Univerzita Karlova v Praze
Přírodovědecká fakulta

Studijní program: Fyzikální chemie
Studijní obor: Fyzikální chemie

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Charakterizace nových chirálních separačních systémů pro použití v HPLC

Characterization of new chiral separation systems for HPLC

Dizertační práce

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Praha, 2014

Předkládaná dizertační práce shrnuje výsledky získané během mého doktorského studia ve Skupině elektroforetických a chromatografických separačních metod (ECHMET) na Katedře fyzikální a makromolekulární chemie Přírodovědecké fakulty Univerzity Karlovy v Praze.

Práce byla financována v rámci řešení následujících projektů: GA UK, č. p. 356411, KONTAKT LH11018, CEEPUS CIII-RO-0010-08-1314-M-69316 a výzkumného záměru MŠMT, projekt MSM0021620857.

Prohlášení:

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V Praze……. ..…….

podpis

Poděkování

Již druhou hodinu sedím nad touto, zatím prázdnou, stránkou a přemýšlím, jak mám na pár řádcích vyjádřit mé díky za to, co pro mě moji úžasní kolegové, přátelé a rodina udělali. Poděkování by mělo být upřímné, neotřelé a vhodné do dizertační práce, což už se mi teďkon nedaří. Děkuji Evě za její mimořádný přístup, cenné rady, zkušenosti a také nadhled. Nikdy nezapomenu na její: „Ó, je to báječné, ale našla jsem tam nějaké drobnosti“ a pak mi odevzdala papíry, které obsahovaly více červených oprav a poznámek než bylo původního textu. Obrovské děkuji patří Květě, za její neskonalou trpělivost, neuvěřitelnou obětavost a za to, že mě neumlátila, ač jsem si to mnohokrát zasloužil. Děkuji Martině a Janě, démonickému duu, za jejich báječný přístup a optimismus. Děkuji i ostatním lidem z laborky za nezapomenutelné chvíle, co jsme společně zažili, strávili a vypili.

Dále děkuji mé mamince a přítelkyni, za jejich lásku a podporu. Ač ani jedna netuší, co jsou to chirální separace a co jsem vlastně ty čtyři roky dělal, vždycky mi stály po boku.

Klíčová slova

Cyklofruktan, chirální stacionární fáze, chirální separace, vysokoúčinná kapalinová chromatografie, superkritická fluidní chromatografie

Keywords

Cyclofructan, chiral stationary phases, chiral separations, high performance liquid chromatography, supercritical fluid chromatography

Souhrn

Dizertační práce je zaměřena na fyzikálně-chemickou charakterizaci interakčních mechanizmů chirálních stacionárních fází na bázi derivatizovaných cyklofruktanů. Správná interpretace retenčních a enantiodiskriminačních interakcí usnadní vývoj, validaci a optimalizaci enantioselektivních metod využívajících tyto fáze.

Nejdříve byly studovány interakční mechanizmy tří komerčně dostupných chirálních stacionárních fází na bázi derivatizovaných cyklofruktanů v podmínkách normálního separačního módu kapalinové chromatografie. Jednalo se o stacionární fáze na bázi dimethylfenyl karbamátu cyklofruktanu 7, *R*-naftylethyl karbamátu cyklofruktanu 6 a izopropyl karbamátu cyklofruktanu 6. Jako výchozí přístup byl zvolen model lineárních vztahů volných energií. Na základě tohoto modelu byly jako hlavní interakce přispívající v různé míře k retenci ve všech separačních systémech s cyklofruktanovými stacionárními fázemi určeny: schopnost poskytovat vodík pro tvorbu vodíkové interakce a dipolarita/polarizibilita, disperzní interakce pak retenci v různé míře snižují. Pro objasnění vlivu sacharidového skeletu na chirální separace byly následně cyklofruktanové stacionární fáze porovnány s cyklodextrinovými analogy. Cyklofruktanové chirální stacionární fáze prokázaly v podmínkách normálního módu mimořádnou enantioselektivitu zejména pro deriváty binaftolu a různé aminy.

V následující části byl zkoumán separační potenciál cyklofruktanových fází, konkrétně fáze tvořené dimethylfenyl karbamátem cyklofruktanu 7, v podmínkách superkritické fluidní chromatografie. Pomocí modelu lineárních vztahů volných energií byly odhaleny rozdílné distribuce retenčních interakcí v kapalinové a superkritické chromatografii a byl také demonstrován jejich vliv na chirální separace.

Přestože se cyklofruktanové chirální stacionární fáze používají zejména v normálním či polárně-organickém módu kapalinové chromatografie, byl prokázán jejich značný enantioselektivní potenciál také v reverzním separačním módu. Navíc byl studován efekt přídavku barnatých iontů do mobilní fáze, který významně ovlivňuje enantioselektivitu separačního systému na bázi derivatizovaných cyklofruktanů pro určité skupiny analytů.

Praktické využití poznatků základního výzkumu je konkrétně ukázáno na dvou příkladech vývoje enantioseparačních metod pro stanovení léčiv.

Abstract

The dissertation thesis is focused on the physico-chemical characterization of interaction mechanisms of chiral stationary phases based on derivatized cyclofructans. Correct interpretation of retention and enantiodiscrimination processes substantially facilitates the development and optimization of new enantioselective methods using cyclofructan-based chiral stationary phases.

At first, the interaction mechanisms of three commercially available cyclofructan-based stationary phases were studied in normal-phase mode of liquid chromatography. Namely, systems using chiral stationary phases based on dimethylphenyl carbamate cyclofructan 7, *R*-naphtylethyl carbamate cyclofructan 6 and isopropyl carbamate cyclofructan 6 were studied. Linear free energy relationship model was used as a basic tool for characterization of interactions on the stationary phases. The mentioned model revealed that the main interactions contributing to retention in cyclofructan-based systems are hydrogen bond acidity and dipolarity/polarizibility, while dispersion interactions cause decrease of retention. The impact of oligosaccharide skeleton of the cyclofructan selector on the enantioselectivity was elucidated by the comparison with seemingly analogous cyclodextrin-based chiral stationary phases. Cyclofructan-based chiral stationary phases performed unique enantioselectivity for binaphthol derivatives and various amines in normal-phase mode.

In the next step, the separation potential of cyclofructan-based chiral stationary phases, namely dimethylphenyl carbamate cyclofructan 7 chiral stationary phase, was studied under the conditions of supercritical fluid chromatography. Different distribution of retention interactions in both methods, liquid and supercritical fluid chromatography, was revealed by the linear free energy relationship model. The impact on chiral separations was also demonstrated.

Despite the fact that cyclofructan-based chiral stationary phases are mostly applied in normal-phase or polar-organic modes, the considerable separation potential in reversed-phase mode was demonstrated. The effect of the addition of Ba^{2+} to the mobile phase was studied. Changes in enantioselectivity of CF CSPs for some analytes were observed.

In order to demonstrate practical impact of the research carried out in the thesis, two methods for the determination of drugs enantiomers were developed, optimized and validated.

Obsah

Seznam použitých zkratek

ACN	acetonitril
BGE	základní elektrolyt
CE	kapilární elektroforéza
CS	chirální selektor
CSP	chirální stacionární fáze
DMP-CD	dimethylfenyl karbamát β-cyklodextrinu
DMP-CF7	dimethylfenyl karbamát cyklofruktanu 7
FT-IR	infračervená spektroskopie s Fourierovou transformací
GC	plynová chromatografie
hex	hexan
HILIC	hydrofilní interakční kapalinová chromatografie
HPLC	vysokoúčinná kapalinová chromatografie
IP-CF6	izopropyl karbamát cyklofruktanu 6
IPA	propan-2-ol
LFER	model lineárních vztahů volných energií
MeOH	methanol
NMR	nukleární magnetická rezonance
NP	normální mód
POM	polárně-organický mód
RN-CD	*R*-naftylethyl karbamát β-cyklodextrinu

RN-CF6	*R*-naftylethyl karbamát cyklofruktanu 6
RP	reverzní mód
SFC	superkritická fluidní chromatografie
TEA	triethylamin
TFA	kyselina trifluoroctová
UPLC	ultra účinná kapalinová chromatografie

Seznam použitých symbolů

α	faktor enantioselektivity
a	regresní koeficient LFER rovnice
A	deskriptor LFER, celková nebo efektivní acidita vodíkové vazby
b	regresní koeficient LFER rovnice
B	deskriptor LFER, celková nebo efektivní bazicita vodíkové vazby
c	konstanta LFER rovnice
$\Delta G°$	změna standardní Gibbsovy energie
e	regresní koeficient LFER rovnice
E	deskriptor LFER, rozsah molární refrakce
θ	fázový poměr
H	výškový ekvivalent teoretického patra
k	retenční faktor
R	univerzální plynová konstanta
Rs	chromatografické rozlišení píků
T	termodynamická teplota
u	lineární rychlost mobilní fáze
v	regresní koeficient LFER rovnice
V	deskriptor LFER, McGowanův objem solutu
X	koeficient vířivé difúze
Y	koeficient axiální difúze
Z	koeficient odporu proti přenosu hmoty ve stacionární a mobilní fázi

1 Úvod

Chirální separace jsou neodmyslitelné v oblasti potravinářského, agrochemického a zejména farmaceutického průmyslu [1,2]. Jednotlivé formy chirálních látek, v závislosti na typu chirality označované jako enantiomery či atropizomery, se svými běžnými fyzikálně-chemickými vlastnostmi neliší, ale jejich rozdílnost se projeví v chirálním prostředí. Takovým prostředím je například lidské tělo, ve kterém každá z enantiomerních forem může vyvolat odlišnou biologickou odpověď [3-5]. Kromě čichových a chuťových vjemů se rozdílnost může projevit mnohem závažněji, a to zejména u léčiv. Je známo velké množství léků s chirálními aktivními složkami. V některých případech podporují oba enantiomery v různé míře léčebný účinek, v jiných působí proti sobě nebo je jeden enantiomer neaktivní. Nejzávažnější jsou případy, kdy jedna forma účinné látky přímo poškozuje zdraví jedince [3-5].

Ke stanovení resp. separaci, jednotlivých enantiomerů se používají základní separační techniky, jako jsou vysokoúčinná kapalinová chromatografie (*high performance liquid chromatography*, HPLC, popř. *ultra performance liquid chromatography*, UPLC), kapilární elektroforéza (*capillary electrophoresis*, CE), plynová chromatografie (*gas chromatography*, GC) nebo superkritická fluidní chromatografie (*supercritical fluid chromatography*, SFC) nejčastěji uspořádané tak, aby přímo vzniklo enantioselektivní prostředí. Enantioseparační systém lze v chromatografických metodách vytvořit přídavkem chirálního selektoru (CS) do mobilní fáze nebo použitím chirální stacionární fáze (CSP).

Vývoj, optimalizace a následná validace separačních metod jsou časově i finančně náročné procesy. Výběr nejvhodnější CSP se v HPLC, v současnosti dominující techniky pro chirální separace, často opírá o experimentální zkušenosti analytického chemika a bývá zdlouhavý. Usnadnění resp. urychlení tohoto procesu přinášejí různé *screeningové* přístupy [6-8], které však nejsou obecné a jsou vyvinuty pouze pro vybrané CSP. Jinou možnost poskytují rozsáhlé databáze obsahující již provedené analýzy [9]. Tyto však nemají velký význam např. pro nově syntetizované látky nebo nově připravené CSP. Dalšími významnými a hojně používanými nástroji, které mohou výběr CSP velmi usnadnit a přinést tak nezanedbatelnou časovou i

finanční úsporu, jsou fyzikálně-chemická charakterizace a interpretace základních retenčních a enantiodiskriminačních procesů probíhajících v separačním systému [10].

Předkládaná dizertační práce se zabývá základní charakteristikou CSP na bázi derivatizovaných cyklofruktanů. Enantioselektivní potenciál cyklofruktanových CSP byl studován jak v HPLC, tak v SFC systémech. Fyzikálně-chemická charakterizace interakcí v cyklofruktanových HPLC/SFC systémech, posouzení vlivu sacharidové kostry cyklofruktanového CS na separace a aplikace těchto CSP v jednotlivých separačních módech přispěje k vývoji separačních metod využívajících tyto CSP a významným způsobem zefektivní proces optimalizace.

2 Cíle práce

Cílem práce bylo charakterizovat interakce, které přispívají k retenčnímu a enantiodiskriminačnímu procesu na cyklofruktanových CSP.

Dílčí cíle:

- Zjištění a vzájemné porovnání chromatografických interakcí poskytovaných CSP na bázi derivatizovaných cyklofruktanů za použití modelu LFER.
- Porovnání cyklofruktanových CSP s cyklodextrinovými analogy. Posouzení vlivu oligosacharidové kostry na enantioselektivní potenciál.
- Charakterizace cyklofruktanových systémů v SFC a jejich základní porovnání s HPLC.
- Posouzení enantiodiskriminačních možností cyklofruktanových CSP v SFC.
- Posouzení enantiodiskriminačních možností cyklofruktanových CSP v reverzním módu HPLC.

3 Chromatografické separační systémy

V rámci předkládané dizertační práce byly cyklofruktanové CSP studovány ve dvou chromatografických systémech, HPLC a SFC. Podkapitola 3.1 obsahuje kromě základního popisu těchto separačních technik také jejich vzájemné srovnání s důrazem na jejich aplikační odlišnosti. Přístup využitý k charakterizaci jednotlivých interakčních mechanizmů cyklofruktanových HPLC/SFC systémů je uveden v podkapitole 3.2.

3.1 Základní srovnání HPLC a SFC

V popředí mezi technikami pro separaci enantiomerů je HPLC. V závislosti na uspořádání je možné ji využít nejen v analytickém měřítku, ale i pro semipreparativní a preparativní účely. Kromě vysoké spolehlivosti, dobré opakovatelnosti a robustnosti patří mezi přednosti této techniky široká variabilita separačních systémů. V HPLC se v současnosti pro chirální separace nejvíce uplatňují systémy CSP. Z hlediska uspořádání separačního prostředí se rozlišují celkem tři módy. Normální mód (NP), ve kterém je chirální stacionární fáze polárnější než fáze mobilní. Reverzní mód (RP), ve kterém je mobilní fáze polárnější než fáze stacionární. Posledním běžně používaným módem specifickým pro chirální separace je polárně-organický mód (POM), ve kterém je polárně-organická mobilní fáze tvořena směsí acetonitrilu (ACN) a methanolu (MeOH), či pouze MeOH, a malými přídavky kyseliny octové a triethylaminu (TEA) pro zlepšení selektivity. Kromě výše zmíněných módů existuje ještě další mód, tzv. *hydrophilic interaction liquid chromatography*, HILIC, který se dostal do oblasti zájmu relativně nedávno, a proto je jeho využití pro chirální separace zatím sporadické [11,12]. Existuje celá řada komerčně dostupných CSP, které jsou vhodné pro jeden určitý separační mód, univerzálnější však jsou multimodální CSP. Podrobnější přehled o používaných CSP bude uveden v kapitole 4. Jiným přístupem je přídavek CS do mobilní fáze za použití achirální stacionární fáze. Tento postup však není v současnosti příliš využíván.

