 |
| 16 | 0 | 0 | 0 | 0 | 0 |

### 2.6. Calculations

Retention factors (k) for the analytes are calculated as $(t_R - t_0)/t_0$, where $t_R$ stands for the retention time of the analyte and $t_0$ for the void time, marked as the first disturbance of the baseline after the sample injection.

The following equation [32] was used for the prediction of the retention factors of the silymarin compounds in coupled column systems:

$$k_{A,B} = \frac{\Phi_A k_A + \Phi_B k_B}{\Phi_A + \Phi_B} \qquad \text{(Eq. 1)}$$

where $k_{A,B}$ is the predicted retention factor of an analyte in a coupled column system, $k_A$ and $k_B$ are the retention factors of the compound on the individual stationary phases, and $\Phi_A$ and $\Phi_B$ represent the effective lengths of the stationary phases. The selectivity $\alpha$ is calculated for each consecutive peak pair applying the $k_{A,B}$ values of different compounds.

## 3. Results and discussion

### 3.1. Selection of stationary phase

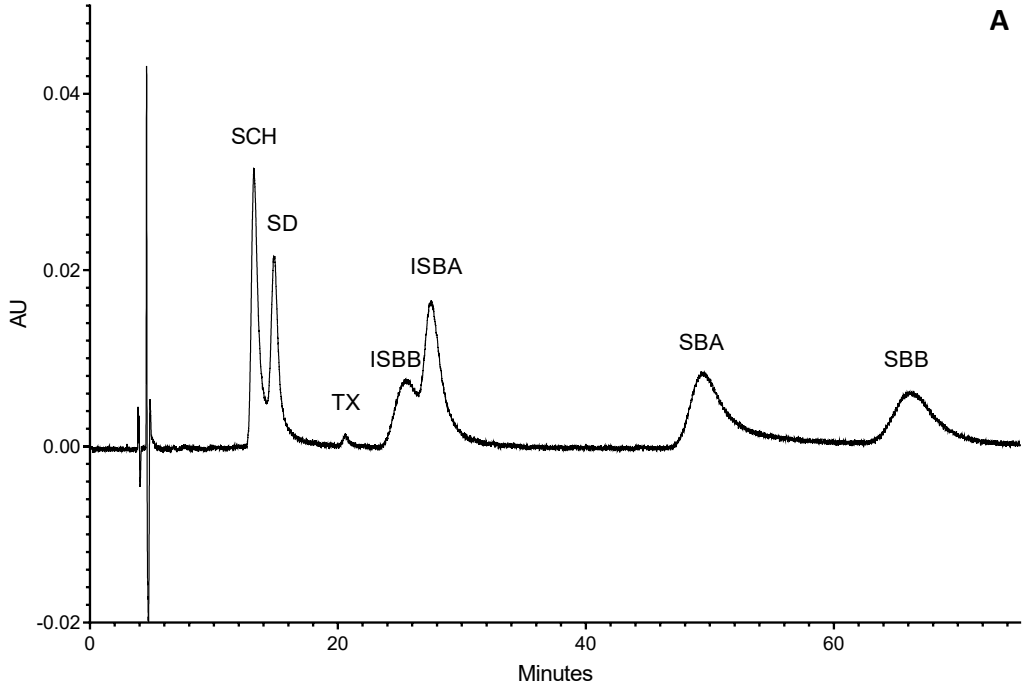
Column combinations with the potential to separate all seven analysed compounds were identified by screening six chiral and four achiral stationary phases separately. The screening was executed with generic chromatographic conditions, with 40% MeOH as organic modifier, containing 0.1% (V/V) IPA and 0.1% (V/V) TFA as additives, a backpressure of 150 bar and a column temperature of 30°C [35]. The flow rate during screening was adjusted for each column to generate the same system pressure of 237 bar with the fixed backpressure of 150 bar. The system pressure of 237 bar was generated by the lowest pressure generating column [32]. Creating the same average pressure contributed to the consistency in separation conditions under which the analytes were separated in different columns.

The separation of all seven analytes was not achieved during the single column screening. The results of the best performing columns selected for coupling are shown in the Fig. S-2.

Based on the single column screening, the retention factors were calculated using Eq. 1 for all column combinations, including at least 1 chiral column (54). The retention factors calculated for each coupled column system were used to estimate the elution sequence of the analytes and the selectivities α. Based on the results of these calculations, the two coupled column systems with the best probability to separate all silymarin compounds in one run, were identified as LC-2 + LC-3, and LC-3 + LA-1.

In the coupled column system, the column positioned first is operating under a higher pressure than the second one. This causes an increase in the mobile phase density and its solvation power, resulting in a decrease in retention compared to the column positioned downstream. To establish the effect of the coupling sequence on the separation performance, four column systems were tested: LC-2 + LC-3, LC-3 + LC-2, LC-3 + LA-1, and LA-1 + LC-3. The flow rate was adjusted to generate the same average pressure as during single column screening, similarly to the approach that was defined in [33].

For the LC-3 and LA-1 stationary phases, slightly better Rs were achieved when the LA-1 column was connected first (Fig. 1A). The elution sequence of the analytes was: SCH, SD, TX, ISBB, ISBA, SBA, and SBB, with Rs 1.81, 7.50, 1.79, 0.82, 5.12 and 2.72, respectively. Resolution of the critical peak pairs in the LC-3 + LA-1 column system, SCH and SD, and ISBB and ISBA, were 1.47 and 0.67, respectively. The best Rs for the combination of LC-2 and LC-3 was achieved when placing the LC-3 column first (Fig. 1C). The analytes eluted in the sequence: TX, SCH, ISBB, SBA, ISBA, SD, SBB with Rs 11.10, 1.11, 0.49, 2.50, 3.10 and 1.28, respectively. Resolution of the critical peak pairs in the LC-2 + LC-3 column system was 1.05 between SCH and ISBB, and 0.30 between ISBB and SBA. The elution sequences of the analytes remained the same in the LC-3 + LA-1 and LC-2 + LC-3 coupled systems (Fig. 1B, D). Compared to the results from single column screening, the coupled columns results provided at least partial separation of all analysed compounds. However, the peak shapes of the analytes in coupled systems were worse, resulting in no baseline separation. The predicted retention factors and retention times were compared to the experimentally obtained ones. The relative deviations of retention factors ranged from 49% to 67% in the LC-3 + LC-2 system and from 70% to 82% for the LA-1 + LC-3 system. The high deviations can be explained by low compressibility of the mobile phase with 40% modifier. This can result, in the longer systems, in different volumes of the mobile phase exposed to different pressures. Because no baseline separation was achieved in any of the coupled systems, the chromatographic conditions, i.e. organic modifier and its content, flow rate, backpressure, column temperature, additives and their concentration, and sample solvent, were further optimized.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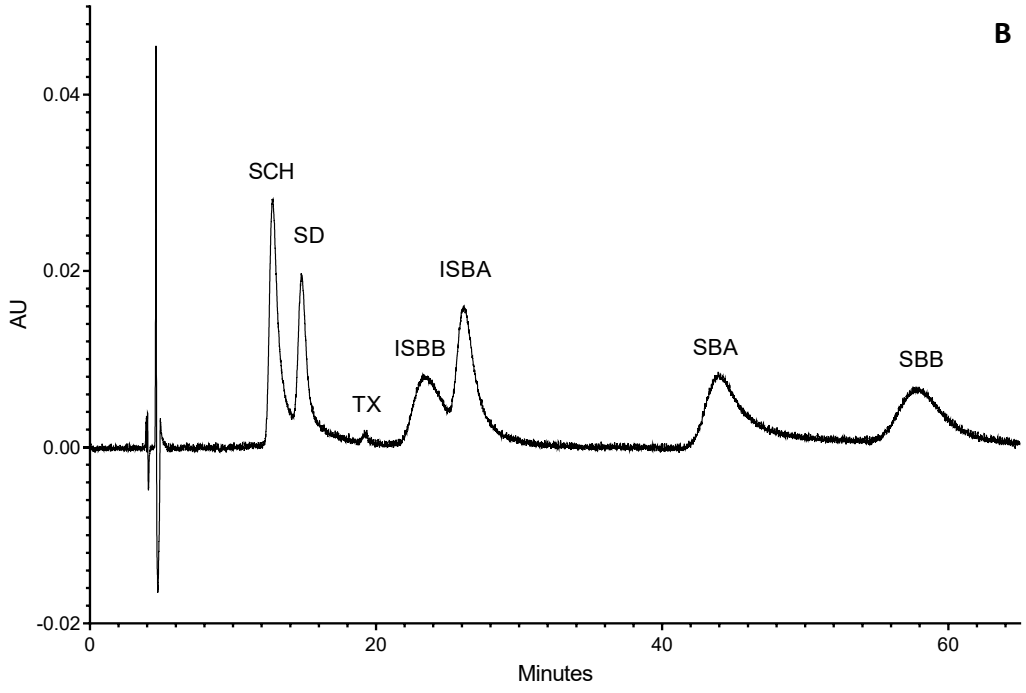
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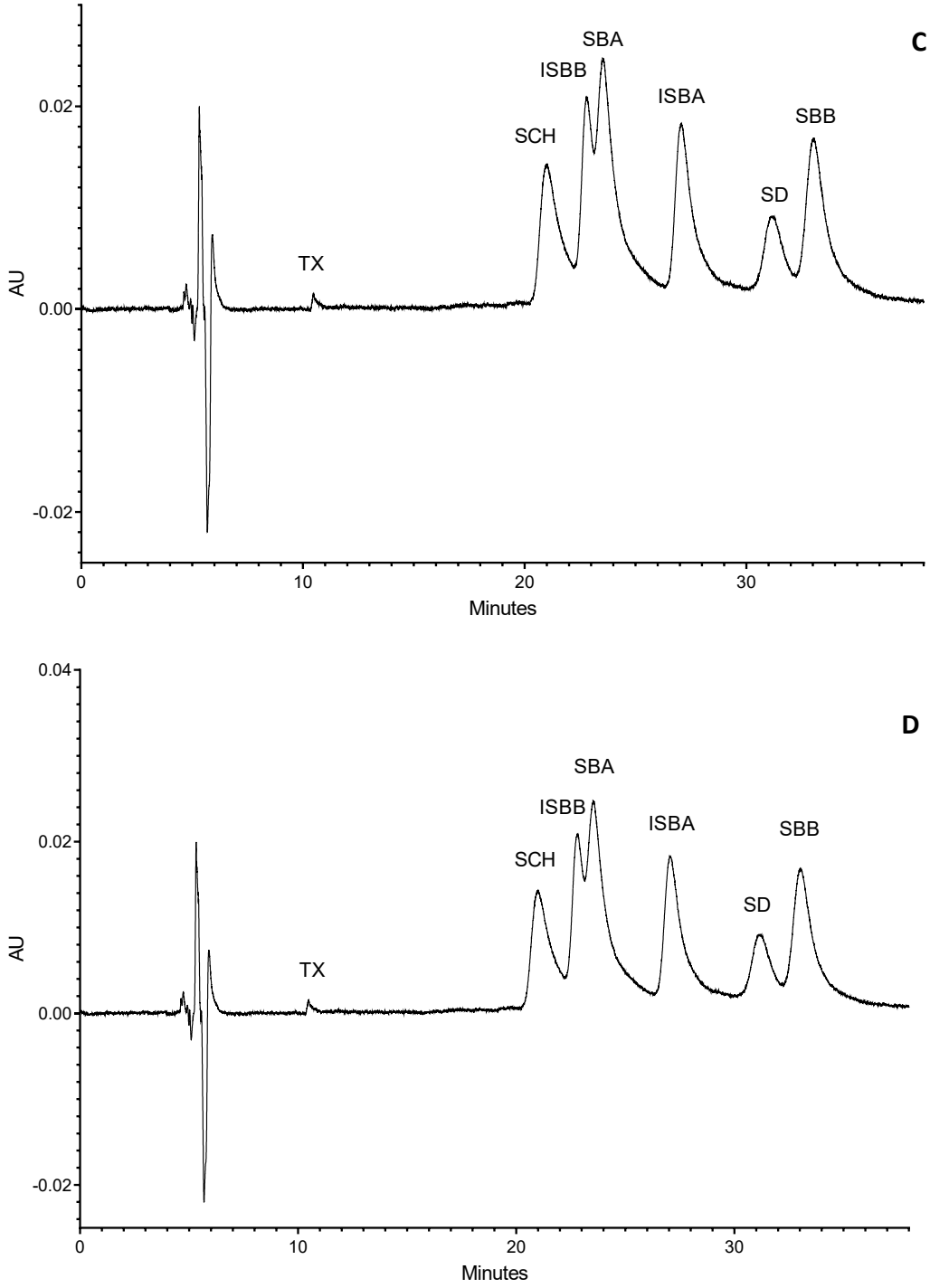