Zatímco achirální separační systémy HPLC byly vzhledem ke zvyšujícím se nárokům na analýzy velmi inovovány (např. zavedení sub-2μm částic), vývoj CSP postupuje v tomto ohledu pomaleji. Výrazné urychlení chirálních separací však přináší SFC, která se v současnosti dostává stále více do popředí. Tato separační technika

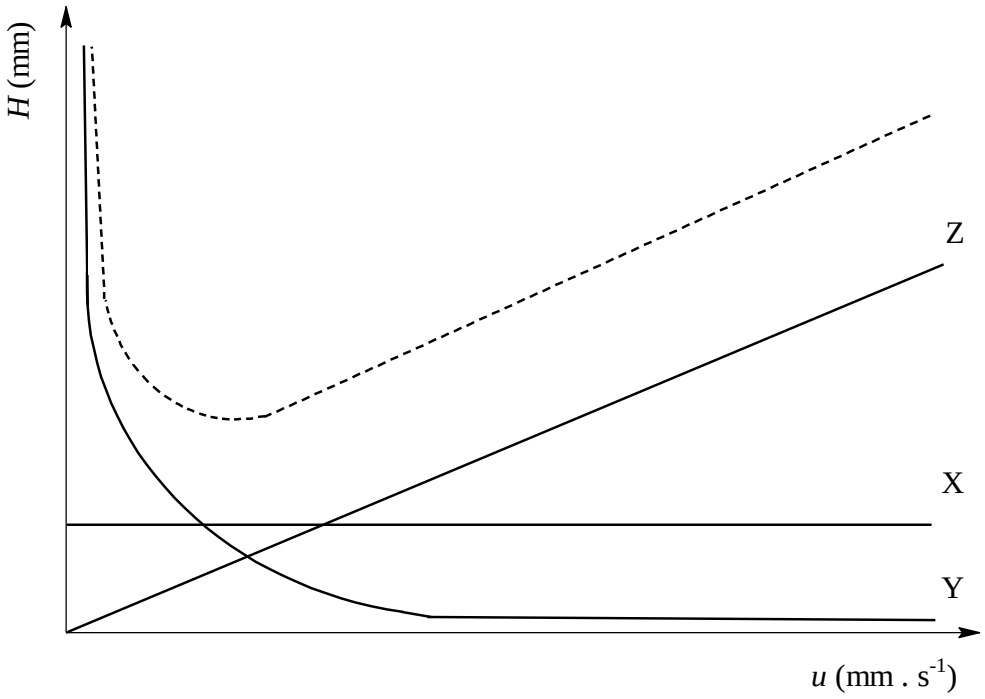
používá jako majoritní složku mobilní fáze superkritickou tekutinu. Superkritické médium obvykle tvoří oxid uhličitý, neboť je relativně levný, netoxický, nehořlavý a jeho kritických hodnot (T_C = 304,12 K, p_C =73,74 bar) [13] lze snadno dosáhnout. Vlastnosti superkritické tekutiny leží mezi vlastnostmi kapalin a plynů. Svojí hustotou a rozpouštěcí kapacitou odpovídá superkritická tekutina kapalinám, zatímco viskozitou a difúzními vlastnostmi odpovídá spíše plynům [13]. Důsledkem těchto unikátních vlastností je nižší tlak v systému, a tedy možnost používat několikanásobně vyšší průtoky mobilní fáze v porovnání s běžnou HPLC bez ztráty separační účinnosti [14].

Teoretické vysvětlení je založeno na van Deemterově křivce vyjádřené rovnicí 1, která udává závislost výškového ekvivalentu teoretického patra H na lineární rychlosti mobilní fáze u.

$$H = X + \frac{Y}{u} + Zu \tag{1}$$

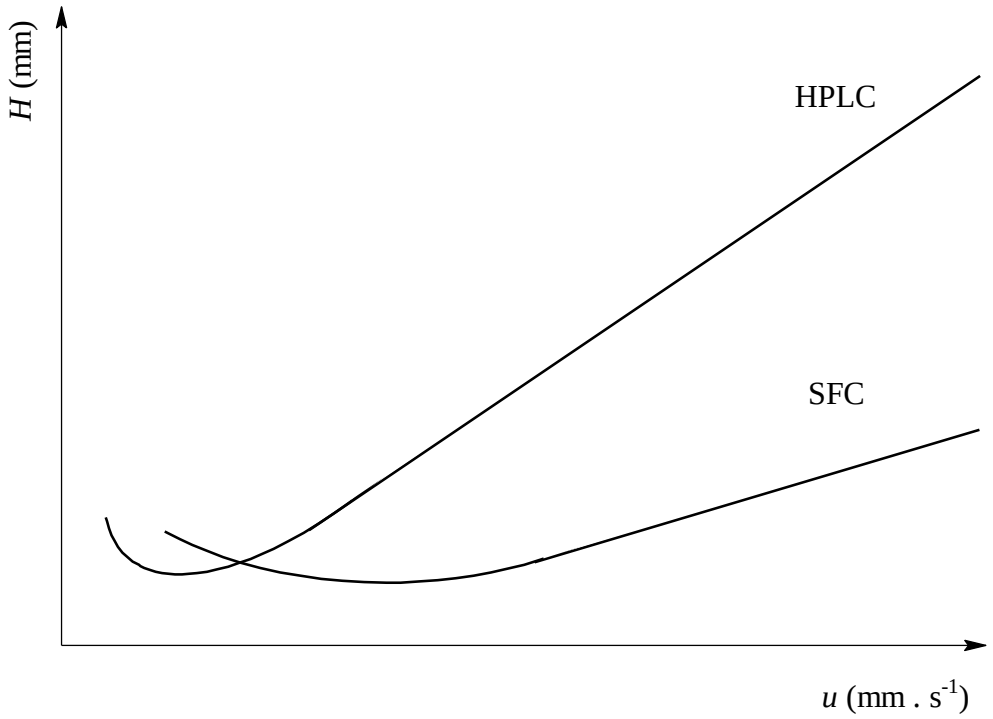
kde koeficient X odpovídá vířivé difúzi, koeficient Y axiální difúzi. Koeficient Z vyjadřuje odpor proti přenosu hmoty ve stacionární a mobilní fázi. H značí výškový ekvivalent teoretického patra a u značí lineární rychlost mobilní fáze.

Závislost jednotlivých příspěvků na lineární rychlosti mobilní fáze je schematicky znázorněna na Obrázku 1. V obrázku je přerušovanou čarou uvedena kompletní Van Deemterova křivka, v jejímž minimu se dosahuje nejvyšších separačních účinností.



Obrázek 1: Van Deemterova křivka zobrazující závislost výškového ekvivalentu teoretického patra na lineární rychlosti mobilní fáze.

Majoritní příspěvek k nárůstu výškového ekvivalentu teoretického patra tvoří koeficient *Z*. Hodnoty tohoto koeficientu, vyjadřujícího odpor proti přenosu hmoty ve stacionární a mobilní fázi, stoupají vlivem vyšší difuzivity analytu v superkritické tekutině v SFC pomaleji než v HPLC. Důsledek tohoto jevu je patrný z Obrázku 2. Zvýšení lineární rychlosti mobilní fáze, tedy zvýšení průtoku mobilní fáze systémem, zapříčiní v HPLC prudký vzestup hodnot výškového ekvivalentu teoretického patra a vede tedy ke snížení separační účinnosti. Pro SFC je tento vzestup minimální, proto se v SFC systémech dosahuje srovnatelné separační účinnosti při násobně vyšších průtocích oproti HPLC [14]. Aplikace vyšších průtoků, kromě zkrácení doby analýzy, také významným způsobem zkracuje dobu potřebnou pro ekvilibraci SFC systému.



Obrázek 2: Srovnání Van Deemterových křivek pro HPLC a SFC systémy.

Další výhodou je kompatibilita superkritického oxidu uhličitého se všemi běžně používanými organickými modifikátory a aditivy. Je však nutné poznamenat, že většina SFC separací probíhá vlivem přídavku organického modifikátoru do mobilní fáze v subkritické oblasti. Vlastnosti subkritické a superkritické tekutiny jsou však v SFC systémech považovány za analogické, a proto nebývají v odborných publikacích tato dvě media odlišována [15].

Přestože bylo zprvu na SFC pohlíženo pouze jako na analogii NP HPLC, ukazuje se, že SFC je schopna plně substituovat i RP HPLC [16]. Přenos separačních metod z HPLC do SFC a obráceně ale nelze považovat za přímočarý a nemusí být jednoznačně úspěšný. Většina komerčně dostupných kolon pro chirální separace v HPLC může být aplikována i v podmínkách SFC. Nezanedbatelnou výhodou SFC oproti HPLC je však skutečnost, že vzorek po analýze zůstává zakoncentrovaný v malém množství organického modifikátoru [17,18].

Kromě do jisté míry nižší variability mobilních fází patří k nevýhodám SFC také náročnější instrumentace. Aby v separačním systému byla superkritická tekutina stabilní, je nutné v celém systému (včetně detektoru) udržovat hodnoty tlaku a teploty nad jejími kritickými hodnotami. Tato podmínka, zejména udržení odpovídajícího

tlaku, činila v minulosti značné problémy. Nedostatečná tlaková regulace způsobovala nízké opakovatelnosti analýz. Nicméně technickým vývojem byly původní nedostatky eliminovány a v současné době se SFC jeví jako efektivnější, ekologicky přijatelnější a levnější varianta oproti HPLC [19,20].

3.2 Charakterizace chromatografických systémů

Charakterizace separačních systémů v HPLC/SFC je velmi obtížný úkol. Na rozdíl například od CE, která se dá interpretovat jako jednodimenzionální děj, protože průměr kapiláry je zanedbatelný oproti její délce a CS je součástí základního elektrolytu, děj v HPLC/SFC systému je tří dimenzionální. Navíc CSP obvykle obsahují více stereogenních interakčních míst, což určení interakčního mechanizmu ještě více komplikuje. Nezanedbatelné jsou také vlivy sterické a interakce mobilní fáze s CSP. Značný přínos k objasnění interakcí v separačních systémech představují termodynamické studie (van´t Hoffovy závislosti, entalpicko-entropické kompenzace, adsorpční izotermy apod.), které však popisují systém jako makroskopický celek, ale nedokáží interpretovat mikroskopické měřítko [10]. Vhled do enantioselektivního procesu na molekulární úrovni s úspěchem poskytují spektroskopické metody, jako jsou nukleární magnetická rezonance (NMR), infračervená spektroskopie s Fourierovou transformací (FT-IR), rentgenová difrakce krystalu selektor-analyt, nebo molekulární modelování. Každá z těchto metod má však svá omezení, např. v krystalu selektor-analyt nemusí prostorové uspořádání odpovídat prostorovému uspořádání během separačního procesu [21].

Dalším z možných a hodně využívaných přístupů jsou modely, ve kterých se obecně empiricky korelují termodynamické veličiny (změny těchto veličin) s retenčními daty. Takové modely mohou být následně využity k posouzení/predikci vlivu jednotlivých molekulárních parametrů v chemické rovnováze, tedy i v chromatografickém procesu [22,23]. Jedním ze základních modelů je model lineárních vztahů volných energií (*linear free energy relationship,* LFER), který je postaven na předpokladu, že změna standardní Gibbsovy energie je lineárně závislá na charakteristikách prostředí [24-27]. Retenční proces - přenos analytu z mobilní fáze do fáze stacionární - je spojen rovnicí 2 se změnou standardní Gibbsovy energie, a proto na něj může být aplikován model LFER.

$$\ln k = \frac{-\Delta G^\circ}{RT} + \ln \theta \quad (2)$$

kde k je retenční faktor příslušného analytu, ΔG° je změna standardní Gibbsovy energie systému, R univerzální plynová konstanta, T termodynamická teplota a θ fázový poměr.

Kromě základního modelu LFER byly odvozeny i další analogické přístupy, které předpovídají např. biologickou aktivitu nebo fyzikálně-chemické vlastnosti látek na základě jejich strukturních parametrů [22,28-30].

Nejpoužívanější rovnice LFER pro HPLC/SFC systémy má tvar

$$\log k = c + e\mathrm{E} + s\mathrm{S} + a\mathrm{A} + b\mathrm{B} + v\mathrm{V} \quad (3)$$

kde log k reprezentuje dekadický logaritmus retenčního faktoru, členy E, S, A B, V jsou deskriptory příslušného analytu, člen c je hodnota úseku na ose y a e, s, a, b, v jsou regresní koeficienty [27].

Rovnice 3 vyjadřuje retenci analytu, přesněji dekadický logaritmus retenčního faktoru, jako součet několika individuálních, vzájemně nezávislých, interakčních příspěvků. Každý z těchto příspěvků je v rovnici LFER vyjádřen součinem fyzikálně-chemického parametru analytu (deskriptor) a vlivu prostředí na danou interakci (regresní koeficient). Základních pět deskriptorů je uvedeno a podrobněji popsáno v Tabulce 1 a dále v referencích [25-27]. Chromatografická data pro LFER analýzu se získávají pomocí sady testovacích analytů. Tato sada musí obsahovat dostatečné množství analytů, jejichž deskriptory jsou známy, a zároveň jsou rovnoměrně distribuovány, aby žádná z interakcí nebyla preferována. Mezi nejčastěji používané deskriptory patří Abrahamovy deskriptory, které byly odvozeny z rovnovážných měření a lze je tedy s určitou chybou považovat za deskriptory termodynamické. Kromě již zmíněných Abrahamových termodynamických deskriptorů existují i deskriptory založené na různých výpočtech, které již nemají nutně termodynamický základ. Mezi takové patří např. deskriptory pro flexibilitu a globularitu, které určitým způsobem charakterizují prostorové chování analytu [31].

Tabulka 1: Základní deskriptory modelu LFER a jejich popis.

Deskriptor	Určení deskriptoru
Rozsah molární refrakce E, popisuje interakce prostřednictvím n-/π-elektronových párů.	Získává se z indexu lomu zkoumané látky.
Dipolarita, polarizibilita S, popisuje interakce typu dipól-dipól, dipól-indukovaný dipól.	Získává se pomocí plynové chromatografie za použití polární stacionární fáze.
Celková nebo efektivní acidita vodíkové vazby A, popisuje schopnost analytu poskytnout atom vodíku pro tvorbu vodíkové vazby s molekulami mobilní a stacionární fáze.	Hodnoty tohoto parametru jsou vztaženy ke změně standardní Gibbsovy energie pro reakce v tetrachlormethanu s referenční bází.
Celková nebo efektivní bazicita vodíkové vazby B, popisuje schopnost analytu přijmout atom vodíku pro tvorbu vodíkové vazby s molekulami mobilní a stacionární fáze.	Deskriptor je stanoven na základě Abrahamovy stupnice. Nebazické soluty mají nulovou hodnotu tohoto deskriptoru.
McGowanův objem solutu V, odpovídá disperzním a kohezivním interakcím analytu s chromatografickým systémem, včetně schopnosti tvorby kavity.	Deskriptor je počítán na základě struktury analytu pomocí tabelovaných hodnot molárních objemů pro jednotlivé prvky a délky vazby.