**Figure 1:** Chromatograms of silymarin mixture on the (A) LC-3 + LA-1, (B) LA-1 + LC-3, (C) LC-2 + LC-3, and (D) LC-3 + LC-2 systems, using a mobile phase containing 40% MeOH with 0.1% (V/V) IPA and 0.1% (V/V) TFA, a backpressure of 150 bar, a column temperature of 30°C, with flow rates 1.23 (A, B) and 1.08 mL/min (C, D).

### 3.2. Selection of organic modifier

Alternative organic modifiers, such as EtOH, 2-PrOH and ACN (40% organic modifier containing 0.1% (V/V) IPA and 0.1% (V/V) TFA), were tested for both column combinations, but this did not result in an improved in separation. When EtOH was used, the peaks of SD and ISBB co-eluted in the LA-1 + LC-3 system, with the peaks of ISBB, SBA and SCH did in the LC-3 + LC-2 system. In case of 2-PrOH, the seven

compounds eluted as three peaks on the LA-1 + LC-3 and as four peaks on the LC-3 + LC-2 coupled column system. When ACN was used as an organic modifier, no separation of the analytes was seen on either of both systems.

In general, MeOH tends to generate better results on the polysaccharide based CSPs. Only when MeOH was used as organic modifier, all seven analytes were at least partially separated. Consequently, the method optimization was continued with a mobile phase containing MeOH as organic modifier.

### 3.3. Screening experimental design approach

The screening design was executed on the two previously selected coupled column systems. The effects of five factors were examined at three levels (Table 2). The ranges of the factors modifier content, flow rate and column backpressure were for both column systems selected to accommodate pressure restrictions imposed by the equipment, while examining a broad range of the levels and keeping the analysis times within acceptable limits. The MeOH content was examined in the range of 35 – 40% to keep the analysis time within 1 hour. The additives, TFA and IPA, were tested in concentrations ranging from 0.05 to 0.1% each. Higher concentrations were not used to avoid salt precipitation problems [36]. Further, column temperatures of 20, 30 and 40°C were selected.

**Table 2:** The levels of the five factors examined in the screening design on the LA-1 + LC-3 and LC-3 + LC-2 column systems.

| Factors | LA-1 + LC-3 | | | LC-3 + LC-2 | | |
|---|---|---|---|---|---|---|
| | **-1** | **0** | **1** | **-1** | **0** | **1** |
| Organic modifier (%) | 35 | 37.5 | 40 | 35 | 37.5 | 40 |
| Flow rate (mL/min) | 2.0 | 2.4 | 2.8 | 1.5 | 1.9 | 2.3 |
| Additives (%) | 0.05 | 0.075 | 0.1 | 0.05 | 0.075 | 0.1 |
| Backpressure (bar) | 105 | 115 | 125 | 110 | 125 | 140 |
| Temperature (°C) | 20 | 30 | 40 | 20 | 30 | 40 |

A stronger retention of the analytes was observed with the column combination LA-1 + LC-3. In comparison to the other chiral columns, the LA-1 column contains 5 µm particles, resulting in a lower system pressure being generated using the same flow rates. Therefore, the flow rates investigated for this system ranged between 2.0 to 2.8 mL/min and the selected backpressure range was from 105 to 125 bar. The separation of the silymarin compounds on the LC-3 + LC-2 system, where both columns contained 3µm particles, was examined within the flow rate range 1.5 -2.3 mL/min. Influence of the backpressure was tested within a broader interval of 110 - 140 bar.

The examined responses were the Rs of the critical peak pairs of the separation, i.e. between SCH and SD, and ISBB and ISBA for the LA-1 + LC-3 column system. In the LC-3 + LC-2 column system, the two critical pairs were SCH and ISBB, and ISBB and SBA. Even if baseline separation of SD and SBB was not achieved, the Rs was higher than between SCH and ISBB, and between ISBB and SBA, with better peak shapes. Therefore, this peak pair was not considered as critical. For both column combinations, analysis time was also considered as response.

#### 3.3.1. Influence of the MeOH content

The effect of the MeOH content was examined for both systems at three levels: 35, 37.5 and 40%. In general, using 35% of organic modifier on the LA-1 + LC-3 system caused co-elution of the SCH and SD peaks. The Rs of the other critical pair, ISBB and ISBA, was also the worst when using the lowest level of MeOH content. This can be attributed to a large peak broadening effect when lower contents of

MeOH were used in the mobile phase. Overall, the highest increase in Rs occurred when using 40% MeOH (Fig. 2A).

On the LC-3 + LC-2 column system, the lowest Rs, resulting in the co-elution of SCH and ISBB, was observed in experiments with 35% MeOH in the mobile phase. With an increase of the MeOH content, the separation of both pairs improved. Between 37.5% and 40% MeOH only moderate changes in Rs were seen for both critical peak pairs (Fig. 2B).

For both systems, an increased MeOH content resulted in a decreased retention of the analytes and hence faster analysis times (Fig. S-3A,B).

### 3.3.2. Influence of the flow rate, concentration of additives and backpressure

In the LA-1 + LC-3 coupled system, the Rs of ISBB and ISBA increased with increasing flow rate, while there was practically no change in Rs of SCH and SD between 2.4 or 2.8 mL/min (Fig. 2C). Similar conclusions were drawn when examining the effect of the additives content. The separation of SCH and SD has negligible changes between 0.075% and 0.1% of additive contents, while the Rs of ISBB and ISBA was the highest at 0.1% level (Fig. 2E). Regarding backpressure, the highest Rs was achieved using 125 bar (Fig. 2G).

For the LC-3 + LC-2 column system, the best separation of the critical pairs was, in general, achieved at the highest examined levels of the flow rate, additives concentration and backpressure (Fig. 2D, F, H).

For both coupled systems, shorter analysis times were achieved with chromatographic conditions set at the highest levels of all three factors (Fig. S-3C-H).

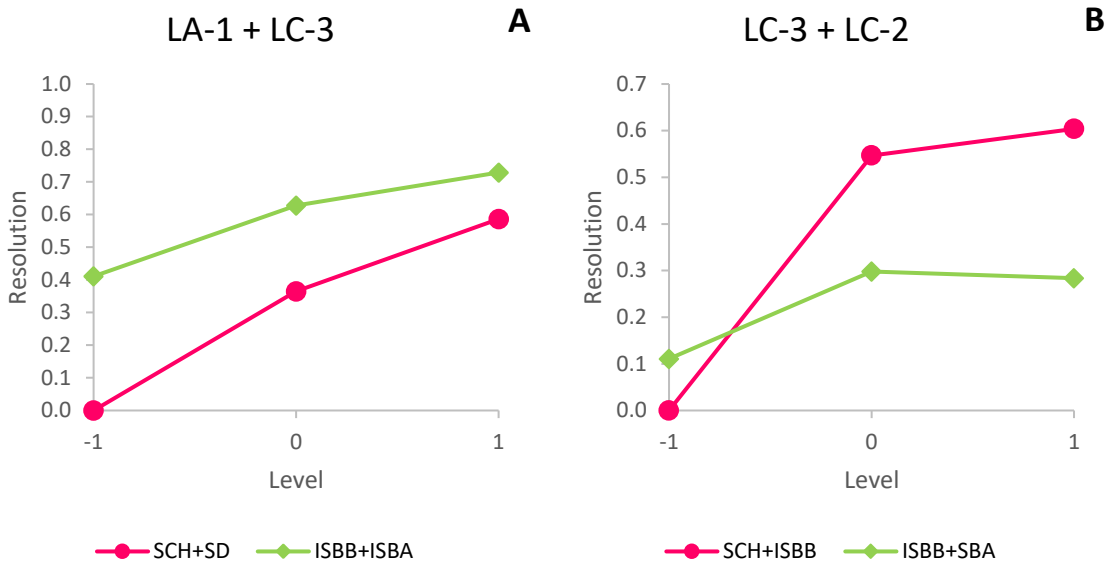
### 3.3.3. Influence of the column temperature

The temperature had a different effect on the separation of the two critical peak pairs using the LA-1 + LC-3 system. While the separation of SCH and SD was the best at 30°C, the highest Rs between ISBB and ISBA was achieved at a column temperature of 40°C (Fig. 2I).