Regresní koeficienty (*e, s, a, b, v*) se získají multidimenzionální lineární regresí a vyjadřují rozdíl mezi stacionární a mobilní fází ve vztahu k dané interakci.

- Koeficient *e* vyjadřuje rozdíl mezi fázemi v možnosti interakce prostřednictvím n-/π- elektronových párů.
- Koeficient *s* vyjadřuje rozdíl mezi fázemi v dipolaritě/polarizibilitě.
- Koeficient *a* vyjadřuje rozdíl ve schopnosti akceptovat vodík pro tvorbu vodíkové interakce.
- Koeficient *b* vyjadřuje schopnost poskytovat vodík pro tvorbu vodíkové interakce.
- Koeficient *v* vyjadřuje rozdíl v disperzních interakcích.

V případě, že regresní koeficient je kladný, pak je daný typ interakce preferován ve stacionární fázi, a tedy přispívá k retenci. Záporný regresní koeficient naznačuje, že daný typ interakce převládá v mobilní fázi a tedy tato interakce snižuje retenční čas. Statisticky nevýznamný regresní koeficient značí, že daná interakce je ekvivalentní pro obě fáze a neovlivňuje retenci. Dále model poskytuje hodnotu úseku *c*, který není spojen s žádnou definovanou interakcí, nicméně je pro daný systém za daných podmínek konstantou. Tento člen reflektuje fázový poměr a také možné interakce, které nejsou v základním modelu obsaženy [26].

Kromě stanovení distribuce interakcí v daném separačním systému může být LFER model použit i ke vzájemnému srovnání stacionárních fází. Pokud budou zachovány všechny separační podmínky (teplota, průtok, fixní složení mobilní fáze), budou získané regresní koeficienty na tyto separační podmínky vztaženy. Případné rozdíly hodnot regresních koeficientů proto odpovídají různým interakčním charakteristikám porovnávaných stacionárních fází. Tento přístup lze využít např. pro posouzení vlivu derivatizačních skupin či uspořádání CS na retenci/enantioselektivitu. Model LFER poskytuje základní kvalitativní i kvantitativní informace o interakčních mechanizmech v daném separačním systému, a proto je neocenitelným a hojně využívaným nástrojem pro studium chirálních i achirálních systémů jak v HPLC, tak v SFC [32-34].

4 CSP používané v HPLC a SFC

CSP tvoří základ většiny enantioselektivních systémů jak v HPLC, tak v SFC. Ideální CS by měl separovat co nejširší škálu enantiomerů, být stabilní během přípravy stacionární fáze, a také za podmínek separace (teplotní stabilita, stabilita vůči pH, stabilita v rámci používaných složek pufrů, kompatibilita s používanými rozpouštědly). Dále by měl být dostupný v dostatečném množství a kvalitě (definované složení a čistota) a za přijatelnou cenu.

V současné době je pro chirální separace v HPLC/SFC k dispozici mnoho komerčně dostupných CSP. Mezi nejvýznamnější patří CSP sacharidového typu. Polysacharidové CSP, které jsou v současnosti nejpoužívanější, a CSP na bázi cyklodextrinů budou podrobněji popsány v podkapitole 4.1, zatímco nověji uvedeným CSP na bázi cyklofruktanů, které byly studovány v rámci dizertační práce, bude věnována samostatná podkapitola 4.2.

4.1 Přehled nejpoužívanějších CSP

Polysacharidové CSP jsou tvořeny lineárními do šroubovice uspořádanými polysacharidy amylosou nebo celulosou, které se vzájemně liší typem spojení glukosových jednotek. Glukosové jednotky spojené β-(1,4) vazbou tvoří celulosu, zatímco amylosa je tvořena glukosovými jednotkami spojenými α-(1,4) vazbou. Enantioselektivní separační vlastnosti polysacharidových CSP se projeví zejména po derivatizaci aromatickými substituenty s různými funkčními skupinami. Derivatizační skupina je připojena k polysacharidové kostře nejčastěji přes karbamátový můstek. Základním enantiodiskriminačním mechanizmem polysacharidových CSP jsou vodíkové vazby a dipólové interakce v součinnosti se sterickými faktory. Dále se uplatňují π-π interakce analytu s derivatizačními skupinami. Starší generace polysacharidových CSP byla připravena pouhým pokrytím (*coatingem,* tzn. fyzikální adsorpcí polysacharidu na silikagelové částice), přičemž každý mód měl k sobě kompatibilní CSP. Nová generace polysacharidových CSP připravených imobilizací, tzn. chemickým navázáním polysacharidu na silikagelové částice, je multimodální [35].

Cyklodextriny jsou oligosacharidy tvořené D-glukopyranosovými jednotkami spojenými α-(1,4) vazbou. V oblasti chirálních separací se nejvíce uplatnily

cyklodextrinové CS složené ze 6, 7, či 8 glukosových jednotek (označované jako α-, β-, či γ-cyklodextriny), nativní nebo s různými derivatizačními skupinami. Zejména pak β-cyklodextriny se využívají v celé řadě analytických aplikací. Prostorové uspořádání spojených glukosových jednotek vytváří uvnitř cyklodextrinového CS hydrofobní kavitu. Inkluze analytu do této kavity CS je primární enantioselektivní interakcí na cyklodextrinových CSP v RP HPLC. Na hydrofilních okrajích kavity se mohou uplatnit další interakce - vodíkové vazby, dipólové interakce, popř. π-π interakce, v závislosti na typu derivatizační skupiny, které přispívají k chirálnímu rozlišení. V NP a POM je kavita "blokována" složkami mobilní fáze, nicméně nadále se uplatňují enantioselektivní interakce analytu s hydroxylovými a derivatizačními skupinami na povrchu cyklodextrinu [36,37].

Ostatní CSP a jejich aplikační využití jsou pro HPLC uvedeny v Tabulce 2 a v následujících referencích [10,21,38,39]. Základní přehled CSP a jejich aplikací v SFC jsou shrnuty v referenci [40] a dále v **Publikaci I**, kde je kromě výčtu CSP používaných v SFC a jejich aplikací uveden i základní teoretický rámec SFC, včetně vlastností a interakčních mechanizmů této separační techniky.

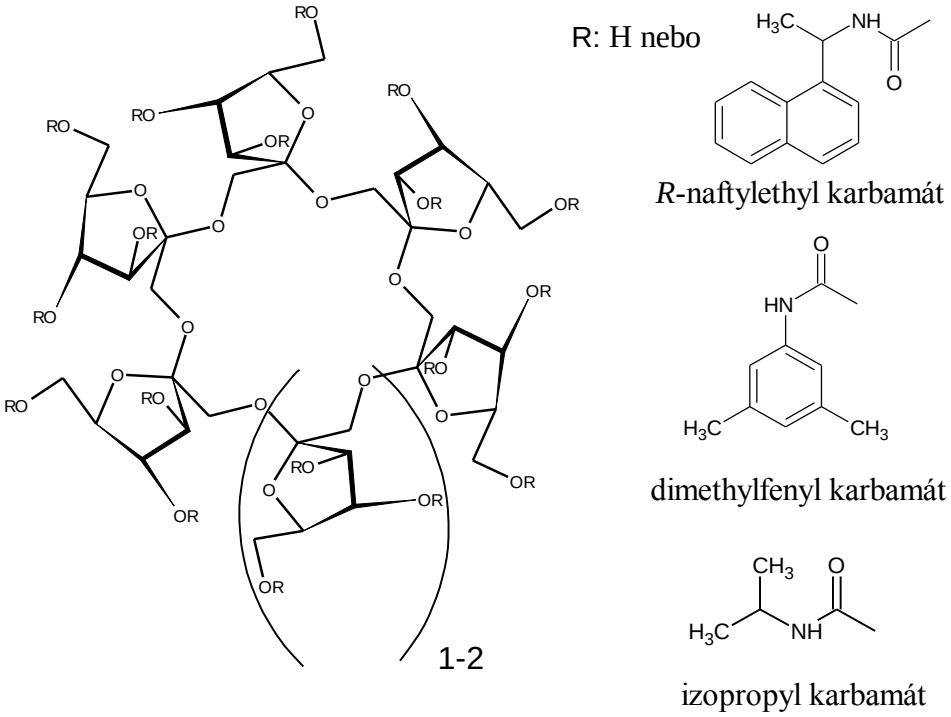
Tabulka 2: Přehled základních typů CSP, jejich nejčastějších aplikačních HPLC módů a pravděpodobných enantiodiskriminačních mechanizmů.

CS/CSP	Aplikační mód	Limitace	Pravděpodobný interakční mechanizmus	Enantioselektivita pro látky	Ref.
proteinové	RP	teplotní a pH stabilita CSP, nízký obsah organického modifikátoru v mobilní fázi	konformační změny CS vlivem složení mobilní fáze a teploty, vodíkové interakce, π-π interakce a iontové interakce	neutrální, kyselé i bazické látky	41 42
makrocyklická antibiotika	RP, NP, POM	pH stabilita CSP	vodíkové interakce, π-π interakce, dipólové a iontové interakce v součinnosti se sterickými faktory	široké spektrum látek	43 44
Pirklovy fáze (donor/akceptorové)	primárně NP, méně RP	nejsou multimodální	vodíkové interakce, π-π interakce, dipól-dipól interakce za přispění sterických vlivů	široké spektrum látek	45 46 47
c*rown*-ethery	primárně RP, méně NP	nízké pH vodných mobilních fází, nízký obsah organického modifikátoru v mobilní fázi (některé typy)	inkluzní komplexace v důsledku vzniku vodíkových interakcí mezi amoniovým kationtem a kyslíky v *crown*-etherovém kruhu, popř. iontové interakce	primární aminy (aminokyseliny a jejich estery, amidy, aminoalkoholy)	48 49
syntetické polymery	NP, POM	nižší enantioselektivita	vodíkové interakce, π-π interakce v součinnosti se sterickými faktory	široké spektrum látek	50 51
chirální měniče iontů	POM, RP	pouze pro ionizovatelné analyty	iontové interakce	obecně látky iontové povahy	52 43
chirální měniče ligandu	RP	nízký obsah organického modifikátoru v mobilní fázi	reverzibilní koordinace analytu do koordinační sféry kovu (nejčastěji Cu^{2+}), který je komplexací imobilizován na CS (obvykle aminokyseliny nebo jejich deriváty)	analyty obsahující dva nebo tři elektron-donorové substituenty (α-aminokyseliny, α–hydroxykyseliny, aminoalkoholy)	54 55

NP – normální mód; POM- polárně-organický mód; RP - reverzní mód

4.2 CSP založené na derivatizovaných cyklofruktanech

Armstrongova skupina představila v roce 2009 nový typ CS založených na derivatizovaných cyklofruktanech [56]. Cyklofruktany jsou, stejně jako velmi úspěšně používané cyklodextrinové CS, makrocyklické oligosacharidy obecně složené ze šesti či více D-fruktofuranosových jednotek spojených β-(2,1) vazbou. Současné komerčně dostupné cyklofruktanové CSP obsahují CS se šesti či sedmi fruktofuranosovými jednotkami. Obrázek 3 ukazuje základní strukturu cyklofruktanů, a dále jsou na něm zobrazeny tři derivatizační skupiny použité v komerčně dostupných cyklofruktanových CSP. Jak je z obrázku patrné, cyklické spojení fruktofuranosových jednotek vytváří základní *crown*-etherový skelet, který je však pro interakce u nativního cyklofruktanu částečně blokován intramolekulovými vodíkovými vazbami.



Obrázek 3: Struktury cyklofruktanových chirálních selektorů.

Na rozdíl od cyklodextrinů cyklofruktany netvoří hydrofobní kavitu [56]. Molekulové uspořádání významným způsobem ovlivňuje enantioselektivní vlastnosti cyklofruktanů. Zatímco nativní cyklofruktany vykazují omezenou selektivitu pro separaci enantiomerů, derivatizací hydroxylových skupin dojde k narušení intramolekulových vodíkových vazeb a následnému rozevření struktury selektoru,

čímž se zpřístupní *crown*-etherový kruh a významně se zlepší enantioselektivní vlastnosti. Typ derivatizační skupiny zásadním způsobem ovlivňuje selektivitu cyklofruktanových stacionárních fází vůči konkrétním skupinám analytů. Alifatické derivatizační skupiny zvyšují enantioselektivitu pro separaci aminů [57]. Aromatické derivatizační skupiny přispívají k retenci dalšími typy interakcí, především π-π interakcemi, a významným způsobem zlepšují enantioselektivitu pro celou řadu látek [58].

Přestože jsou cyklofruktanové CSP multimodální, jejich uplatnění se v současnosti nachází především v NP a POM HPLC [59-62]. V RP HPLC jsou zatím cyklofruktanové CSP využívány minimálně [63,64]. Dále byly publikovány aplikace cyklofruktanů jako CS pro GC [65], CE [66,67] nebo jako stacionární fáze pro HILIC systémy [68-70].

Publikace I

Supercritical Fluid Chromatography as a Tool for Enantioselective Separation; A Review

Kalíková K., Šlechtová T., **Vozka J.**, Tesařová E.

Analytica Chimica Acta **2014**, 821, 1-33

Analytica Chimica Acta 821 (2014) 1–33

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Review

Supercritical fluid chromatography as a tool for enantioselective separation; A review

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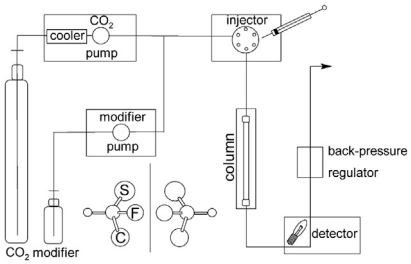
HIGHLIGHTS

- Review article on enantioselective separations in supercritical fluid chromatography.
- The review covers the period from 2000 up to August 2013.
- Both theoretical studies and applications are covered.
- Applications along with separation conditions are arranged in tables.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 2 December 2013
Received in revised form 31 January 2014
Accepted 22 February 2014
Available online 28 February 2014

Keywords:
Review
Supercritical fluid chromatography
Chiral stationary phase
Enantioselective separation
Enantiomers

ABSTRACT

Supercritical fluid chromatography (SFC) has become popular in the field of enantioselective separations. Many works have been reported during the last years. This review covers the period from 2000 till August 2013. The article is divided into three main chapters. The first one comprises a basic introduction to SFC. The authors provide a brief explanation of general principles and possibilities of this method. The advantages and drawbacks are also listed. Next part deals with chiral separation systems available in SFC, namely with the commonly used chiral stationary phases. Properties and interaction possibilities of the chiral separation systems are described. Recent theoretical papers are emphasized in this chapter. The last part of the paper gives an overview of applications of enantioselective SFC in analytical chemistry, in both analytical and preparative scales. Separation systems and conditions are summed up in tables so that they provide a helpful tool for analysts who search for a particular method of analysis.