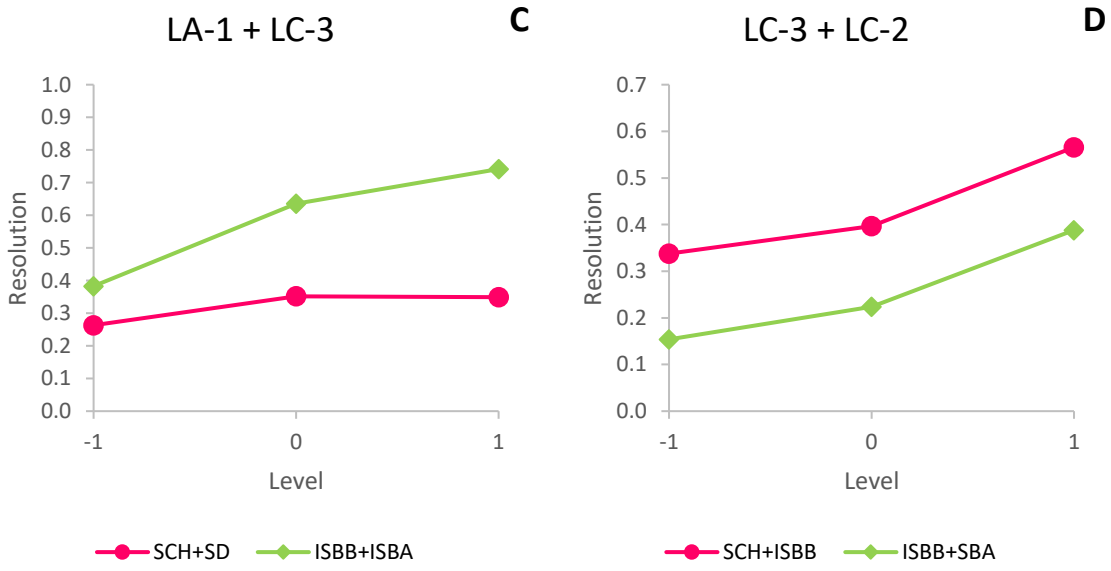
In the LC-3 + LC-2 column system, the Rs between the critical pairs was the lowest at 20°C, resulting in co-elution of ISBB and SBA. Only a minimal change in Rs between SCH and ISBB was observed between 30°C and 40°C, while the best separation of ISBB and SBA was achieved at a column temperature of 40°C (Fig. 2J).

An increase in column temperatures always resulted in faster analysis time in both coupled systems (Fig. S-3I,J).

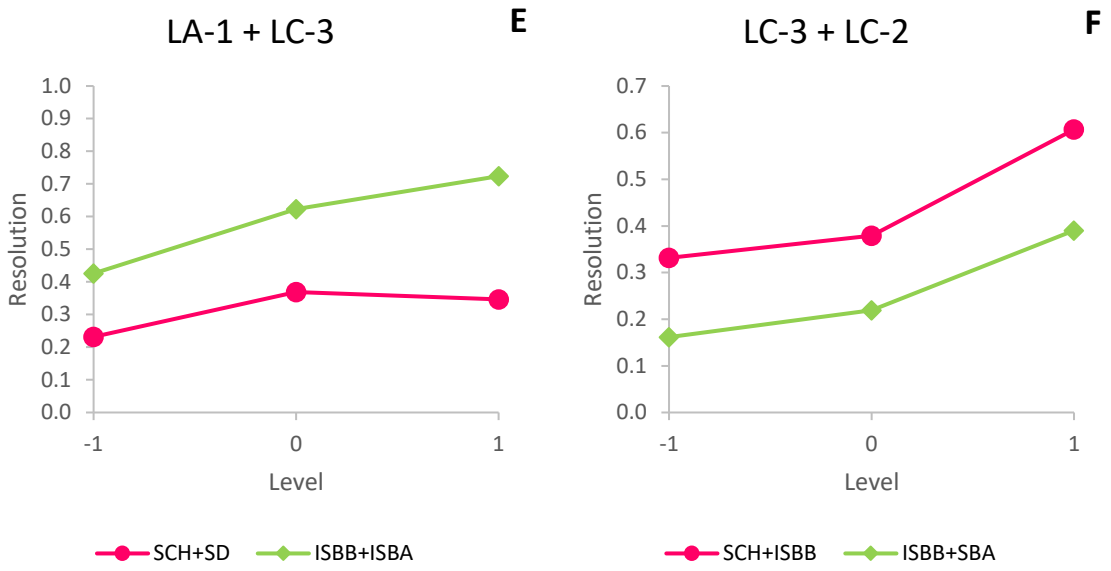
**Content of MeOH**



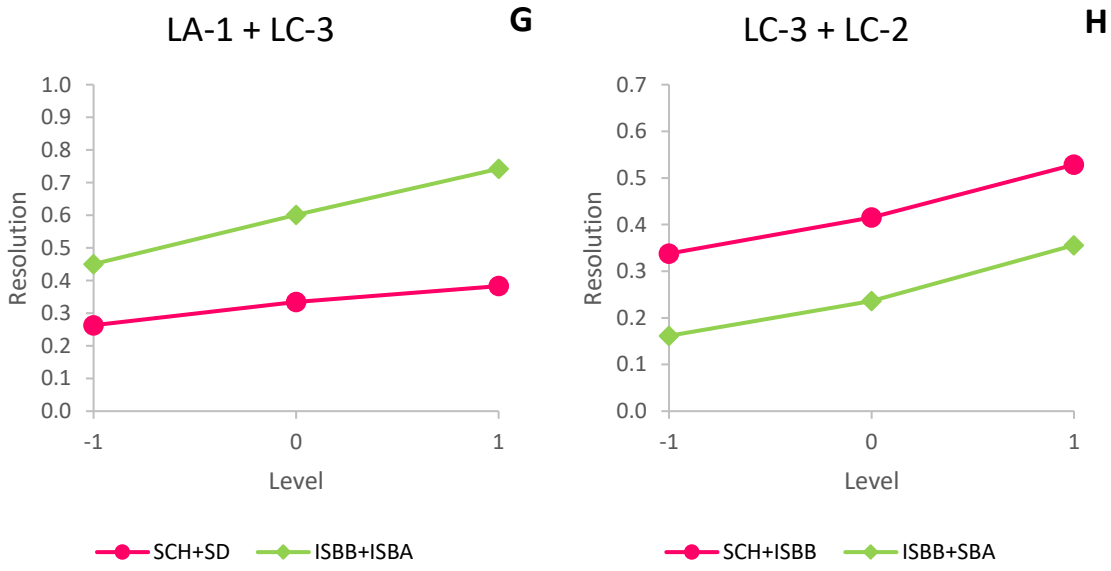

**Flow rate**



**Concentration of additives**




**Backpressure**



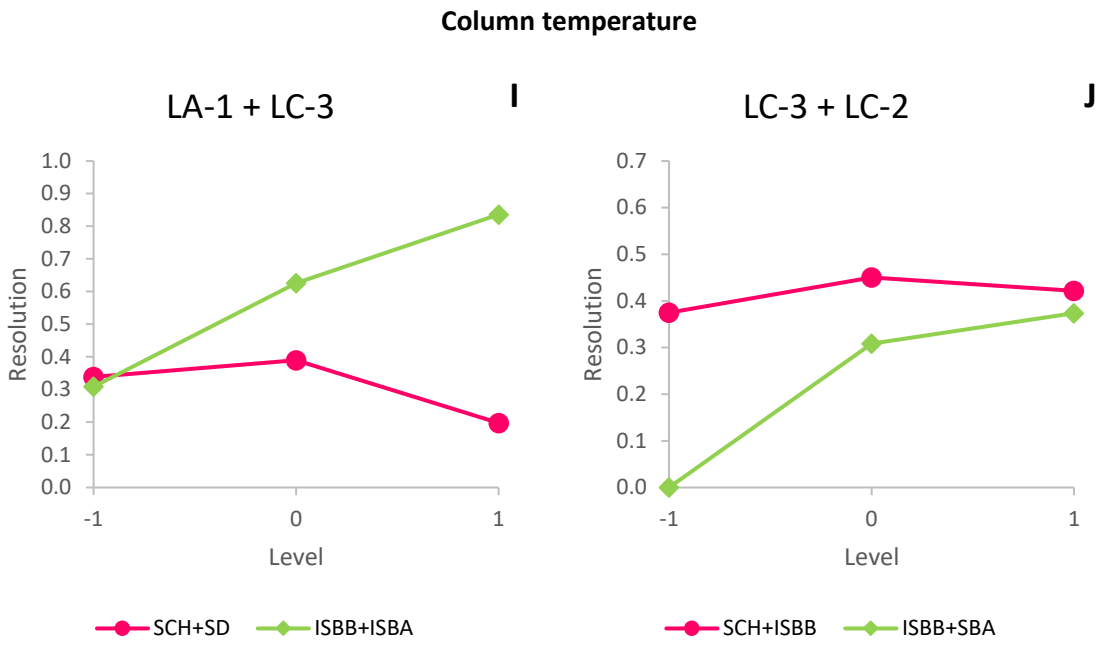



**Figure 2:** Effect plots of the MeOH content (A, B), flow rate (C, D), concentration of additives (E, F), backpressure (G, H), and column temperature (I, J) on the Rs of SCH+SD and of ISBB+ISBA on the LA-1 + LC-3 system (A, C, E, G, I) and the Rs of SCH+ISBB and of ISBB+SBA on the LC-3 + LC-2 coupled system (B, D, F, H, J).