Abbreviations: 2-BuOH, 2-butanol; 2-PrOH, 2-propanol; ACN, acetonitrile; BRTM, binary retention time method; CBZ, carboxybenzyl; CD, cyclodextrin; CFs, cyclofructans; CSPs, chiral stationary phases; DCM, dichloromethane; DEA, diethylamine; DMEA, dimethylethylamine; DMOA, N,N-dimethyloctylamine; DNS, dansylchloride; ECP, elution characteristic point; ED, equilibrium dispersive; ESA, ethanesulfonic acid; EtOH, ethanol; FA, formic acid; HOAc, acetic acid; HPLC, high performance liquid chromatography; IBA, isobutylamine; IPA, isopropylamine; L-PA, phenylalanine anilide; MeCD, dimethylated-β-cyclodextrin; MeOH, methanol; MIP, molecularly imprinted polymer; NH_4OAc, ammonium acetate; NH_4TFA, ammonium trifluoroacetate; NPLC, normal phase liquid chromatography; OTC, open tubular columns; POPLC, polar organic phase liquid chromatography; POSC, polar organic solvent chromatography; QN, quinine; QD, quinidine; R, ristocetin A; SMC, simulated moving columns; SFC, supercritical fluid chromatography; SF-SMB, supercritical fluid simulated moving bed chromatography; T, teicoplanin; TAG, teicoplanin aglycone; TEA, triethylamine; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol; UHPLC, ultra high performance/pressure liquid chromatography; V, vancomycin; THF, tetrahydrofuran.

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http://dx.doi.org/10.1016/j.aca.2014.02.036
0003-2670/

Contents

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1. Introduction

Routine qualitative and quantitative analyses, separations of complicated matrices, preparative scale separations or chiral separations are common and irreplaceable part of pharmaceutical, agrochemical and food productions. Nowadays, high performance liquid chromatography (HPLC) predominates as separation technique in both analytical and preparative scale. However, demands of the market on the limits of detection/quantification and analysis time rapidly increase. To meet these requirements producers of HPLC apparatus lowered dwell volumes and made the devices trouble-free as much as possible. Producers of HPLC columns minimized the particle size to dimensions, which were not applicable 20 years ago due to unacceptable increase of the system pressure that could not be maintained at that time. (Contemporary modern systems working with extremely high pressures are termed therefore ultra high performance/pressure liquid chromatography (UHPLC).) Moreover, shell particles and monolithic columns were invented and improved. Nevertheless, there still remained the possibility for improving the properties of mobile phases. This made a space for supercritical fluid chromatography (SFC).

Supercritical fluid is formed if temperature and pressure of a gas or liquid exceed their critical values. Supercritical fluids have unique features lying between gas and liquid states. Liquid-like densities and dissolving capabilities together with gas-like viscosities and diffusion properties make them ideal candidates for major mobile phase components [1,2]. In general, critical values of the majority of substances cannot be routinely achieved but critical temperature and critical pressure of CO_2 (T_C = 304.12 K, p_C = 73.74 bar) are easily attainable. Moreover, CO_2 has other favorable properties as it is non-toxic, non-flammable, can be easily purified and is relatively cheap. Its high molecular diffusivity

considerably enhances mass transfer [1]. The separation technique, which uses supercritical fluid as the main component of the mobile phase, is widely accepted as SFC, despite the fact that the majority of SFC separations take place in subcritical region due to the addition of organic modifiers [1,2]. It is worth noticing that SFC can substitute both normal phase and reversed phase HPLC separation modes, despite the fact that it was often incorrectly considered to be only a normal phase system [2]. Three independently changeable and strictly controlled conditions, namely pressure, temperature and mobile phase composition enable separation of a large number of compounds in reasonable analysis time. Wide variety of possible organic modifiers facilitates the method development and significantly accelerates the optimization of separation. Principal features of the SFC separation systems enable also trouble-free column coupling suitable for analyses of complicated mixtures. The SFC mobile phases enable high flow rates and therefore fast analyses. Post-analysis evaporation of CO_2 keeps products concentrated in the organic modifier. Last but not least, compounds are usually better soluble in mixtures of supercritical fluids and organic modifiers than in pure organic solvents [1,3].

SFC has been introduced more than 50 years ago [4]. However, only few papers were published during the next two decades, despite the fact that SFC pioneers substantially improved the instrumentation [*e.g.* 5–7]. The rebirth of SFC started in early 80s of the 20th century. Since then there were keen disputations between the supporters of packed and open tubular SFC columns. Open tubular columns (OTC) were considered to provide better chromatographic efficiency due to the lower pressure drop. These columns were also compatible with common GC detectors and furthermore they were supported by strong marketing strategy mainly on the US market. The described situation caused a temporary abatement of more user-friendly packed columns [8,9]. However, technical difficulties and poor reproducibilities/repeatabilities of the methods resulted within a few years in the extinction of OTC in SFC [8,9]. SFC as a method regained its popularity in the 90s. This was supported by the boom of packed SFC columns that proved to have bigger separation potential than the OTC. Consequently, SFC could substitute or even surpass HPLC in many applications. The main difficulties with back-pressure regulation, consistent flow rates, modifier addition and automation have been resolved. Modern SFC apparatus are compatible with common chromatographic detectors including MS detector [10,11]. Moreover, first attempts to employ sub-2-μm and shell particles in SFC have succeeded [12,13].

In 2013 a new SFC apparatus was introduced by Waters as ultraperformance convergence chromatography UPC^2, which opens a new possible dimension of analytical instrumentation. SFC is becoming a widely accepted and used technique in both academic and commercial spheres. A number of interesting reviews, which cover history [8,12], applications [3,9,14] and also the physicochemical point of view [1,2,15] has emerged recently.

The aim of our work is to provide a comprehensive literature overview focused on chiral SFC separations covering the time period from 2000 till August 2013 according to Web of Science. As fast and efficient enantioselective separations are essential demands mainly in pharmaceutical industry, SFC using chiral stationary phases seems to be a good solution. For the right choice of a SFC method some basic knowledge of the stationary phases is fundamental. Therefore, we start with basic description of available chiral stationary phases (CSPs) and properties of separation systems in SFC, and then we summarize applications that are clearly arranged in tables.

2. Chiral stationary phases and chiral SFC separation process

Open tubular, packed capillary, and packed column formats were utilized in chiral SFC, although the field has been dominated by applications involving packed columns in recent years [16,17]. The unique properties of supercritical fluids make packed column SFC the most favorable choice for fast enantioselective separations among all possible separation techniques [18]. For chiral SFC separations most HPLC chiral stationary phases can be directly used [3] and new types of CSPs are still being developed. While many papers are focused on particular applications by chiral SFC (see Tables 1 and 2) only few papers deal with fundamental SFC studies [9]. In this chapter we give an overview of CSPs used in SFC and review some theoretical aspects that can be useful in method development and optimization of the enantioselective SFC separations.

2.1. Polysaccharide-based CSPs

CSPs based on derivatized polysaccharides are most popular in SFC nowadays [19,20]. Recent development in their synthesis and application was reviewed by Chankvetadze [21] in 2012. As native polysaccharides showed only weak chiral recognition ability, various derivatives, particularly of cellulose and amylose, were developed [22,23]. These derivatives behave differently in terms of enantioselectivity. For example, amylose benzoates show much lower recognition abilities than the cellulose derivatives. This may be a consequence of lower conformational stability of the amylose derivatives [23]. Tris(phenylcarbamates) of cellulose and amylose differ in their higher-order structure, *i.e.* left-handed 3/2 and 4/1 helical chain conformations, respectively. The difference in their helical structures may result in different chiral recognition ability [23–26].

Amylose-based Chiralpak AD column (amylose tris(3,5-dimethylphenylcarbamate) CSP) was applied for basic determination of interactions responsible for retention and chiral discrimination of thiazolbenzenesulfonamide compound [27]. The results revealed that while the main adsorbing interactions are formed between the hydroxyl group of the analyte (OH group is located on alkyl chain connected to pyridine ring) and the carbamate group of the CSP, chiral discrimination was achieved through an inclusion mechanism within the chiral cavity created along the amylose chains. It was demonstrated that the process is enthalpy-driven. A full factorial design with three center points was used for study of the influence of chromatographic conditions (column temperature, column back-pressure, and methanol content in the mobile phase) on the chromatographic behavior, *i.e.* retention and enantioselectivity of amino alcohols on Chiralcel OD (cellulose tris(3,5-dimethylphenylcarbamate) CSP) and Chiralpak AD columns [28]. The centerpoint conditions were set as Chiralcel OD column, 10 mM DMOA and 50 mM acetic acid in MeOH/CO_2 20/80 (v/v), column temperature 30 °C, column back-pressure 200 bar. The column temperature and concentration of MeOH had a greater impact on chromatographic performance than column back-pressure. Differences between predicted and experimental data were less than 10%. A mixed theoretical and empirical isotherm was used to describe the adsorption behavior of 1-phenyl-1-propanol enantiomers as a function of temperature, density and modifier concentration at the same time on Chiralcel OD column [29]. The authors found that the separation performance was better at 30 °C than at 40 °C if the other conditions were kept the same. Consequently, the same column was used under nonlinear adsorption conditions. A binary Langmuir isotherm was applied to describe adsorption of 1-phenyl-1-propanol enantiomers as a function of density and

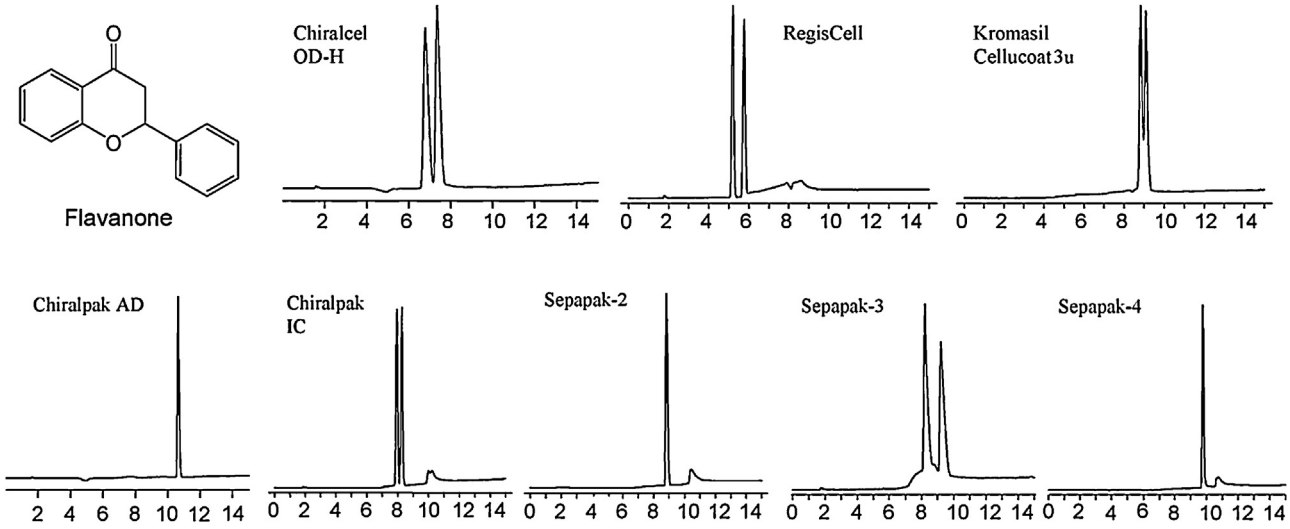

Fig. 1. Comparison of enantioseparation of flavanone on the generic OD-H columns and the Chiralpak AD-H and five other polysaccharide-based CSPs. Separation conditions: gradient elution, CO_2 and 4% (MeOH with 25 mM IBA) for 4 min, then ramp at 4% min^{-1} to 40%, hold for 2 min at 40%, 200 bar, 2 mL min^{-1}, 35 °C, detection wavelength 215 nm, injection volume 10 μl.

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modifier concentration [30]. Bao et al. used the equilibrium dispersive (ED) model to describe the chromatographic process [31]. For the estimation of single-component adsorption isotherms of trans-(−)- and -(+)-paroxol, overloaded single-component elution profiles were used to calculate adsorption capacity by the elution characteristic point (ECP) method. The isotherms obtained were further validated by comparing experimental elution profiles on Chiralpak AD-H (amylose tris (3,5-dimethylphenylcarbamate) CSP) column with the predictions based on the ED model. Inversion of elution order of paroxol enantiomers when using 2-PrOH instead of MeOH or EtOH suggested that chiral recognition mechanism on Chiralpak AD-H column could be quite different in SFC and HPLC. The enantioseparation of flurbiprofen on Chiralpak AD-H column was studied under linear and non-linear conditions [32]. The linear isotherms showed characteristics typical of SFC systems, *i.e.* the Henry constants decreased with increasing density and modifier concentrations. Non-linear isotherms were obtained by matching experimental elution profiles of pure enantiomers with calculated ones that were based on competitive Langmuir and bi-Langmuir isotherms. It was found that mass transfer coefficients were rather high, which was reflected in the sharp elution profiles. The saturation capacities did not show regular trends with neither the density nor the modifier concentration. A binary retention time method (BRTM) for measurement of competitive Langmuir isotherm parameters was developed [33]. The method utilizes measured retention times of the two shock fronts of binary injections and the Henry constants to estimate the isotherm parameters. Two schemes, G-BRTM and V-BRTM were introduced. The G-BRTM can be used for both mass and volume overload conditions, while the V-BRTM can be used only for volume overload injections. However, the V-BRTM is expected to be more robust as no bounds for the ranges of the decision variables are required and hence is independent of user's input.

Retention data were previously obtained on Chiralcel OD and Chiralcel OB-H (cellulose tribenzoate) columns [30,34]. Wenda et al. presented multi-objective optimization analysis and experimental implementation of a single column isocratic SFC process for the enantioseparation of flurbiprofen using Chiralpak AD-H column [35]. Optimization problems can be sorted into two kinds with respect to the number of objective functions, namely single and multi-objective. These two kinds of optimization problems are conceptually different. Single objective problems seek to maximize or minimize one objective function and thus result in unique set of decision variables. In the case of multi-objective optimization there may not exist an unique optimum (*i.e.* a single point) with respect to all the objectives. Instead, there would be an entire set of optimal solutions (*i.e.* a curve known as Pareto curve) when the objectives conflict with each other. Simulation of the process was carried out using a detailed model with equilibrium description by a competitive Langmuir isotherm. West et al. investigated factors participating in the chiral recognition on two polysaccharide-based columns, Chiralcel OD-H (cellulose tris(3,5-dimethylphenylcarbamate) CSP) and Chiralpak AD-H [36,37]. The reasons for successful enantioseparation were shown to be clearly different on the two CSPs. Indeed, steric fit along with hydrogen bonding seemed to be the most important for good enantiorecognition on Chiralpak AD-H. However, enantiorecognition on Chiralcel OD-H column required not only hydrogen bonding but also dipole–dipole and π–π interactions. Two chlorinated polysaccharide CSPs, cellulose tris-(3-chloro-4-methylphenylcarbamate) and amylose tris-(5-chloro-2-methylphenylcarbamate) (Lux Cellulose-2 and Lux Amylose-2 columns) were used for investigating effects of molecular structure of chiral fluoro-oxoindole-type compounds, temperature, modifier nature and its content on retention and enantioresolution in SFC [38]. The effect of temperature was shown to be of less significance than the other factors studied. However, the temperature was strongly dependent on the stationary phase, the mobile phase and structure of analytes. De Klerck et al. compared the enantioselectivity of twelve polysaccharide-based CSPs from different manufactures [39]. They confirmed the presumption that CSPs containing the same selector do not always display the same enantioselectivity. Many works using various polysaccharide-based CSPs are focused also on thermodynamic studies (using van't Hoff plots, dependences of $\ln k$ on reciprocal thermodynamic temperature) of enantioseparation processes: Chiralpak IB (cellulose tris(3,5-dimethylphenylcarbamate) immobilized onto silica gel CSP) column [40]; Chiralcel OD-H, Chiralpak AD, Lux Cellulose-2, Lux Amylose-2 columns [41–44]; Lux Cellulose 1 (tris(3,5-dimethylphenylcarbamate) CSP), Lux Cellulose 2 columns [45,46]; Chiralcel OD-H [47] and Chiralpak IC (cellulose tris (3,5-dichlorophenylcarbamate) immobilized on silica gel) columns [48]; Sino-Chiral OJ (cellulose tris(4-methylbenzoate) CSP) column [49]; Chiralpak AD-H column [50] were used for these studies.

Various screening strategies were developed and applied for different polysaccharide-based columns [51–59]. For detailed information, *i.e.* columns and mobile phase compositions see Table 1 (Fig. 1).

2.2. Cyclic oligosaccharides

2.2.1. Cyclodextrins

Cyclodextrins (CDs) used in enantioselective separation systems are composed of 6-8 D-glucopyranose units. These units linked together form a relatively hydrophobic cavity while hydrophilic hydroxyl groups on the rim can serve for additional interactions or can be further derivatized [3]. Formation of enantioselective inclusion complexes of analytes with the hydrophobic cavity of CDs can be hindered by the apolar carbon dioxide in SFC [60]. The majority of works deals with syntheses of new CD derivatives and their evaluation.

Synthesis and application of some novel cyclodextrin (CD) CSPs applied also in SFC appeared in some review papers dealing with chromatographic separations in general [61,62]. The structure of β-cyclodextrin is depicted in Fig. 2A.

Four mono-2 and mono-6-O-pentenyl-β-CD-CSPs were compared in terms of their enantioselectivity for aminoglutethimide and thalidomide [63]. The influence of the nature of heteroatom functionality in the spacer arm between CD and support and regioselectivity of the pentenyl spacer in position 2 or 6 on the glucopyranosidic unit were evaluated. The impact of the position of the pentenyl moiety was of crucial importance in the chiral discrimination phenomenon. SFC with these CSPs is suitable for the enantioseparation of aminoglutethimide but not effective for thalidomide.

Sumichiral OA-7500 column composed of heptakis(2,3,6-tri-O-methyl)-β-CD was compared with amylose-based CSP (Chiralpak AD-H column) in terms of enantioselectivity [64]. The effects of various separation conditions were investigated and compared for both columns. It was found that lower alcohol content in the mobile phase improved enantioseparation of α-tetralol on the Sumichiral column and 1-phenylethylamine on the Chiralpak AD-H column, while this effect was not observed with either α-tetralol or 2-phenylpropionic acid on the Chiralpak AD-H column. Four cationic β-CD derivatives, namely mono-6-(3-methylimidazolium)-6-deoxy-perphenylcarbamoyl-β-CD chloride (MPCCD) [65], mono-6-(3-methylimidazolium)-6-deoxyper(3,5-dimethylphenylcarbamoyl)-β-CD chloride (MDPCCD), mono-6-(3-octylimidazolium)-6-deoxyperphenylcarbamoyl-β-CD chloride (OPCCD) and mono-6-(3-octylimidazolium)-6-deoxyper(3,5-dimethylphenylcarbamoyl)-β-CD chloride (ODPCCD), were synthesized and physically coated onto porous spherical silica gel to obtain CSPs [66]. Obtained results revealed that the CSPs containing an *n*-octyl group on imidazolium moiety and phenylcarbamoyl groups on the CD ring provided enhanced analyte – chiral substrate interactions over CSPs bearing methyl group on the imidazolium moiety and 3,5-dimethylphenylcarbamoyl groups on the CD ring. OPCCD CSP showed the best separation abilities for tested analytes. Vinyline-functionalized cationic β-CD was co-polymerized with vinylized silica in the presence of azobisisobutyronitrile and conjugated monomers to form chemically immobilized CSP applicable in SFC [67]. The results showed that analytes undergoing good chiral resolution contained ionizable moieties (forming anions), which take part in electrostatic attractions with the cationic moiety on the CSP. Other cationic β-CD CSPs were prepared for application in SFC, *i.e.* cationic β-CD perphenylcarbamoylated derivatives chemically bonded onto vinylized silica using a radical co-polymerization [68]. Authors found out that electrostatic forces between enantiomers and the cationic moiety of β-CD are important for retention and enantioseparation. Aromatic cationic moiety on β-CD derivative enabled better enantioseparations than an aliphatic one.

2.2.2. Cyclofructans

Cyclofructan-based CSPs were introduced in 2009 by Armstrong' group [69]. Cyclofructans (CFs) are macrocyclic oligosaccharides that consist of six or more β-(2→1) D-fructofuranose units. According to the number of fructofuranose units in the macrocyclic ring the common abbreviations for these compounds are CF6, CF7 and CF8 [69]. In contrary to CDs the central core of CFs is hydrophilic, has the crown ether like structure – see Fig. 2B. Derivatization of CFs significantly increases their enantioselectivity. Preliminary results showed enantioselective separation power of *R*-naphthylethyl CF6 CSP (Larihc CF6-RN column) in SFC [69]. More detailed study was performed with dimethylphenyl carbamate CF7 CSP (Larihc CF7-DMP column) in SFC and the results were compared with those obtained in HPLC [70]. The interactions contributing to retention in various mobile phase compositions were revealed by linear free energy relationship in both separation systems. The distribution and strength of individual interaction types varied with the mobile phase compositions. The results suggested that adsorption of certain components of the mobile phases plays more important role in SFC than in HPLC. Dispersion interactions showed similar negative values using both techniques. The main contribution of hydrogen bond acidity was also comparable for both methods. However, the propensity to interact with *n*- and/or π-electron pairs of solutes was significant only in SFC. The effect of column back-pressure on enantioseparation using binary mobile phases was tested on *R*-naphthylethyl CF6 CSP and other nine columns in the mobile phase composed of MeOH/CO_2 20/80 (v/v) [71]. Increased apparent dead time (t_0) was observed at an increased column back-pressure. The analysis of the experimental data indicated that t_0 depends not only on the relative density change along the column length but also on the adsorption of the modifier (MeOH) onto the stationary phase. The measured retention (k) over pressure was found to follow a linear relationship. As the column back pressure increased from 100 to 200 bar, resolution decreased only slightly, on average 6%, mainly due to the retention and efficiency decrease. The higher the retention of a compound the more sensitive was its retention to pressure changes. This empirical observation was validated based on the SFC separation of 11 pairs of enantiomeric drug-like molecules on all tested columns.

2.3. Ion exchange CSPs

Quinine (QN) and quinidine (QD) are alkaloids of the *Cinchona* family [72]. The QN- and QD-based CSPs (see Fig. 3) can possess besides the ion-pairing interactions, a combination of hydrogen bond formation, π–π and van der Waals interactions [73]. Quinine and quinidine-derived anion-exchanger CSPs showed good enantioselective potential for separation of acidic enantiomers also in SFC [72,74]. It was found that a carbamoyl modification of the secondary hydroxyl group at C9 position of the alkaloid significantly enhanced the enantiorecognition capabilities of the resulting chiral selector. The *tert*-butyl carbamates of QN and QD immobilized on spherical silica gel turned out to be the most versatile compromise of structure variations (QN-AX and QD-AX columns).

A novel strong cation exchange type CSP based on a syringic acid amide derivative of trans-(*R*,*R*)-2-aminocyclohexanesulfonic acid was prepared [75]. The results point to the existence of carbonic and carbamic acid salts formed as a consequence of reactions occurring between carbon dioxide, the alcoholic modifiers and the amine species present in the sub/supercritical fluid medium, respectively. The authors proved that retention on this CSP is predominantly

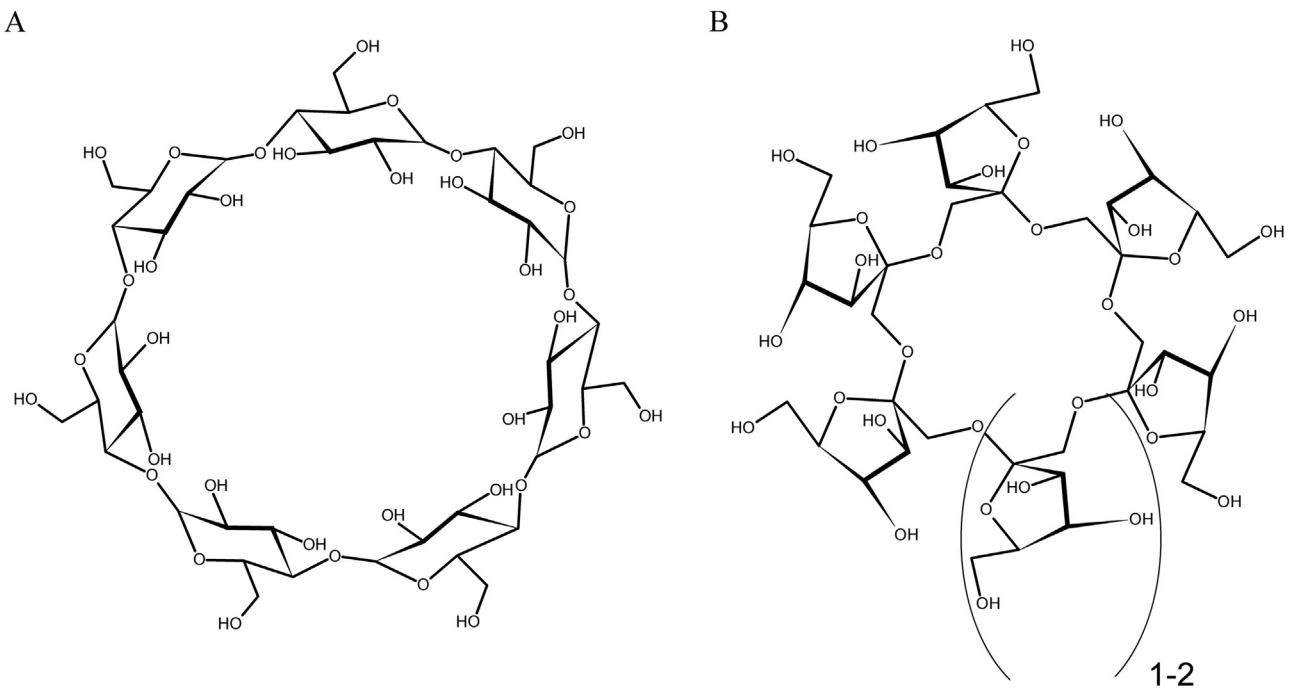

Fig. 2. Structure of cyclic oligosaccharides. (A) β-Cyclodextrin and (B) cyclofructan.

based on an ion exchange mechanism, according to the stoichiometric displacement model.

2.4. Macrocyclic glycopeptide CSPs

To this family of CSPs belong mainly teicoplanin (T), teicoplanin aglycone (TAG), ristocetin A (R) and vancomycin (V)-based phases. Glycopeptide antibiotics consist of an aglycone "basket" and pendent carbohydrate moieties, which are missing, of course, in teicoplanin aglycone structure [76]. The aglycone portion of these compounds is made up of 3 or 4 macrocyclic rings, which contain ether, amide and peptide linkages. In addition, one or more carbohydrate moieties are attached at various locations to each of the aglycones. Macrocyclic glycopeptide CSPs are used less frequently for chiral separation in SFC nowadays. Teicoplanin-based (Chirobiotic T) column was used in a set of other nine columns in simulated moving bed SFC [77]. The chiral recognition capabilities of three macrocyclic glycopeptide-based chiral columns, namely Chirobiotic T, Chirobiotic TAG and Chirobiotic R, were evaluated with supercritical and subcritical fluid mobile phases [78]. All separations were performed with an outlet pressure regulated at 100 bar, temperature 31 °C and at flow rate of 4 mL min^{-1}. Various amounts of MeOH ranging from 7 to 67% (v/v) were added to the CO_2 along with small amounts (0.1–0.5%, v/v) of TEA and/or TFA dependent on the analyte structure. Chirobiotic TAG column was the most effective, closely followed by the Chirobiotic T column. Both teicoplanin-based CSPs were able to separate, partially or fully, 92% of the enantiomers tested. The ristocetin chiral selector could partially or baseline resolve 60% of the enantiomers. Three macrocyclic glycopeptides CSPs, namely Chirobiotic T, Chirobiotic V and Chirobiotic TAG columns were compared in terms of their enantioselectivity for twenty-four structurally related coumarin derivatives [79]. The relationship between the analyte structure and CSPs' enantioselectivity was discussed. The majority of these derivatives could be separated in less than 10 min on the Chirobiotic columns. Another paper was focused on the influence of variation of separation conditions on enantioseparation on ristocetin A-based CSP (Chirobiotic R) [80]. Seven of the set of nine analytes studied were enantioseparated in SFC, while all could be separated using different modes of HPLC. The authors found out that varying conditions and structures did not allow identification of the interactions responsible for chiral recognition. The effect of additives (isopropylamine and triethylamine) concentrations on the chromatographic behavior of vancomycin-based CSP (Chirobiotic V) was examined [81]. Many analytes failed to elute from the vancomycin-based CSP in the absence of an additive and the most noticeable effect of increasing additive concentration was a significant decrease in retention. Chirobiotic V column was used as one of a set of ten chiral columns for new SFC tandem column screening tool [82]. The modification of SFC instrument enabled to screen ten different columns and twenty-five different tandem column arrangements. The resulting setup could be useful for screening

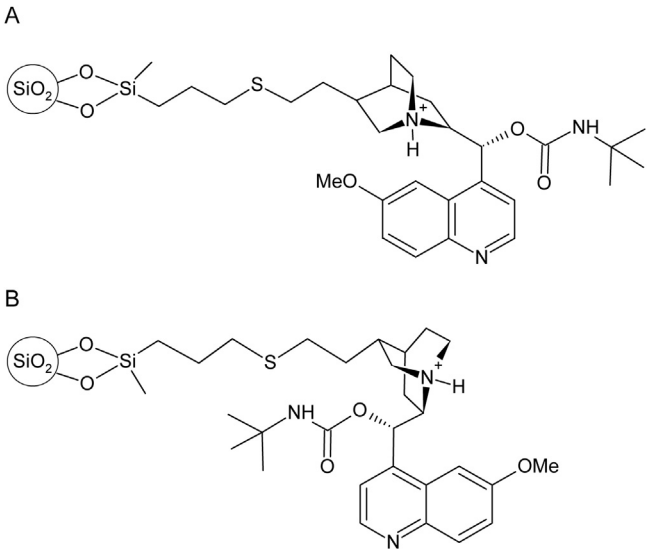

Fig. 3. Structures of weak anion exchangers. (A) QN-AX (quinine-based) and (B) QD-AX (quinidine-based).

of multicomponent separation problems in general. The effect of column back-pressure on SFC enantioseparation was tested on teicoplanin-based CSP (Chirobiotic T column) [71]. The results of this work are described in more detail in Section 2.2.2 as CF-based column was also tested.