### 3.3.4. Selection of the optimal chromatographic conditions

The significance of the effects of the five studied factors was evaluated based on Dong's Algorithm [37–39]. The MeOH content showed a significant effect on the separation of SCH and SD on the LA-1 + LC-3 system. The effect of temperature, although having opposite effect on the two critical peak pairs, was also determined as significant.

On the LC-3 + LC-2 system, an increase of MeOH content from 35% to 37.5% had a significant effect on the separation of SCH and ISBB. This was also the case on the separation of both critical peak pairs when increasing the column temperature from 20°C to 30°C. However, a further increase of the MeOH content or the temperature had no important effect on the separations.

To establish the most suitable column temperature, analyses on both column systems were performed at 30°C and 40°C. The other chromatographic conditions were all selected at high level, based on the effect plots.

For the LA-1 + LC-3 coupled systems, the best chromatographic conditions were as follows: 40% MeOH, 2.8 mL/min, 0.1% IPA and TFA, 125 bar and 30°C (Fig. 3A). The Rs achieved with these conditions were 0.62 for both SCH and SD, and ISBB and ISBA. The Rs with 40°C as column temperature were worse, with Rs 0.28 and 0.72 for SCH and SD, and ISBB and ISBA, respectively. Compared to the initial separation conditions, the analysis was faster, but the resolution of the critical pairs was lower. Additionally, considerable peak broadening and lower peak heights were observed.

The best separation conditions for the LC-3 + LC-2 column systems were: 40% MeOH, 2.3 mL/min, 0.1% IPA and TFA, 140 bar and 40°C. The Rs of SCH and ISBB, and ISBB and SBA was 0.64 and 0.38, respectively (Fig. 3B). The analysis was faster, while the separation of the critical pairs did not approve, compared to the initial separation conditions. The Rs achieved with 30°C as column temperature for SCH and ISBB was 0.73, while ISBB and SBA co-eluted.

To summarize, the best column temperature was established with additional experiments, the MeOH content in the mobile phase was not increased beyond 40% and no other factors had a significant effect on the separation on either column system. However, after initial optimization, while the analyses on both column systems were faster, the Rs of the critical peak were lower than with the initial method, so no improvement in separation was obtained. The method development therefore further focused on the optimization of other analysis conditions, such as sample solution solvent and mobile phase additives.

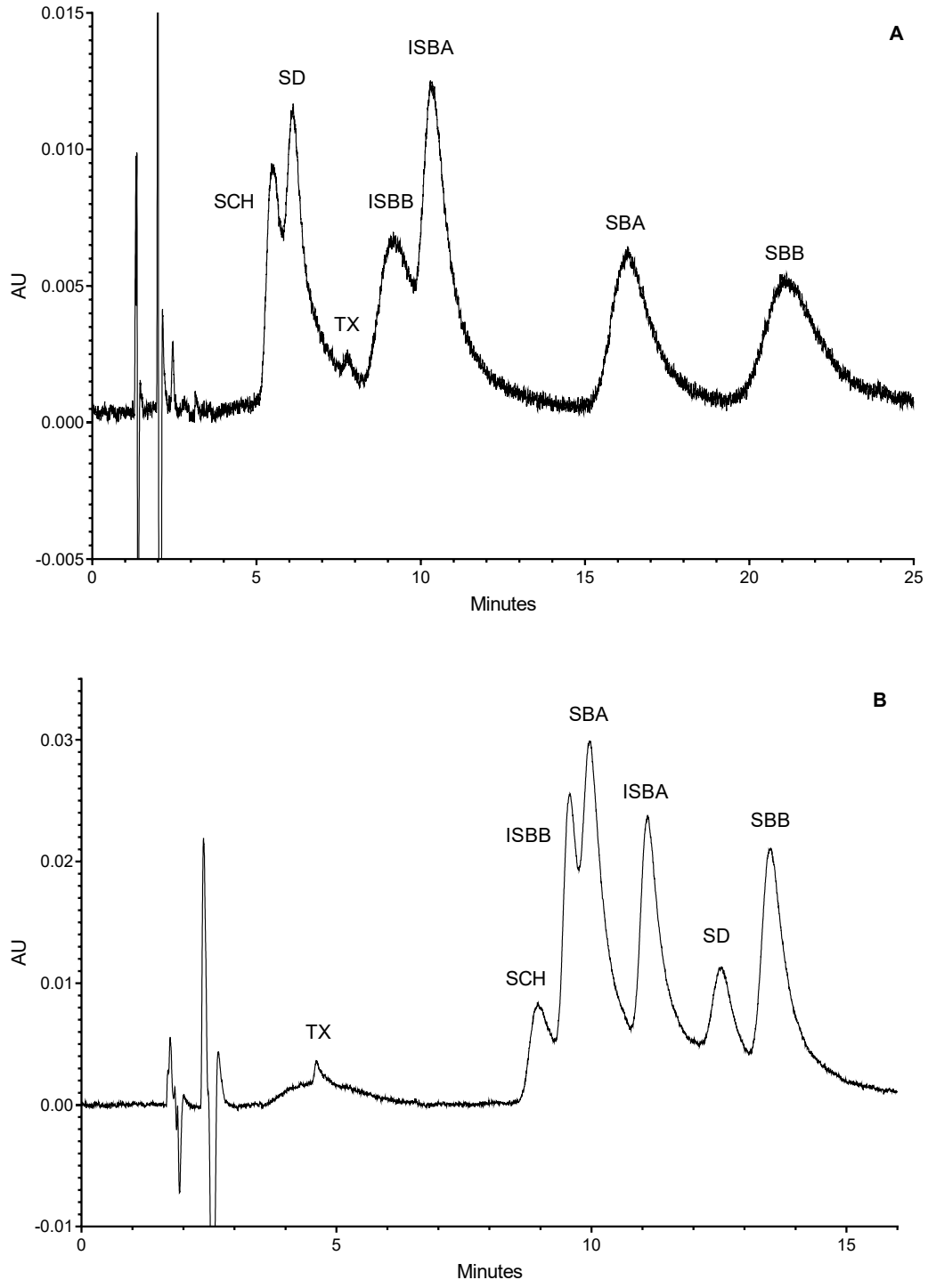



**Figure 3:** Separation of silymarin compounds (A) on the LA-1 + LC-3 system: 40% of MeOH, 0.1% IPA and 0.1% TFA, 2.8 mL/min, 125 bar and 30°C and (B) and the LC-3 + LC-2 system: 40% MeOH, 0.1% IPA and 0.1% TFA,2.3 mL/min, 140 bar and 40°C.

### 3.4. Optimization of the sample solution solvent

The effect of the sample solvent on the peak shapes in SFC was evaluated in the research of Desfontaine et al. [40]. Based on their results, the use of aprotic solvents with a limited adsorption on the stationary phase, such as methyl tert-butyl ether, dichloromethane, cyclopentyl methyl ether or ACN, is recommended as sample solvent. MeOH, as sample solvent, did not provide satisfactory results for the peak shapes.

Silymarin has a limited solubility in polar protic solvents, but it is highly soluble in polar aprotic solvents, such as acetone, dimethylformamide or tetrahydrofuran [10]. However, in this study, coated chiral columns were used, and the use of these aprotic solvents would lead to dissolution of the polysaccharide derivates, and therefore must be avoided [41].

Because of the limited solubility of silymarin and the character of columns used, only a limited selection of sample solvents was tested. The following were selected: EtOH, PrOH, ACN, Hep/PrOH/EtOH (2:1:2) and Hep/PrOH/MeOH (2:1:2). Because of the limited amounts of standards available, the effects of different sample solvents on the peak shapes were tested with silymarin extract and compared with the results when the extract was dissolved in MeOH. Analysed were the best chromatographic conditions.

The best overall separation, in terms of resolution and peak shapes, was achieved with MeOH as the sample solvent with the LA-1 + LC-3 system. Introducing different sample solvents to the LC-3 + LC-2 system provided similar results to MeOH, so this was finally kept as sample solvent (for both systems, data not shown).

### 3.5. Optimization of the mobile phase additives

The effect of several individual additives, i.e. 0.3% formic acid, 0.3% acetic acid, 0.1% TFA, 10 mM ammonium formate, 10 mM ammonium acetate and 2% H2O, on the separation of the silymarin compounds was determined at the best chromatographic conditions. The outcomes were compared to the results achieved by using both 0.1% TFA and 0.1% IPA in the mobile phase.

On the LA-1 + LC-3 system, the Rs between SCH and SD, and ISBB and ISBA were improved from 0.62 to 0.85 and from 0.62 to 0.70, respectively, when using 0.1% TFA as additive (Fig. 4A). However, the same additive resulted in practically no difference in the separation of the critical pairs on the LC-3 + LC-2 column combination the Rs between SCH and ISBB was slightly improved from 0.64 to 0.70 and between ISBB and SBA from 0.38 to 0.42 (Fig. 4B).

The separation of the analysed compounds was worse when using any of the other tested additives. Using TFA as an additive improved the separation compared to the best optimized method. However, the resolution of the critical peaks still remains worse than the initial separation (Fig. 1B, D). Moreover, the use of combined basic and acidic additives is preferred over a single additive in order to reduce memory effects on the CSPs.

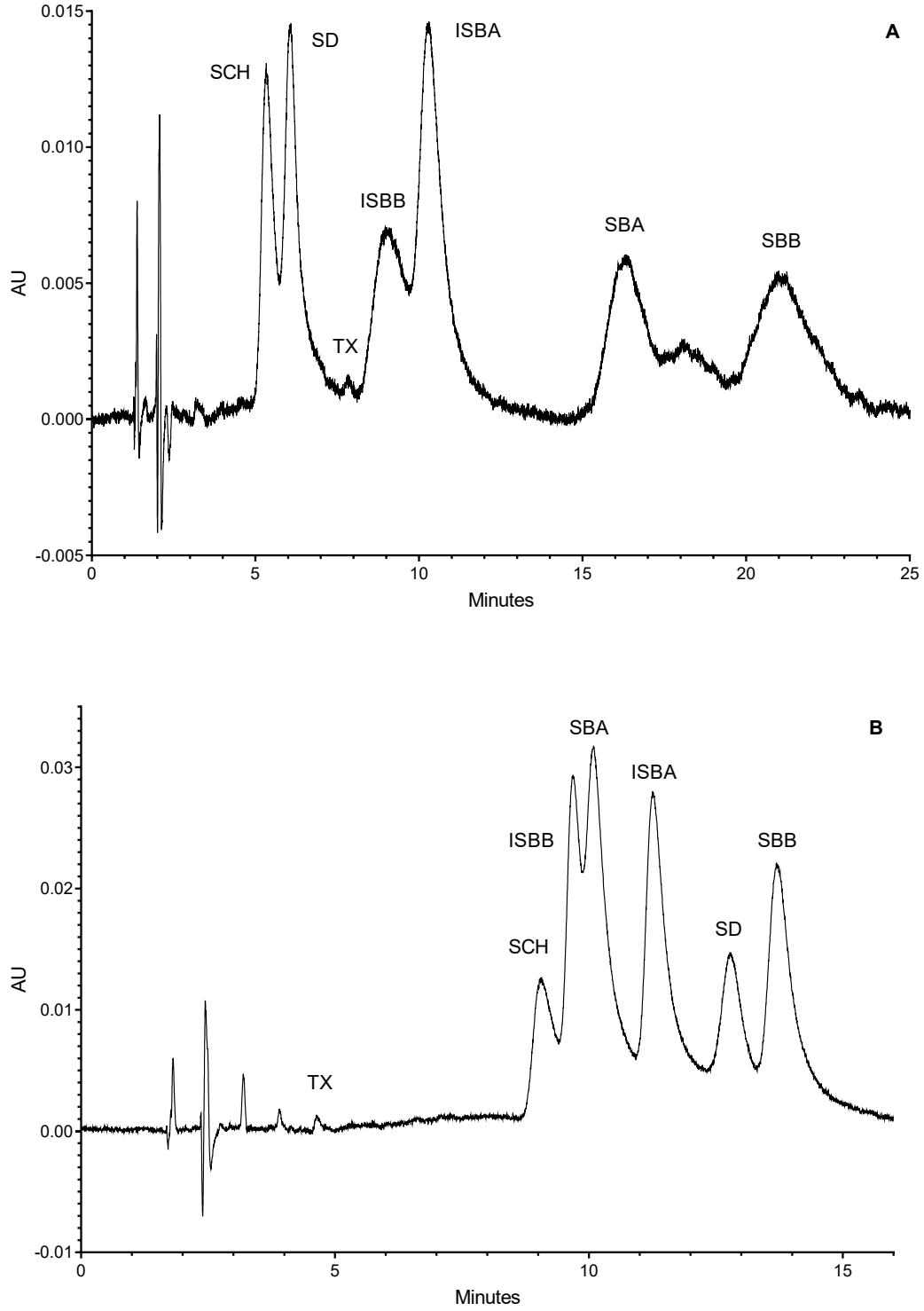



**Figure 4:** Separation of silymarin compounds (A) on the LA-1 + LC-3 system: 40% MeOH, 2.8 mL/min, 0.1% TFA, 125 bar, and 30°C and (B) on the LC-3 + LC-2 system: 40% MeOH, 2.3 mL/min, 0.1% TFA, 140 bar and 40°C.

**4. Conclusions**

The separation of silymarin was studied on a selection of achiral and chiral columns. The single column screenings always resulted in co-elution of at least two silymarin compounds. A partial separation of all tested analytes was only obtained when two columns were serially coupled. An SFC method for the

separation of SBA, SBB, ISBA, ISBB, SCH, SD and TX on two serially coupled chiral column systems, Lux Amylose - 1 + Lux Cellulose - 3 and Lux Cellulose - 3+ Lux Cellulose - 2, was further optimized. The most significant effect on the separation of the analytes was seen after coupling the stationary phases. Other chromatographic parameters had only minor influences on the Rs between the analytes. After comprehensive method optimization, baseline separation could not be achieved for all analysed compounds. The method providing the best separation of silymarin was obtained on the LA-1 + LC-3 system, under initial chromatographic conditions resulting from the column screening: 40% MeOH containing 0.1% IPA and 0.1% TFA as additives, a flow rate 1.23 mL/min, a backpressure of 150 bar, and a column temperature of 30°C. The elution sequence of analytes was SH, SD, TX, ISBB, ISBA, SBA, and SBB, with Rs between adjacent peak pairs of 1.81, 7.50, 1.79, 0.82, 5.12 and 2.72, respectively. Baseline separation was not achieved for the SCH and SD, and ISBB and ISBA pairs. The SCH and SD pair, with Rs 1.81, did not reach baseline separation due to their peak shapes. The Rs between the isosilybin diastereomers was only 0.82. For this method, the analysis time was over one hour and considerable band broadening is observed for later eluting peaks. Methods achieving baseline separation of silymarin flavonolignans within much shorter analysis times were already developed in UPLC and CE [18,20].

The method developed by Gros et al. [21] used the same chiral selector in immobilized columns for the SFC analysis as in LC-3 column used in this study. Compared with the silymarin screening on the LC-3 (Fig. S-2 C), the separation on LC-3 column was faster but tailing of the peaks contributed to the overall worse separation. The peak tailing can be caused by the nature of the coated stationary phases, secondary interactions of analytes with silanol groups, extra-column effects or a sample solvent effect, but this was not examined further in this study.

After selection of the stationary phases, influential options for further method optimization were limited for this case study. It is clear that column coupling improved the separation of analytes, but in the coupled systems considerable peak broadening was observed, resulting in a loss of resolution and overall low quality chromatography. Peak broadening, while to be expected on coupled column systems, was contributed to further by using system with external column oven and coupling of peaks using PEEK connectors, both of which introduced additional dead volume.

The future work in SFC for the separation of silymarin compounds could focus on testing a broader selection of chiral columns. Future research should also consider employing shorter columns, allowing for coupling more than two columns in various orders and gradient elution positively affecting peak shapes. Finally, immobilized chiral columns are also to be investigated, because they allow to use of a wider range of solvents, which might be beneficial given the limited solubility of silymarin and observed peak broadening.

**Conflict of Interest**

The authors have declared no conflict of interest.

**Acknowledgements**

The authors gratefully acknowledge financial support by the grant project SVV No. 260 662.