2.5. Pirkle or brush type CSPs

Pirkle CSPs were developed to be either π-electron acceptors or π-electron donors and later also columns with both π-donor and π-acceptor phase attributes [83]. Chirex 3005 column consisting of (*R*)-1-naphthylglycine and 3,5-dinitrobenzoic acid found its applicability also in chiral SFC/tandem mass spectrometry [84]. The conditions of analysis of ketoprofen enantiomers providing a good balance among sensitivity, resolution, and sample throughput used for further validation were: flow rate of 5 mL min^{-1} and 55% MeOH in CO_2 as mobile phase. The first order kinetic equation was used to determine the enantiomerization barrier of some of 3-hydroxy-1,4-benzodiazepine enantiomers and N-(*p*-methoxybenzyl)-1,3,2-benzodithiazol-1-oxide in SFC with Whelk-O1 (*R*,*R*) column [85,86]. Chirex 3022 column, (*S*)-indoline-2-carboxylic acid and (*R*)-1-(α-naphthyl) ethylamine with urea linkage CSP and Whelk-O1 (*S*,*S*) column were used in a unique column switching technique called "Simulated Moving Columns" (SMC) [87]. SMC use two or three short chiral columns connected in series, and enable the unresolved enantiomers to separate repeatedly and exclusively through each of the columns until sufficient resolution is attained. Unlike the traditional closed-loop recycling chromatography where analytes are cycled through a single column and pump, SMC works independently on the pumps and therefore loss in resolution (due to band broadening) is avoided. In SMC, there is no increase of the system back-pressure in the process, since the total physical length of the columns remains constant, regardless of how many cycles are required to achieve resolution. So, SMC allows improvement of separation by virtually multiplying the column length which provides increased resolution at a constant pressure. The Whelk-O1 (*S*,*S*) column and polysaccharide-based CSPs were used for evaluating new mobile phase modifier 2,2,2-trifluoroethanol [88]. This modifier was used as an alternative to alcohols for enantioseparation of alcohol sensitive compounds. It was shown that trifluoroethanol exhibits ability to resolve a variety of enantiomers when conventional alcohol modifiers should not be used for the analytical application or the preparative separation. Szczerba and Wrezel tested effects of varying co-solvents for chiral SFC method development on Whelk-O1 column [89]. The authors found out that increasing polarity of alcohol correlates with decreasing selectivity and retention for tested analytes. The Whelk-O1 (*R*,*R*) column was used for testing of column back-pressure effects on enantioseparation [71]. The results of this work are described in more detail in Section 2.2.2. This CSP combines both π-electron donor (tetrahydrophenanthrene moiety) and π-electron acceptor (3,5-dinitrobenzoyl group) with amide hydrogen donor–acceptor site in a semi-rigid scaffold [90].

2.6. Synthetic polymeric columns

Despite the fact that synthetic polymeric CSPs can be prepared according to the requests of analysts, they did not find wide routine use in any of the chromatographic separation systems. Nevertheless, polymeric chiral columns also found applications in SFC. Kromasil CHI-TBB column composed of (*o*,*o*′-bis-4-tert-butylbenzoyl)-N,N′-diallyl-L-tartar diamide was found to be suitable for enantioseparation in SFC [91–93]. For more details see Table 1. Adsorption isotherms for the ibuprofen enantiomers were determined on a Kromasil CHI-TBB column at a temperature of 40 °C and pressures of 15.6 and 17.0 MPa [94]. The porosity of the stationary phase was calculated from chromatograms of pure *n*-hexane. The measured overall porosity of the analytical column was $\varepsilon = 0.703$. Han and coworkers proposed empirical equations for calculation of retention factor and resolution values of ibuprofen enantiomers using Kromasil CHI-TBB column [95]. Two polymeric CSPs based on trans-(1*S*,2*S*)-cyclohexanedicarboxylic acid bis-4-vinylphenylamide, and trans-N,N′-(1*R*,2*R*)-cyclohexanediyl-bis-4-ethenylbenzamide monomers were prepared and evaluated in SFC [96]. Authors found out that different orientation of the amide group of monomer used for synthesis of the two CSPs resulted in significant differences in their enantioselectivities. The CSPs were highly complementary to each other. Only 8 enantiomers from a total of 42 were separated on the both CSPs. Most chiral molecules tested were separated just on one column. P-CAP (poly(trans-1,2-cyclohexanediyl-bis acrylamide) column was found to be beneficial in the separation of a complex mixture of enantiomers and achiral impurities. A key advantage of this type of CSP is the fact that it is available in both enantiomeric forms, allowing reversal of elution order of enantiomers [97]. The polymeric N,N′-[(1*S*,2*S*)-1,2-cyclohexanediyl] bis-2-propenamide (P-CAP), the polymeric N,N′-[(1*R*,2*R*)]-1,2-diphenyl-1,2-ethanediyl] bis-2-propenamide (P-CAP-DP), the polymeric trans-9,10-dihydro-9,10-ethanoanthracene-(11*S*,12*S*)-11,12-dicarboxylic acid bis-4-vinylphenylamide (DEABV) and the polymeric N,N′-[(1*R*,2*R*)-1,2-diphenyl-1,2-ethanediyl] bis-4-vinylbenzamide (DPEVB) were bonded to 5 μm silica particles and used for preparation of four chiral columns [98]. Their enantioselectivity was tested with a set of 88 structurally different chiral compounds. All enantiomers were separated on one or more of the prepared CSPs. However, the DPEVB CSP was significantly less efficient while the DEABV CSP seemed to be the most broadly applicable of these CSPs. Three CSPs were synthesized based on (1*S*,2*S*)-1,2-bis(2,4,6-trimethylphenyl) ethylenediamine, (1*S*,2*S*)-1,2-bis(2-chlorophenyl) ethylenediamine, and (1*S*,2*S*)-1,2-di-1-naphthylethylenediamine *via* a simple free-radical-initiated polymerization in solution [99]. All three CSPs showed enantioselectivity for a large number of racemates with a variety of functional groups, including amines, amides, alcohols, amino acids, esters, imines, thiols, and sulfoxides. Their performances were compared with that of P-CAP-DP commercial polymeric column (the chiral monomer used is (1*S*,2*S*)-1,2-diphenylethylenediamine). P-CAP-DP CSP added π–π interaction possibilities [99,100] that were not available in the P-CAP phase. The new polymeric CSPs showed similar or better enantioselectivities and faster separation capability compared with the commercial column.

2.7. Molecularly imprinted polymers CSPs

Molecularly imprinted polymer (MIP)-based CSPs are composed of chiral "receptor" selective for one enantiomer of the pair. High selectivity arises from shape-selective recognition sites, generated by the imprinting process [101]. MIPs are often prepared in form of monoliths. If the monolith swells, its through pores will decrease in size resulting in lower permeability, and consequently leading to reduced reproducibility [102]. Physical properties of mobile phases and the polymer swelling will depend on the CO_2/organic modifier ratio, temperature and pressure. Manipulation of these variables should enable the polymer swelling to be "tuned" [103]. While MIP CSPs were applied for many years in chiral HPLC separations [101] only few publications deal with their use in SFC. MIPs as CSPs in SFC were first used in 2000 [104]. Two types of MIP CSPs were prepared, for the templates free base racemic propranolol and L-enantiomer of phenylalanine anilide (L-PA) were used. After several days under SFC conditions, the performance of the photochemically initiated L-PA MIP was

Table 1
Summary of CSPs, mobile phase compositions, separation conditions (back-pressure, flow rate and temperature) and applications of SFC methods for enantioselective separation. Main mobile phase component was CO_2.

CSP/column	Analyte	Separation conditions	Note	References
Amylose tris(3,5-dimethylphenylcarbamate)				
Chiralpak AD	2-Bromo-methyl-2-(2,4-dichlorophenyl)-1,3-dioxolan-4-yl methylbenzoate	8% ACN	Comparison of SFC and HPLC	[144]
	2-Oxatetracyclo [5.4.0.0^{1,8}.0^{5,11}] undec-9-ene derivatives	Various amounts of MeOH or EtOH 200 bar, 1 or 2 mL min^{-1}, 20 or 30 °C	Method development	[145]
	Albendazole sulfoxide	30% 2-PrOH 200 bar, 3 mL min^{-1}, 35 °C	Method development	[146]
	Cetirizine	30% (2-PrOH + 0.1% TEA + 0.1% TFA) 20 MPa, 2 mL min^{-1}, 35 °C	Method development	[147]
	Citadiol	10% (MeOH + 0.25% TEA) 120 bar, 4 mL min^{-1}, 40 °C	HPLC method validation and comparison to SFC	[148]
	Cyclic ditryptophan	MeOH + H_2O (98/2) + 20 mM ammonium formate Gradient: 0.2–1.2 mL min^{-1} at 20 min 1.8 mL min^{-1} CO_2	Interface for analytical pyrolysis	[149]
	Bifonazole	30% EtOH 20 MPa, 2 mL min^{-1}, 35 °C	Method development, effect of temperature	[43]
	Econazole, miconazole, sulconazole	15% or 20% MeOH 20 MPa, 2 mL min^{-1}, 35 °C	Method development, effect of temperature	[150]
	Itraconazole	40% [EtOH + 2-PrOH (15/85)] 20 MPa, 2 mL min^{-1}, 35 °C		
	Ketoconazole	30% (EtOH + 0.1% TEA + 0.1% TFA) 300 bar, 3 mL min^{-1}, 35 °C	Method development, effect of temperature	[151]
	Ketoconazole	Various amounts of EtOH + 0.1% TEA + 0.1% TFA 200 bar, 2 mL min^{-1}, 35 °C	Comparison of AD and OD columns	[152]
	4 Dioxolane compounds ketoconazole	Various amounts of ACN or EtOH or MeOH or 2-PrOH (+0.1% TEA + 0.1% TFA as needed) 200 bar, 2 mL min^{-1}, 35 °C		
	1,3-Dioxolane derivatives	Various amounts of EtOH or MeOH or 2-PrOH or ACN (+0.1% TEA + 0.1% TFA as needed) 200 bar, 2 mL min^{-1}, 35 °C	Method development, effect of temperature; 3 out of 4 compounds separated	[153]
	Triadimefon	10% EtOH 200 bar, 2 mL min^{-1}, 35 °C	Method development	[154]
	Triadimenol	EtOH		
	Triadimefon + triadimenol	Gradient: 5% up to 2 min, rise to 25% at 1.8% min^{-1} 200 bar, 2 mL min^{-1}, 35 °C		
	Lansoprazole, omeprazole, pantoprazole, rabeprazole	Various amounts of EtOH or MeOH or 2-PrOH 20 MPa, 2 mL min^{-1}, 35 °C	Method development, effect of temperature	[155]
	Lansoprazole, omeprazole, pantoprazole, rabeprazole	30% EtOH or MeOH or 2-PrOH, all with 0.1% TEA 30% [MeOH + ACN + TEA (50/50/0.1)] 50% [MeOH + 2-PrOH + TEA (90/10/0.1)] 210 bar, 4 mL min^{-1}, 40 °C	Screening for preparative separation	[156]
	Omeprazole, pantoprazole	Various amounts of 2-PrOH 20 MPa, 2 mL min^{-1}, 35 °C	Comparison of SFC and HPLC	[157]
	Oxfendazole	40% (EtOH + 0.1% TEA + 0.1% TFA) 20 MPa, 2 mL min^{-1}, 35 °C	Method development	[147]
	Metoprolol and 18 aminoalcohols	20% (MeOH + 10 mM DMOA + 50 mM HOAc) 200 bar, 2 mL min^{-1}, 30 °C	Comparison of AD and OD columns	[28]
	Mianserin, propranolol, trans-stilbene oxide	MeOH (+0.1% NH_4OH) Gradient: 10–65% in 1.8 min, hold for 0.67 min 120 bar, 5 mL min^{-1}, 40 °C	Ammonium hydroxide as mobile phase additive	[136]

Table 1 (*Continued*)

CSP/column	Analyte	Separation conditions	Note	References
	Ibuprofen	15% 2-PrOH 160 bar, 2 mL min^{-1}, 40 °C	Development of method suitable for SMB	[158]
	Flurbiprofen, ibuprofen, ketoprofen, naproxen	15% or 10% MeOH or EtOH or 2-PrOH or 2-PrOH + 5 mM citric acid 150 bar, 1 or 1.5 mL min^{-1}, 30 °C	Reversal elution order	[137]
	Ketoprofen, N-CBZ-phenylalanine, propranolol, warfarin	Various amounts of MeOH + 0.5% IPA 15 MPa, 2 mL min^{-1}, 30 °C	Evaluation of standard reference material	[159]
	Flurbiprofen, hexobarbital, mianserin, oxprenolol, suprofen	10% MeOH	Comparison of SFC, POSC, NPLC	[160]
	Fenoprofen	25% 2-PrOH		
	Praziquantel	30% 2-PrOH		
	Acenocoumarol, sulpiride	25% MeOH		
	Propiomazine	5% 2-PrOH		
	Promethazine, verapamil	20% 2-PrOH		
	Warfarin	30% EtOH 24 MPa, 7 mL min^{-1}, room temperature	Human plasma sample	[161]
	Trans-3-ethoxycarbonyl-4-(4′-fluorophenyl)-1-methyl piperidine-2,6-dione	9.5% 2-PrOH 15 MPa, 2 mL min^{-1}, 308.15 K	Method development, effect of temperature	[42]
	Thiazolbenzenesulfonamide, tetrazolbenzenesulfonamide	50% (EtOH + 0.5% TEA) 250 bar, 2 mL min^{-1}, 40 °C	Method development, effect of temperature	[27]
	6 triazole pesticides	Various amounts of MeOH or EtOH or 2-PrOH (+0.1% TEA + 0.1% TFA as needed) 200 bar, 2 mL min^{-1}, 35 °C	Method development	[162]
	6 benzimidazole sulfoxides	Various amounts of EtOH or MeOH or 2-PrOH or ACN 20 MPa, 2 mL min^{-1}, 35 °C	Method development, effect of temperature	[44]
	12 chiral drugs	10% MeOH (+0–1% IPA or TEA) 20 MPa, 2 mL min^{-1}, 30 °C	Comparison of TEA and IPA additives	[81]
	20 pharmaceutical racemates	EtOH + 0.1% IPA Gradient: t_0 = 20%, $t_{(5\ min)}$ = 20%, $t_{(10\ min)}$ = 35%, $t_{(15\ min)}$ = 35% 18 MPa, 2.5 mL min^{-1}	Evaluation of new SFC/MS experimental arrangement	[163]
	20 compounds (10 commercial + 10 Pfizer)	MeOH + 0.1% IPA or TFA or both EtOH + 0.1% ESA Gradient: 5–40% at 2.5% min^{-1} 150 bar, 2 mL min^{-1}, 35 °C	Screening method development	[56]
	23 commercial + 23 proprietary compounds	MeOH or EtOH or 2-PrOH (+0.1% DEA or TEA as needed) Gradient: 10–55% over 1.5 min at 30% min^{-1}, 55% kept for 1 min 120 bar, 5 mL min^{-1}, 40 °C	Comparison of chiral columns by screening	[54]
	32 compounds (alcohols, amines, amino acid esters)	10% EtOH with or without 2% amine additive Various amounts of MeOH or EtOH or 2-PrOH, all with 0–1% cyclohexylamine 200 bar, 1.5 mL min^{-1}, 40 °C	Effect of amine mobile phase additives	[131]
	40 chiral drugs	Various amounts of MeOH or EtOH or 2-PrOH, all with 0.5% IPA or TFA 200 or 100 bar, 1.5 or 3 mL min^{-1}, 30 °C	Screening method development	[57]
	50 compounds	EtOH (+0.1% TFA or TEA as needed) Gradient: 0–50% at 3.3% min^{-1}, hold for 5 min 3000 psi, 2 mL min^{-1}, 40 °C	Comparison of HPLC, SFC, CE	[59]
	72 commercially available racemates + 44 Amgen compounds	MeOH or EtOH or 2-PrOH, all with 0.2% DEA Gradient: 5–55% over 8 min 100 bar, 5 mL min^{-1}	Evaluation of unusual modifiers; 30 commercial and 38 Amgen samples separated	[133]