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**Supplementary material**

**Figure S-1:** Chemical structures of the silymarin compounds.

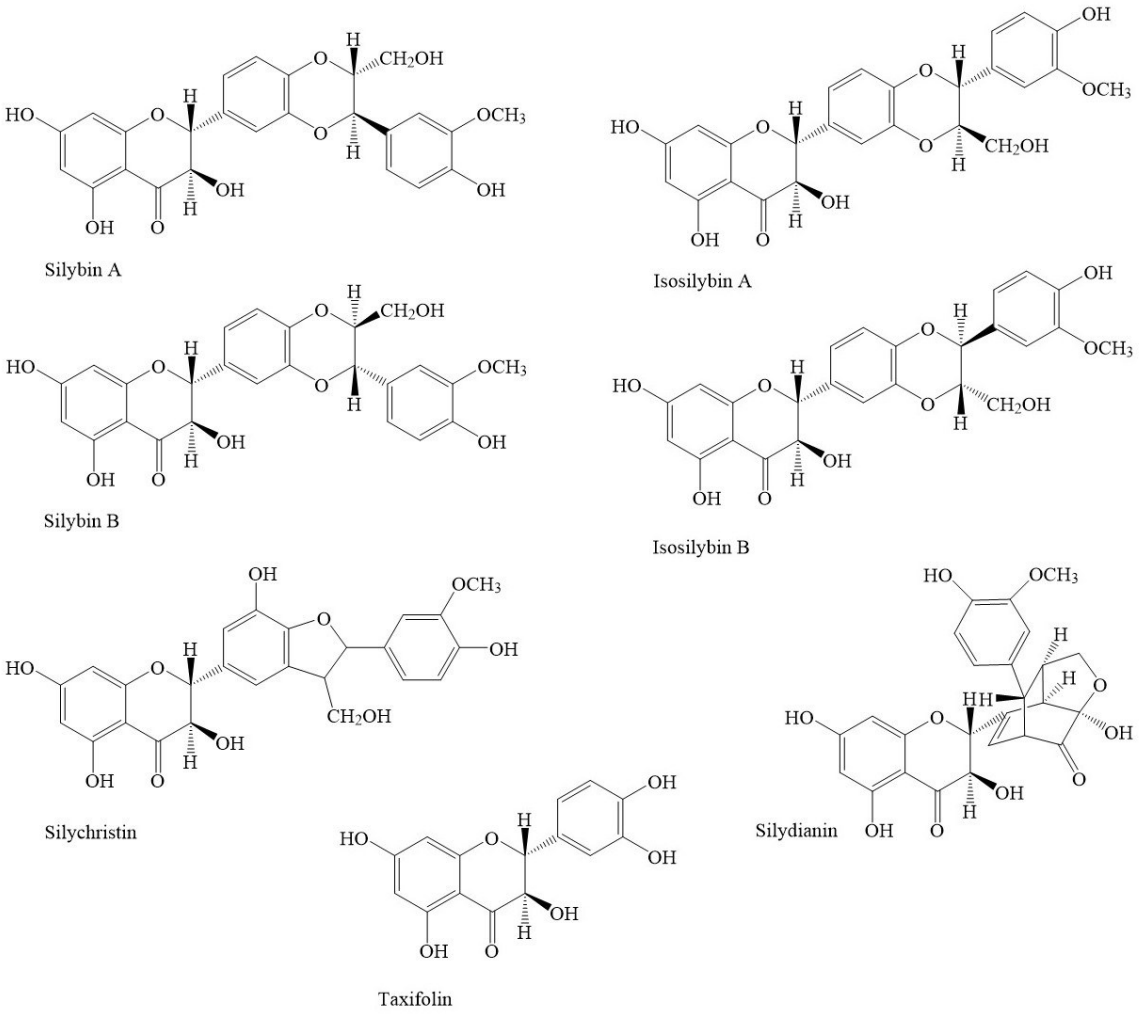
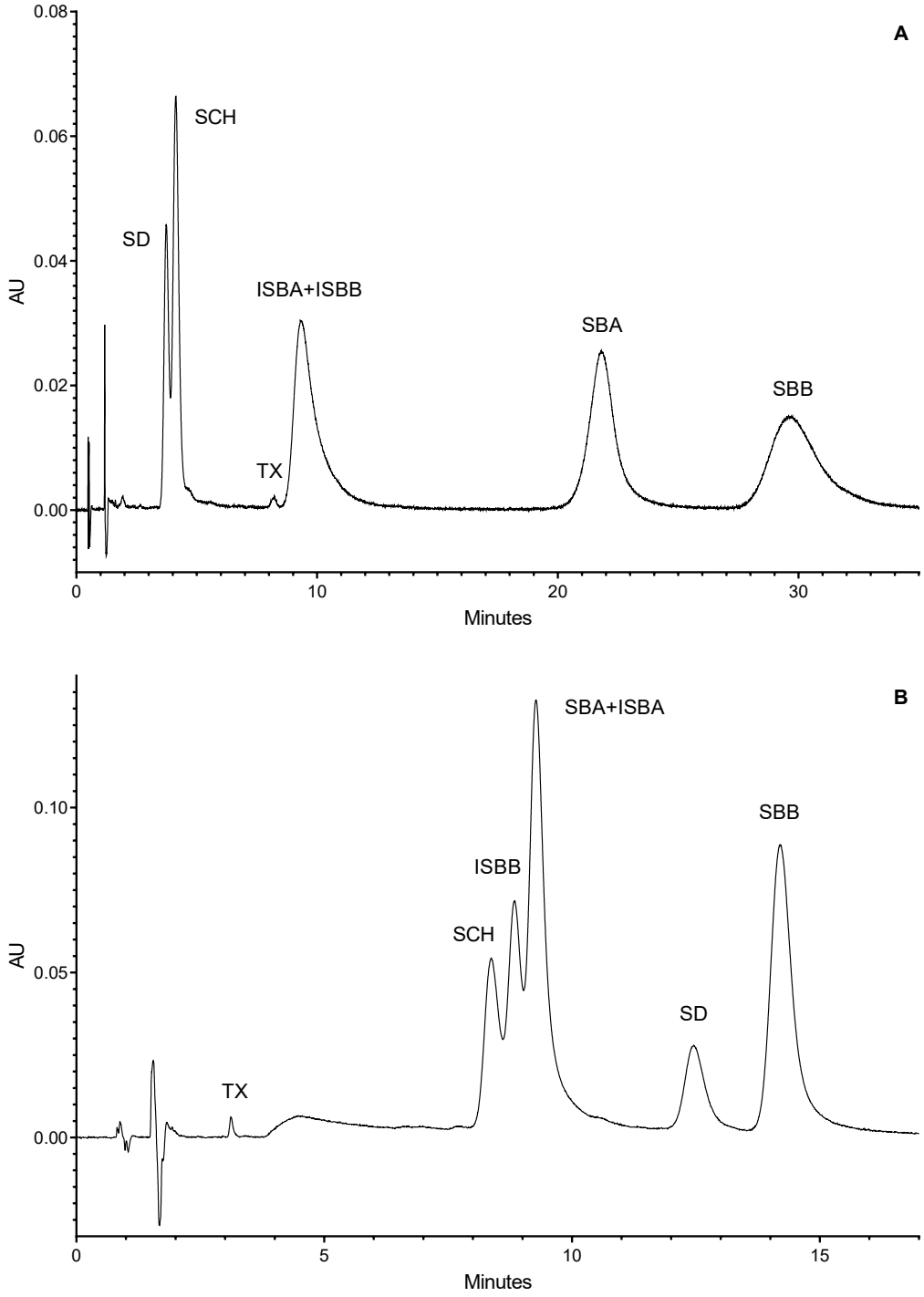


**Figure S-2:** Chromatograms of silymarin screening on LA-1 (A), LC-2 (B) and LC-3 (C) columns, using mobile phase containing 40% MeOH with 0.1% (V/V) IPA and 0.1% (V/V) TFA, a backpressure of 150 bar and a column temperature of 30°C with flow rates 2.32, 1.82 and 1.82 mL/min, respectively.



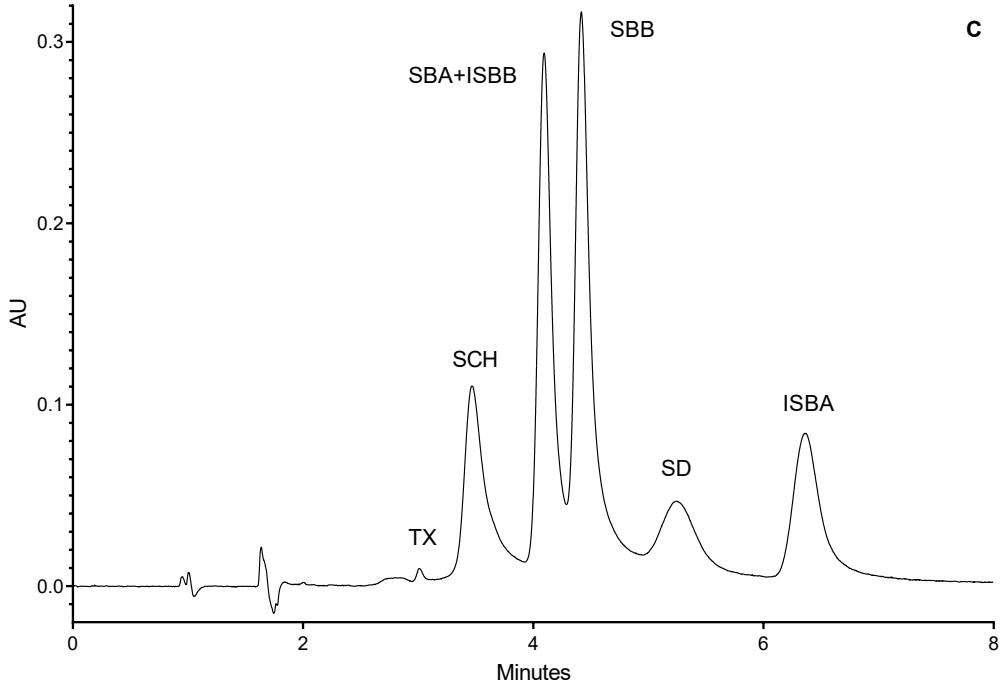
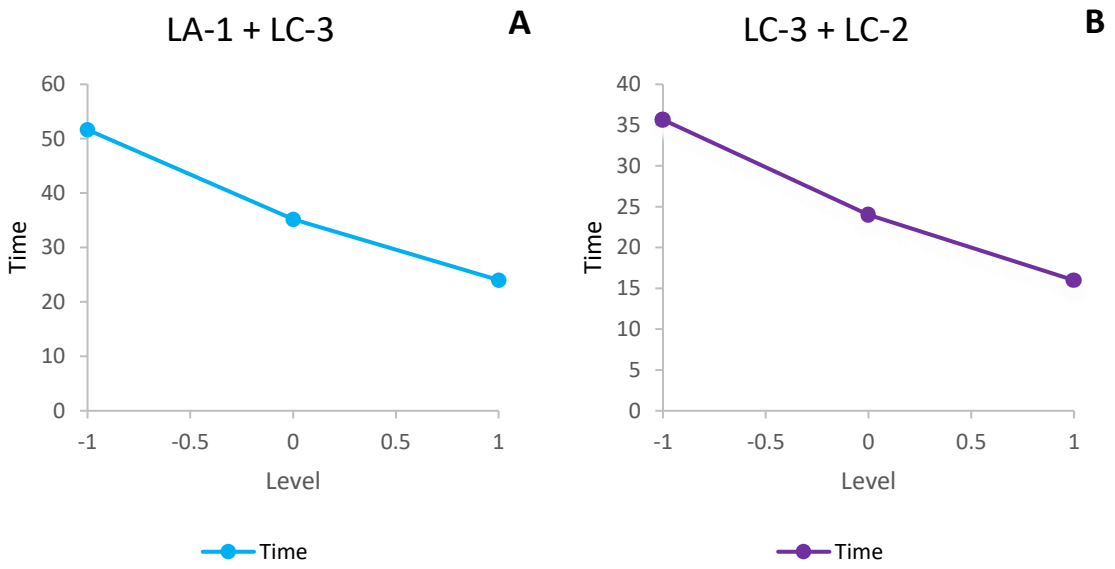
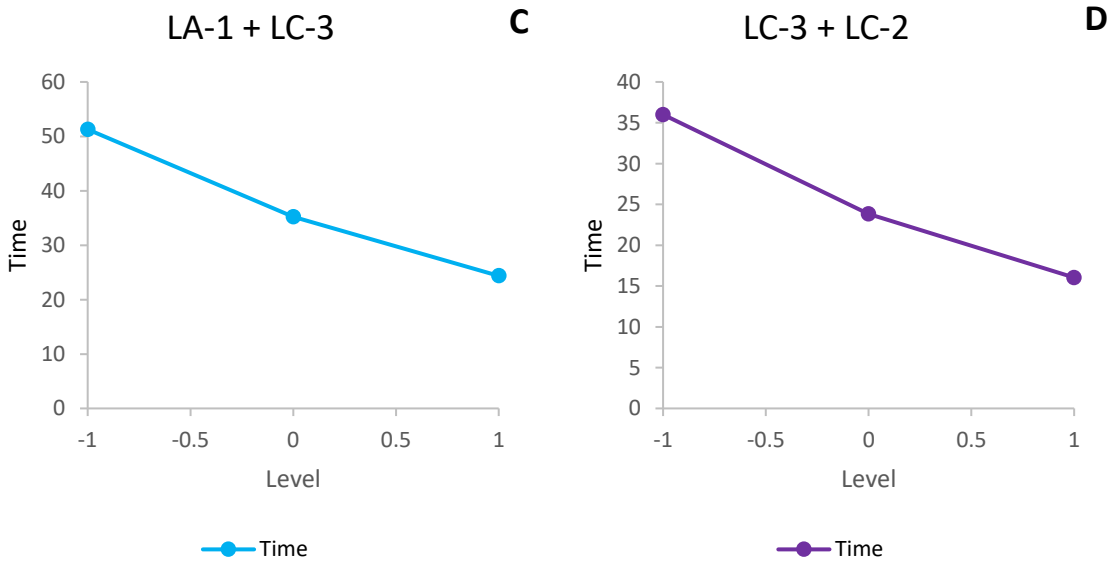