Table 1 (*Continued*)

CSP/column	Analyte	Separation conditions	Note	References
Chiralpak AD-H	1-Phenylethylamine	4% (2-PrOH + 0.1% DEA) 11.8 MPa, 5 mL min^{-1}, 25 °C	Method development, effect of pressure	[64]
	2-Phenylpropionic acid	4% MeOH 7.9 MPa, 5 mL min^{-1}, 40 °C		
	α-Tetralol	6% 2-PrOH 13.7 MPa, 4 mL min^{-1}, 40 °C		
	β-Methylphenylalanine-*N*-benzylcarbamate methyl ester	20% [MeOH + EtOH (50/50)] 100 bar, 2 mL min^{-1}, 35 °C	Method development	[164]
	Amphetamine + methamphetamine	10% (2-PrOH + 0.5% cyclohexylamine) 150 bar, 5 mL min^{-1}	Effect of amine mobile phase additives	[131]
	Ibuprofen + 1-phenylethylamine	10% MeOH 200 bar, 1.5 mL min^{-1}, 35 °C	Chiral column coupling (AD-H + IA)	[82]
	Flurbiprofen, propranolol HCl, thioridazine HCl, tramadol HCl	15–40% MeOH 5 mL min^{-1}, 25 or 40 °C	SFC with polarimetric detection	[119]
	Flurbiprofen	19% MeOH 135 bar, 0.97 mL min^{-1}	Methodology to design SFC separation	[35]
	Flurbiprofen	0.07 mL min^{-1} EtOH, 1 mL min^{-1} CO_2 120 bar, 30 °C	Effect of pressure and modifier concentration	[32]
	Fenoterol, thioridazine	20% (MeOH + 20 mM NH_3) 100 bar, 4 mL min^{-1}, 40 °C	NH_3 as mobile phase additive	[135]
	Fulvestrant	25% [MeOH + ACN (95/5)] 2.5 mL min^{-1}, 55 °C	Method development	[165]
	Naringenin	Various amounts of MeOH with 20 mM NH_3 or 0.2% DEA or 0.2% DMEA 100 bar, 5 mL min^{-1}, 40 °C	NH_3 as mobile phase additive	[135]
	Neonicotinoid insecticides	Various amounts of EtOH 150 bar, 2 mL min^{-1}, 35 °C	Method development, comparison to HPLC	[47]
	Paroxol	5% MeOH 15 MPa, 2 mL min^{-1}, 35 °C	Method development, adsorption isotherm	[31]
	Piperidine derivative, warfarin	Various amounts of MeOH + 10 mM NH_4OAc 100 bar, 2 mL min^{-1}	Intelligent 4 column screening	[53]
	Primaquine diphosphate	20% (MeOH + 0.4% DEA) 4 mL min^{-1}, 35 °C	Contaminant analysis	[166]
	Proline derivatives	5–10% (EtOH + 0.1% TFA) 100 bar, 2.5 mL min^{-1}, 35 °C	Comparison of SFC and HPLC	[50]
	Sotolon	2.5% MeOH 8 MPa, 1.5 mL min^{-1}, 28 °C	Method development, effect of pressure and temperature	[121]
	Six 3-substitued-4-arylquinolinones	Various amounts of MeOH or EtOH or 2-PrOH 150 bar, 2 mL min^{-1}, 35 °C	Screening for preparative method development	[167]
	Seven γ-lactones (C_6-C_{12})	1.5–3% 2-PrOH 7–14 MPa, 1.3–2.35 mL min^{-1}, 30–40 °C	Method development, effect of temperature	[168]
	8 Pyrazinones	10% MeOH or EtOH or 2-PrOH 150 bar, 2 mL min^{-1}, 35 °C	Preclinical testing	[169]
	9 Amides	MeOH (+0.1% IPA) MeOH + ACN + FA + IPA (75/25/0.3/0.3) Gradient: 5–50% at 6.5% min^{-1}, hold for 1 min 150 bar, 4 mL min^{-1}, 40 °C	Effect of additives and column temperature; 7 amides separated, 5 baseline	[48]
	12 compounds, including 4 alcohol sensitive compounds	20% or 40% of MeOH or EtOH or 2-PrOH or ACN or TFE 100 bar, 3 mL min^{-1}, 40 °C	TFE as mobile phase modifier	[88]
	20 compounds (10 amines and their CBZ-derivatives)	20% or 40% ACN or MeOH or EtOH or 2-PrOH or 2-BuOH (+0.1% DEA) 100 bar, 2.5 mL min^{-1}, 40 °C	CBZ derivatization	[170]
	36 compounds (amino acid esters, amino acids, β-blockers, amines)	20% (EtOH + 0.1% ESA) 180 bar, 2 mL min^{-1}, room temperature	ESA as mobile phase additive	[132]
	40 commercial compounds + 100 proprietary compounds	MeOH (+0.2% DEA as needed) Gradient: 7% held for 2 min, increased to 50% at 7% min^{-1}, held for 2 min 100 bar, 4 mL min^{-1}, 35 °C	Comparison to P-CAP column by screening; 86% neutral, non-nitrogen containing compounds, 83% acidic compounds and 85% basic and neutral compounds separated	[97]

Table 1 (*Continued*)

CSP/column	Analyte	Separation conditions	Note	References
	48 compounds	MeOH + 25 mM IBA Gradient: 4% for 4 min, ramp to 40% at 4% min^{-1}, hold for 2 min 200 bar, 2 mL min^{-1}, 35 °C	Screening approach for CSP evaluation; Illustrative separation in Fig. 1	[171]
	57 pharmaceutical compounds	Various amounts of 2-PrOH or MeOH with IPA or TFA or both 150 bar, 3 mL min^{-1}, 30 °C	Chiral screening approach	[172]
	59 pharmaceutical compounds (basic, amphoteric, neutral, acidic)	10% MeOH (+0.5% IPA or TFA or both) 150 bar, 3 mL min^{-1}, 30 °C	Comparison of polysaccharide columns	[39]
	135 compounds	10% MeOH 170 bar, 3 mL min^{-1}, 25 °C	Factors contributing to enantiomer separation	[37]
Chiralpak IA	Lansoprazole, omeprazole, pantoprazole, rabeprazole	30% EtOH or MeOH or 2-PrOH, all with 0.1% TEA 30% [MeOH + ACN + TEA (50/50/0.1)] 210 bar, 4 mL min^{-1}, 40 °C	Screening for preparative separation	[156]
	Mephobarbital, warfarin	10% MeOH	Guideline for mobile phase selection	[51]
	12 compounds, including 4 alcohol sensitive compounds	Various amounts of DCM or ethyl acetate or THF or TFE (+0.1% DEA) 100 bar, 3 mL min^{-1}, 40 °C	TFE as mobile phase modifier	[88]
	20 compounds (10 commercial + 10 Pfizer)	MeOH + 0.1% IPA or TFA or both EtOH + 0.1% ESA Gradient: 5–40% at 2.5% min^{-1} 150 bar, 2 mL min^{-1}, 35 °C	Screening method development	[56]
	23 commercial + 23 proprietary compounds	MeOH or EtOH or 2-PrOH (+0.1% DEA or TEA as needed) Gradient: 10–55% over 1.5 min at 30% min^{-1}, 55% kept for 1 min 120 bar, 5 mL min^{-1}, 40 °C	Comparison of chiral columns by screening	[54]
	72 commercially available racemates + 44 Amgen compounds	MeOH or EtOH or 2-PrOH, all with 0.2% DEA MeOH + DCM + DEA (80/20/0.2) MeOH + THF + DEA (80/20/0.2) MeOH + DCM + THF + DEA (80/10/10/0.2) Gradient: 5–55% over 8 min 100 bar, 5 mL min^{-1}	Evaluation of unusual modifiers; 10 commercial and 27 Amgen samples separated by screening	[133]
RegisPack	Fluoxetine	7.5% (MeOH + 0.1% TEA) 120 bar, 3 mL min^{-1}, 30 °C	Comparison of Regis columns	[174]
	Flurbiprofen	30% MeOH 120 bar, 3 mL min^{-1}, 30 °C		
	Naringerin	25% MeOH	Development of method suitable for SMB	[175]
	Vitamin K_1	5% MeOH 150 bar, 2 mL min^{-1}, 30 °C	Method development; 7 of 8 isomers separated	[176]
	9 chiral drugs	Various amounts of 2-PrOH + 0.5% DEA 125 bar, 4 mL min^{-1}, 40 °C	Comparison of Regis columns; 6 compounds separated	[177]
	130 compounds screened	10% MeOH 150 bar, 3 mL min^{-1}, 25 °C	Comparison of Regis columns; 64% compounds separated	[178]
Amylose tris((*S*)-α-methylbenzylcarbamate)				
Chiralpak AS	Binaphthol	10% 2-PrOH 16 MPa, 2 mL min^{-1}, 40 °C	Development of method suitable for SMB	[77]
	Ephedrine	10% EtOH	Comparison of SFC, POSC and NPLC	[160]
	Lansoprazole, omeprazole, pantoprazole, rabeprazole	30% EtOH or MeOH or 2-PrOH, all with 0.1% TEA 30% [MeOH + ACN + TEA (50/50/0.1)] 210 bar, 4 mL min^{-1}, 40 °C	Screening for preparative separation	[156]

Table 1 (*Continued*)

CSP/column	Analyte	Separation conditions	Note	References
	Tramadol HCl	MeOH Gradient: 0% for 0.25 min, rise to 50% over 3.5 min, hold for 2.5 min 40 °C	SFC + polarimetric detection	[119]
	20 compounds (10 commercial + 10 Pfizer)	MeOH + 0.1% IPA or TFA or both EtOH + 0.1% ESA Gradient: 5–40% at 2.5% min^{-1} 150 bar, 2 mL min^{-1}, 35 °C	Screening method development	[56]
	20 compounds (10 amines and their CBZ-derivatives)	20% or 40% of ACN or MeOH or EtOH or 2-PrOH or 2-BuOH (+0.1% DEA) 100 bar, 2.5 mL min^{-1}, 40 °C	CBZ derivatization	[170]
	23 commercial + 23 proprietary compounds	MeOH or EtOH or 2-PrOH (+0.1% DEA or TEA as needed) Gradient: 10–55% over 1.5 min at 30% min^{-1}, 55% kept for 1 min 120 bar, 5 mL min^{-1}, 40 °C	Comparison of chiral columns by screening	[54]
	40 chiral drugs	Various amounts of MeOH or EtOH or 2-PrOH, all with 0.5% IPA or TFA 100 or 200 bar, 1.5 or 3 mL min^{-1}, 30 °C	Screening method development	[57]
	50 compounds	EtOH Gradient: 0–50% at 3.3% min^{-1}, hold for 5 min 3000 psi, 2 mL min^{-1}, 40 °C	Comparison of HPLC, SFC, CE	[59]
	72 commercially available racemates + 44 Amgen compounds	MeOH or EtOH or 2-PrOH, all with 0.2% DEA Gradient: 5–55% over 8 min 100 bar, 5 mL min^{-1}	Evaluation of unusual modifiers; 10 commercial samples separated	[133]
Chiralpak AS-H	1-(1-*tert*-Butoxyvinyl)-4-methoxy pyrrolidine-2-carboxylic acid	3% (MeOH + 0.2% TFA) 120 bar, 3 mL min^{-1}, 40 °C	Enantiomeric purity	[179]
	β-Methylphenylalanine-N-benzylcarbamate methyl ester	20% MeOH 100 bar, 2 mL min^{-1}, 35 °C	Method development	[164]
	Piperidine derivative, warfarin	Various amounts of MeOH + 10 mM NH_4OAc 100 bar, 2 mL min^{-1}	Intelligent 4 column screening	[53]
	Six 3-substitued-4-arylquinolinones	Various amounts of MeOH or EtOH or 2-PrOH 150 bar, 2 mL min^{-1}, 35 °C	Screening for preparative method development	[167]
	8 pyrazinones	10% MeOH or EtOH or 2-PrOH 150 bar, 2 mL min^{-1}, 35 °C	Preclinical testing; 1 compound separated	[169]
	9 amides	MeOH (+0.1% IPA) MeOH + ACN + FA + IPA (75/25/0.3/0.3) Gradient: 5–50% at 6.5% min^{-1}, hold for 1 min 150 bar, 4 mL min^{-1}, 40 °C	Effect of additives and column temperature; 1 amide baseline separated	[48]
	12 compounds, including 4 alcohol sensitive compounds	20%or 40% of MeOH or EtOH or 2-PrOH or ACN or TFE 100 bar, 3 mL min^{-1}, 40 °C	TFE as mobile phase modifier	[88]
	40 commercial compounds + 100 proprietary compounds	MeOH (+0.2% DEA as needed) Gradient: 7% held for 2 min, increased to 50% at 7% min^{-1}, held for 2 min 100 bar, 4 mL min^{-1}, 35 °C	Comparison to P-CAP column by screening; 33% of acidic compounds and 65% of basic and neutral compounds separated	[97]
	57 pharmaceutical compounds	Various amounts of 2-PrOH or MeOH, all with IPA or TFA or both 150 bar, 3 mL min^{-1}, 30 °C	Chiral screening approach	[172]
	59 pharmaceutical compounds (basic, amphoteric, neutral, acidic)	10% MeOH (+0.5% IPA or TFA or both) 150 bar, 3 mL min^{-1}, 30 °C	Comparison of polysaccharide columns	[39]