C
SBB
SBA+ISBB
SCH
ISBA
SD
TX
AU
0.3
0.2
0.1
0.0
0
2
4
6
8
Minutes

**Figure S-3:** Effect plots of MeOH content (A, B), flow rate (C, D), concentration of additives (E, F), backpressure (G, H), and column temperature (I, J) on the analysis time on the LA-1 + LC-3 system (A, C, E, G, I) and the LC-3 + LC-2 coupled system (B, D, F, H, J).

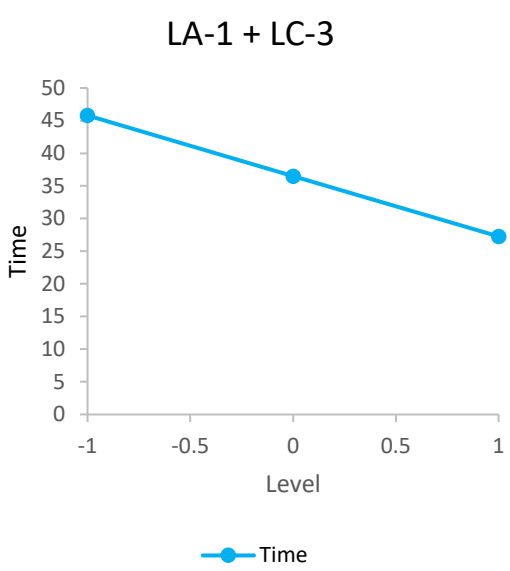
**Content of MeOH**

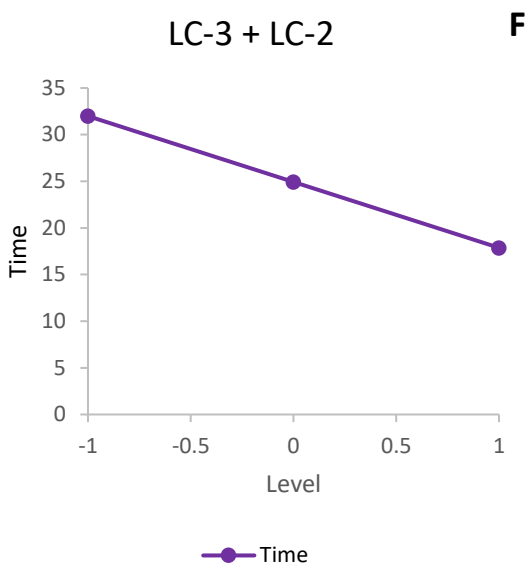



**Flow rate**

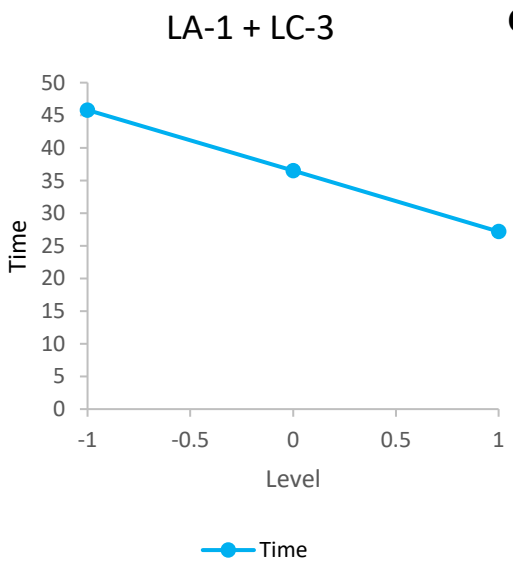


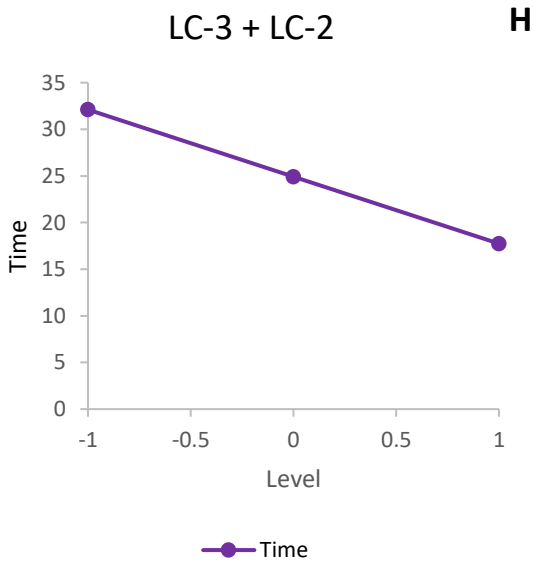
**Concentration of additives**



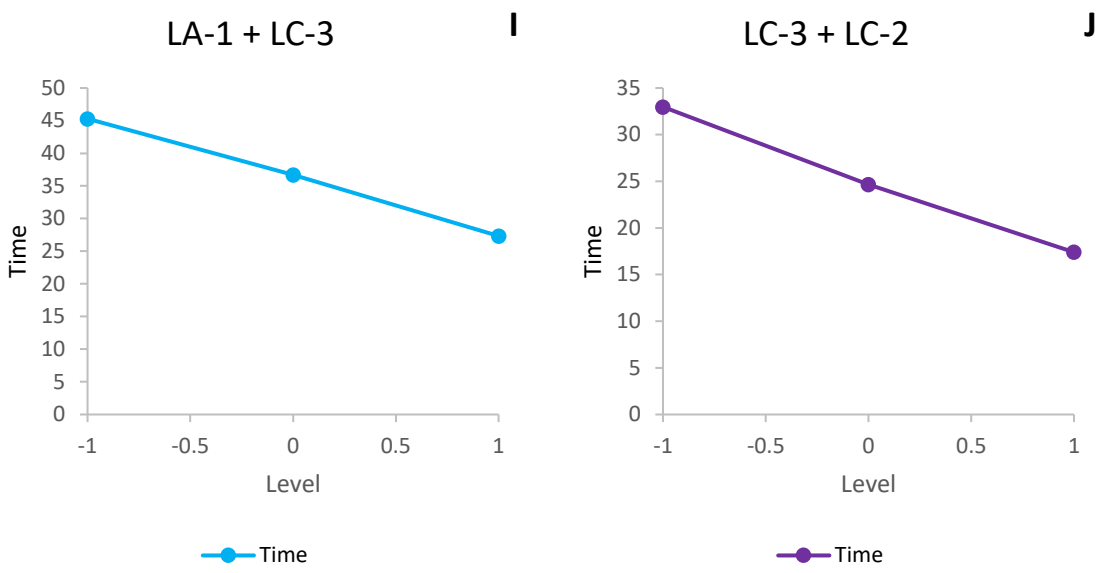




**Backpressure**



**Column temperature**



# 5 Summary, conclusions, and future perspectives

This thesis is based on experimental work dealing with different approaches of method development for the separation of structurally similar compounds using CE and SFC. The presented studies, except for the last, are published in scientific journals and are listed in the Web of Knowledge. Publications dealing with development of CE methods were carried out at the Charles University, Faculty of Pharmacy in Hradec Králové, Department of Analytical Chemistry. The publications studying column coupling in SFC, its application and method optimization for silymarin were carried out at the Vrije Universiteit Brussel, Department of Analytical Chemistry, Applied Chemometrics and Molecular Modelling.

The first part of the thesis focussed on the development and validation of an MEKC method for the determination of three impurities of indomethacin. The multivariate DoE approach used during the screening and method optimization enabled to identify the most influential factors affecting the separation and to select the optimal separation conditions based on a limited number of experiments. Baseline separation of IND, three impurities and the internal standard was achieved within 10 minutes. The method was validated for the limits of impurities set in the European Pharmacopoeia and exhibited good validation characteristics for precision and accuracy. The MEKC method was successfully applied on the purity control of IND gel and IND bulk drug, showing its good potential for quality control purposes. The novel method presents a more environmentally friendly alternative for the determination of indomethacin impurities compared to the conventionally applied HPLC methods. Additionally, the method was validated for DCHIND for the first time. In future studies, the MEKC method can be optimized for the separation of additional impurities, if they become available in a quality and amount that is suitable for method development. Additionally, the range of IND formulations on which the method is applied can be further extended with suppositories, sprays, or ophthalmic solutions. However, sample preparation would need to be optimized for each type of formulation.

The second part of the thesis described the development of a CE method for the separation of the main active compounds of silymarin complex. The previous method development in the first part was difficult, as the analytes were structurally related. In this study, the challenge was to separate structurally similar flavonolignans, including two pairs of diastereomers, originating from the same plant. Two different separation modes were chosen for optimization: CD-MEKC, which was not pursued further for its poor repeatability and robustness, and EKC. The latter optimized method provided baseline separation for all seven compounds. The analysis time was 25 minutes, which is shorter than that of the chromatographic method used in the European Pharmacopoeia. The validated method was applied for the analysis of two dietary supplements containing silymarin. In this study, univariate optimization in the method development, allowing only one factor to be optimized at a time, resulted in a labour-intensive optimization process. Additionally, more time should have been dedicated to the testing of various cyclodextrins, because the optimal separation conditions were reached with a BGE containing TM-β-CD, which was not examined in the first approach. Nevertheless, the proposed method is the first CE method enabling baseline separation of all seven analytes, demonstrating that electrophoretic methods can be a

valuable alternative to chromatographic, including for the analysis of structurally similar secondary plant metabolites.

The third part of the thesis focused on evaluating and improving of the retention prediction of model analytes on coupled column systems in SFC. A selection of chiral and achiral columns was screened using generic chromatographic conditions. Data of retention behaviour of model analytes on single columns were used for predicting the retention behaviour of these compounds in coupled column systems. The prediction accuracy of three equations was evaluated against experimental results. The equation providing the most accurate prediction was selected, and the prediction was further improved by adjusting flow rate and backpressure to achieve the same average column pressure during the analysis. Although adjusting flow rate resulted in longer analysis times, the prediction of elution sequence was more reliable and the calculated retention factors were consistently more accurate. The strategy was applied for a selection of columns for the separation of the silymarin complex. This study provided a simple strategy for the selection of the most suitable combination of stationary phases based on a limited number of experiments. This is especially needed in the field of chiral separations and in separations of structurally similar compounds (e.g., drug and its impurities, structural isomers), where the stationary phase selection is based mainly on a trial-and-error approach. In future studies, the applicability of this strategy on a wider selection of analytes and columns, or its suitability for mobile phases with different compositions, could be tested.

In the last part of this thesis, the selection of coupled column systems for the separation of the silymarin complex is studied more in detail. The optimization then continued by studying the effects of the MeOH content in the mobile phase, flow rate, type and concentration of additives, backpressure, column temperature, and solvent solution on the method performance. Thanks to the multivariate DoE optimization, the factors with a significant effect on the separation were identified within a relatively small number of experiments. As the stationary phase is the main determinant of selectivity, the selection of the suitable columns is the most important step in the method development of separations of isomeric compounds, including diastereomers. The optimization of the other factors did not provide a considerable improvement in the resolution, and a baseline separation of the analytes was not achieved.

Compared to the optimized SFC conditions, the CE method provided better results. The peaks observed on the coupled SFC systems were also very broad. Currently there is no SFC method described for the separation of silymarin complex compounds. The method presented in this work can therefore be considered as a first attempt. Future optimization of the SFC method for the separation of silymarin complex could focus on screening a broader selection of the columns, both in terms of their length and stationary phase chemistry. Additionally, immobilized columns could be considered for screening, as they are compatible with a wider range of solvents, which possibly are more suitable for the SFC separation of the silymarin than the coated columns.

The development of novel methods and approaches for the CE and SFC separation of structurally similar compounds was demonstrated in this work, contributing to the knowledge field of separation science.

# 6 Research output

## 6.1 List of first author publications

1 P. Riasová, D. Doubková, L. Pincová, O. Jung, M. Polášek, P. Jáč, Development of micellar electrokinetic chromatography method for the determination of three defined impurities in indomethacin, Electrophoresis 39(20) (2018) 2550-2557.
https://doi.org/10.1002/elps.201800080
IF = 2.754, Q2 (category Analytical Chemistry); $Q_{AIS}$ 2
Contribution: methodology, validation, formal analysis, investigation, writing – original draft, writing – review and editing.

2 P. Riasová, J. Jenčo, D. Moreno-González, Y. Vander Heyden, D. Mangelings, M. Polášek, P. Jáč, Development of a capillary electrophoresis method for the separation of flavonolignans in silymarin complex, Electrophoresis 43 (2022) 930-938.
https://doi.org/10.1002/elps.202100212
IF = 2.9, Q2 (category Analytical Chemistry); $Q_{AIS}$ 3
Contribution: methodology, validation, formal analysis, investigation, writing – original draft, writing – review and editing, visualization.

3 P. Riasová, P. Jáč, M. Polášek, Y. Vander Heyden, D. Mangelings, Coupling of chiral and achiral stationary phases in supercritical fluid chromatography: evaluating and improving retention prediction, Journal of Chromatography A 1667 (2022) 462883.
https://doi.org/10.1016/j.chroma.2022.462883
IF = 4.1, Q2 (category Analytical Chemistry); $Q_{AIS}$ 2
Contribution: conceptualization, methodology, formal analysis, investigation, writing – original draft, writing – review and editing, visualization.

4 P. Riasová, P. Jáč, M. Polášek, Y. Vander Heyden, D. Mangelings, Chromatographic behaviour of selected flavonolignans from silymarin in supercritical fluid chromatography with coupled chiral columns, manuscript in preparation.
Contribution: methodology, formal analysis, investigation, writing – original draft, writing – review and editing, visualization.

## 6.2 List of publications with co-authorship

1 Y. Grooten, P. Riasová, I. Salsinha, D. Mangelings, Y. Vander Heyden, Stationary-Phase Optimized Selectivity in Supercritical Fluid Chromatography using a customized Phase Optimized Liquid Chromatography kit: Comparison of different prediction approaches, Analytical and Bioanalytical Chemistry 412 (2020) 6553 – 6565.
https://doi.org/10.1007/s00216-020-02739-w
IF = 4.157, Q2 (category Analytical Chemistry); $Q_{AIS}$ 2
Contribution: investigation, writing – review and editing.

2 D. Moreno-González, P. Jáč, P. Riasová, L. Nováková, In-line Molecularly Imprinted Polymer Solid Phase Extraction-Capillary Electrophoresis Coupled with Tandem Mass Spectrometry for the Determination of Patulin in Apple-based food, Food Chemistry 334 (2021) 1-8.
https://doi.org/10.1016/j.foodchem.2020.127607
IF = 9.231, Q1 (category Applied Chemistry); $Q_{AIS}$ 1
Contribution: investigation, writing – review and editing.

3 D. Kosolapov, P. Jáč, P. Riasová, J. Poušková, M. Polášek, L. Nováková, Advances and Challenges in the Analysis of Boswellic Acids by Separation Methods, Critical Reviews in Analytical Chemistry (2024) 1-27.
https://doi.org/10.1080/10408347.2024.2312502
IF = 4.2 (2023), Q1 (category Analytical Chemistry); $Q_{AIS}$ 1
Contribution: methodology, investigation, writing – original draft, writing – review and editing.

## 6.3 List of oral presentations at national and international conferences

1 P. Riasová, P. Jáč, M. Polášek, *Development of CE method for the separation of flavonolignans occuring in milk thistle (Silybum marianum),* 7th Postgraduate and 5th Postdoc conference, Section: Pharmaceutical Analysis, 7th – 8th February, 2017, Hradec Králové, Czech Republic.

2 P. Riasová, P. Jáč, M. Polášek, *Development of capillary electrophoresis method for the characterization of pharmaceuticals containing silymarin complex*, 8th Postgraduate and 6th Postdoc conference, Section: Pharmaceutical Analysis, 24th – 25th January, 2018, Hradec Králové, Czech Republic.

3 P. Riasová, Y. Vander Heyden, D. Mangelings, *Coupling of chiral and achiral stationary phases in SFC*, 2nd STARSS Conference on Separation Science, 27th – 29th November, 2018, Hradec Králové, Czech Republic.

4 P. Riasová, Y. Vander Heyden, D. Mangelings, *Column coupling in supercritical fluid chromatography*, 9th Postgraduate and 7th Postdoc Conference, Section: Pharmaceutical Analysis, 23rd – 24th January, 2019, Hradec Králové, Czech Republic.

5 P. Riasová, P. Jáč, M. Polášek, Y. Vander Heyden, D. Mangelings, Coupling of chiral and achiral columns in supercritical fluid chromatography, BSPS Forum of Pharmaceutical Sciences, 20th May, 2019, Brussels, Belgium.

6 P. Riasová, P. Jáč, M. Polášek, Y. Vander Heyden, D. Mangelings, Optimization of Capillary Electrophoresis and Supercritical Fluid Chromatography Methods for the Separation of Silymarin, 11th Postgraduate and 9th Postdoc Conference, Section: Pharmaceutical Analysis, 27th – 28th January, 2021.

## 6.4 List of poster presentations

1 P. Riasová, P. Jáč, *Electrophoretic behavior of flavonolignans in milk thistle (Silybum Marianum),* 13th International Interdisciplinary Meeting on Bioanalysis – CECE, 17th – 19th October, 2016, Brno, Czech Republic.

2 P. Riasová, P. Jáč, M. Polášek, *Development of capillary electrophoretic method for the separation of milk thistle flavonolignans,* Czech School of Chromatography – HPLC 2017, 12th – 15th March, 2017, Rožnov pod Radhoštěm, Czech Republic.

3 P. Riasová, D. Mitlenerová, L. Pincová, P. Jáč, M. Polášek, *Development of a micellar electrophoretic capillary chromatography method for the determination of impurities in indomethacin,* 45th International Symposium on High Performance Liquid Phase Separations and Related Techniques HPLC 2017, 18th – 22nd June, 2017, Prague, Czech Republic.

4 P. Riasová, P. Jáč, M. Polášek, *Development of capillary electrophoretic method for the separation of isomeric components in a silymarin complex*, 32nd International Symposium on Chromatography ISC 2018, 23rd – 27th September, 2018, Cannes-Mandelieu, France.

5 P. Riasová, P. Jáč, M. Polášek, Y. Vander Heyden, D. Mangelings, *Column coupling in supercritical fluid chromatography using chiral and achiral stationary phases*, 48th International Symposium on High-Performance Liquid Phase Separations and Related Techniques HPLC 2019, 16th – 20th June, 2019, Milan, Italy.