Table 1 (*Continued*)

CSP/column	Analyte	Separation conditions	Note	References
Amylose tris(3,5-dichlorophenylcarbamate)				
Chiralpak IE	Norphenylephrine HCl	EtOH + 0.1% NH_4OH Gradient: 5–60% in 1.8 min, hold for 0.6 min, 105 bar, 4 mL min^{-1}, 40 °C	CSP comparison by screening	[173]
Amylose tris(5-chloro-2-methylphenylcarbamate)				
Lux Amylose-2	(4*S*-Trans)-4-(ethylamino)-4-(N-acetamide)-5,6-dihydro-(6*S*)-methyl-4H-thieno-[2,3-b]thiopyran-7,7-dioxide	15% EtOH 200 bar, 2 mL min^{-1}, 35 °C	Method development, effect of temperature	[41]
	Fluoro-oxindole derivatives	Various amounts of EtOH or MeOH 150 bar, 3 mL min^{-1}, 25 °C	Effect of modifier and temperature	[38]
	23 commercial + 23 proprietary compounds	MeOH or EtOH or 2-PrOH (+0.1% DEA or TEA as needed) Gradient: 10–55% over 1.5 min at 30% min^{-1}, 55% kept for 1 min 120 bar, 5 mL min^{-1}, 40 °C	Comparison of chiral columns by screening	[54]
	47 basic, neutral, amphoteric and 12 acidic compounds	Various amounts of MeOH with IPA or TFA or both 150 bar, 3 mL min^{-1}, 30 °C	Screening and evaluation of mobile phase additives	[134]
	57 pharmaceutical compounds	Various amounts of 2-PrOH or MeOH, all with IPA or TFA or both 150 bar, 3 mL min^{-1}, 30 °C	Chiral screening approach	[172]
	59 pharmaceutical compounds (basic, amphoteric, neutral, acidic)	10% MeOH (+0.5% IPA or TFA or both) 150 bar, 3 mL min^{-1}, 30 °C	Comparison of polysaccharide columns	[39]
Chiralpak AY	Acebutolol HCl, ketoprofen	EtOH + 0.1% NH_4OH Gradient: 5–60% in 1 min, hold for 0.4 min, 105 bar, 1.5 mL min^{-1}, 40 °C	CSP comparison by screening	[173]
Chiralpak AY-H	57 pharmaceutical compounds	Various amounts of 2-PrOH or MeOH, all with IPA or TFA or both 150 bar, 3 mL min^{-1}, 30 °C	Chiral screening approach	[172]
Sepapak-3	12 compounds, including 4 alcohol sensitive compounds	20%or 40% of MeOH or EtOH or 2-PrOH or ACN or TFE 100 bar, 3 mL min^{-1}, 40 °C	TFE as mobile phase modifier	[88]
	48 compounds	MeOH + 25 mM IBA Gradient: 4% for 4 min, ramp to 40% at 4% min^{-1}, hold for 2 min 200 bar, 2 mL min^{-1}, 35 °C	Screening approach for CSP evaluation; Illustrative separation in Fig. 1	[171]
	59 pharmaceutical compounds (basic, amphoteric, neutral, acidic)	10% MeOH (+0.5% IPA or TFA or both) 150 bar, 3 mL min^{-1}, 30 °C	Comparison of polysaccharide columns	[39]
Amylose tris(5-chloro-2-methylphenylcarbamate)				
RegisPack CLA-1	9 chiral drugs	Various amounts of 2-PrOH + 0.5% DEA 125 bar, 4 mL min^{-1}, 40 °C	Comparison of Regis columns; 9 compounds separated	[177]
	130 compounds screened	10% MeOH 150 bar, 3 mL min^{-1}, 25 °C	Comparison of Regis columns; 52% compounds separated	[178]
Cellulose tris(3,5-dimethylphenylcarbamate)				
Chiralcel OD	1-Phenylethanol, bupicavaine, verapamil	10% MeOH 150 bar, 3 mL min^{-1}, 25 °C	Comparison of polysaccharide columns	[180]
	1-Phenylethanol	MeOH Gradient: 2–30% in 1.2 min, hold 1.8 min 150 bar, 2 mL min^{-1}, 25 °C	Enzymatic reaction	[181]
	1-Phenyl-1-propanol	2.7% MeOH 17 MPa, 1 mL min^{-1}, 30 °C	Development of method suitable for SMB	[129]
		2.4% MeOH 150 bar, 1 mL min^{-1}, 30 °C	Method development, effect of pressure and temperature	[29]
		4.9% MeOH 170 bar, 1 mL min^{-1}, 30 °C	Non-linear adsorption isotherm	[30]

Table 1 (*Continued*)

CSP/column	Analyte	Separation conditions	Note	References
	2-*tert*-Butyldimethyl-siloxy methyl-5-(8′-ethoxycarbonyl-7′-nonenyl)-3-methylfuran	0.15 mL min^{-1} EtOH, 3 mL min^{-1} CO_2 100 kg cm^{-2}, 45 °C	Comparison of SFC, HPLC, GC	[182]
	2-*tert*-Butyldimethyl-siloxy methyl-5-(9′-hydroxy-8′-methyl-7′-enyl)-3-methylfuran	0.1 mL min^{-1} EtOH, 3 mL min^{-1} CO_2 120 kg cm^{-2}, 45 °C		
	anti-3-Isopropenyl-12-methyl-13-oxabicyclo[8.2.1]trideca-1(12),10-dien-2-ol	0.2 mL min^{-1} EtOH, 3 mL min^{-1} CO_2 200 kg cm^{-2}, 45 °C		
	Albendazole sulfoxide	10% MeOH 200 bar, 2 mL min^{-1}, 35 °C	Method development	[146]
	Binaphthol	5% 2-PrOH 16 MPa, 2 mL min^{-1}, 40 °C	Development of method suitable for SMB	[77]
	Diltiazem hydrochloride	13% (2-PrOH + 0.5% DEA) 180 kg cm^{-2}, 2 mL min^{-1}, 50 °C	Comparison to HPLC	[183]
	Alprenolol, atropine, mandelic acid, etoprolol, warfarin	10% MeOH	Comparison of SFC, POSC, NPLC	[160]
	Nadolol	10% EtOH		
	Metoprolol and 18 aminoalcohols	20% (MeOH + 10 mM DMOA + 50 mM HOAc) 20% (MeOH + 10 mM DMOA + 50 mM TFA) 200 bar, 2 mL min^{-1}, 25 or 30 °C	Comparison of AD and OD columns	[28]
	Indapamide, N-CBZ-phenylalanine, ropranolol, warfarin	Various amounts of MeOH + 0.5% IPA 15 MPa, 2 mL min^{-1}, 30 °C	Evaluation of standard reference material	[159]
	Nutlin-3	35% EtOH 100 bar, 2 mL min^{-1}, 30 °C	Development of purification method	[184]
	Polychlorinated biphenyls	100% CO_2 150 bar, 2 mL min^{-1}, 36 °C	Method development, effect of temperature	[49]
	Tetralol	5.4% EtOH 150 or 200 bar, 40 °C	Development of method suitable for SMB	[125]
	Four cis-2-(2,4-dichlorophenyl)-1,3-dioxolanes	Various amounts of EtOH or MeOH	Comparison of SFC and HPLC	[144]
	4 Dioxolane compounds ketoconazole	Various amounts of ACN or EtOH or MeOH or 2-PrOH 200 bar, 2 mL min^{-1}, 35 °C	Comparison of AD and OD columns	[152]
	20 compounds (10 amines and their CBZ-derivatives)	20%or 40% of ACN or MeOH or EtOH or 2-PrOH or 2-BuOH (+0.1% DEA) 100 bar, 2.5 mL min^{-1}, 40 °C	CBZ derivatization	[170]
	20 compounds (10 commercial + 10 Pfizer)	MeOH + 0.1% IPA or TFA or both EtOH + 0.1% ESA Gradient: 5–40% at 2.5% min^{-1} 150 bar, 2 mL min^{-1}, 35 °C	Screening method development	[56]
	20 pharmaceutical racemates	EtOH + 0.1% IPA Gradient: t_0 = 20%, $t_{(5\ min)}$ = 20%, $t_{(10\ min)}$ = 35%, $t_{(15\ min)}$ = 35% 18 MPa, 2.5 mL min^{-1}	Evaluation of new SFC/MS experimental arrangement	[163]
	40 chiral drugs	Various amounts of MeOH or EtOH or 2-PrOH, all with 0.5% IPA or TFA 200 or 100 bar, 3 or 1.5 mL min^{-1}, 30 °C	Screening method development	[57]
	50 compounds	EtOH Gradient: 0–50% at 3.3% min^{-1}, hold for 5 min 3000 psi, 2 mL min^{-1}, 40 °C	Comparison of HPLC, SFC, CE	[59]
	72 commercial and 44 proprietary Amgen racemates	MeOH or EtOH or 2-PrOH, all with 0.2% DEA Gradient: 5–55% over 8 min 100 bar, 5 mL min^{-1}	Evaluation of unusual modifiers; 14 commercial and 6 Amgen racemates separated	[133]

Table 1 (*Continued*)

CSP/column	Analyte	Separation conditions	Note	References
Chiralcel OD-H	(4*S*-trans)-4-(ethylamino)-4-(N-acetamide)-5,6-dihydro-(6*S*)-methyl-4Hthieno-[2,3-b]thiopyran-7,7-dioxide	20% 2-PrOH 200 bar, 2 mL min^{-1}, 35 °C	Method development, effect of temperature	[41]
	β-Methylphenylalanine-N-benzylcarbamate methyl ester	20% MeOH 100 bar, 2 mL min^{-1}, 35 °C	Method development	[164]
	Cinnamonitrile and hydrocinnamonitrile intermediates	15% MeOH 120 bar, 1 mL min^{-1}, 30 °C	Achiral and chiral column coupling	[113]
	Citalopram	5% 2-PrOH 120 bar, 4 mL min^{-1}, 40 °C	HPLC method validation and comparison to SFC	[148]
	Ketamin, trichlormethiazide	20% (EtOH + 0.5% DEA) 210 bar, 4 mL min^{-1}, 40 °C	Comparison of cellulose-based columns	[185]
	Metoprolol + 9 structure analogues	20% MeOH or EtOH or 2-PrOH, all with 0.5% DEA 20% [MeOH + ACN + DEA (50/50/0.5)] 210 bar, 4 mL min^{-1}, 40 °C		
	Pindolol, propranolol	30% MeOH 100 bar, 4 mL min^{-1}, 45 °C	Metabolic stability	[186]
		35% (MeOH + 0.2% IPA) 100 bar, 3 mL min^{-1}, 45 °C	Determination in blood sample	[187]
	Neonicotinoid insecticides	10% EtOH 150 bar, 2 mL min^{-1}, 35 °C	SFC method development, comparison to HPLC	[47]
	Warfarin + indapamide	20% MeOH 200 bar, 3 mL min^{-1}, 35 °C	Effect of column back-pressure	[71]
	5 β-Blockers	20% EtOH or MeOH or 2-PrOH, all with 0.1% ESA 180 bar, 2 mL min^{-1}, room temperature	ESA as mobile phase additive	[132]
	Six 3-substitued-4-arylquinolinones	Various amounts of MeOH or EtOH or 2-PrOH 150 bar, 2 mL min^{-1}, 35 °C	Screening for preparative method development	[167]
	8 pyrazinones	10% MeOH or EtOH or 2-PrOH 150 bar, 2 mL min^{-1}, 35 °C	Preclinical testing	[169]
	9 amides	MeOH (+0.1% IPA) MeOH + ACN + FA + IPA (75/25/0.3/0.3) Gradient: 5–50% at 6.5% min^{-1}, hold for 1 min 150 bar, 4 mL min^{-1}, 40 °C	Effect of additives and column temperature; 8 amides separated, 7 baseline	[48]
	12 compounds, including 4 alcohol sensitive compounds	20% or 40% of MeOH or EtOH or 2-PrOH or ACN or TFE 100 bar, 3 mL min^{-1}, 40 °C	TFE as mobile phase modifier	[88]
	40 commercial compounds + 100 proprietary compounds	MeOH (+0.2% DEA as needed) Gradient: 7% held for 2 min, increased to 50% at 7% min^{-1}, held for 2 min 100 bar, 4 mL min^{-1}, 35 °C	Comparison to P-CAP column by screening; 86% of neutral, non-nitrogen containing compounds, 17% of acidic compounds and 80% of basic and neutral compounds separand	[97]
	48 compounds	MeOH + 25 mM IBA Gradient: 4% for 4 min, ramp to 40% at 4% min^{-1}, hold for 2 min 200 bar, 2 mL min^{-1}, 35 °C	Screening approach for CSP evaluation; Illustrative separation in Fig. 1	[171]
	57 pharmaceutical compounds	Various amounts of 2-PrOH or MeOH, all with IPA or TFA or both 150 bar, 3 mL min^{-1}, 30 °C	Chiral screening approach	[172]
	59 pharmaceutical compounds (basic, amphoteric, neutral, acidic)	10% MeOH (+0.5% IPA or TFA or both) 150 bar, 3 mL min^{-1}, 30 °C	Comparison of polysaccharide columns	[39]
	135 compounds	10% MeOH 170 bar, 3 mL min^{-1}, 25 °C	Factors contributing to enantiomer separation	[37]
Chiralpak IB	1-phenylethanol, 1-(2-naphthyl)-ethanol, bupicavaine	10% MeOH 150 bar, 3 mL min^{-1}, 25 °C	Comparison of polysaccharide columns	[180]
	Acetofenate, benalaxy, diclofop-methyl, difenoconazole, myclobutanil	3% or 10% 2-PrOH 150 bar, 2 mL min^{-1}, 35 °C	Method development, effect of temperature	[40]

Table 1 (*Continued*)

CSP/column	Analyte	Separation conditions	Note	References
	Ketamin, trichlormethiazide	20% EtOH + 0.5% DEA 210 bar, 4 mL min^{-1}, 40 °C	Comparison of cellulose-based columns	[185]
	Metoprolol + 9 structure analogues	20% MeOH or EtOH or 2-PrOH, all with 0.5% DEA 20% [MeOH + ACN + DEA (50/50/0.5)] 210 bar, 4 mL min^{-1}, 40 °C		
	Polychlorinated biphenyls	100% CO_2 or 1% EtOH 150 bar, 2 mL min^{-1}, 36 °C	Method development, effect of temperature	[49]
	20 compounds (10 commercial + 10 Pfizer)	MeOH + 0.1% IPA or TFA or both EtOH + 0.1% ESA Gradient: 5–40% at 2.5% min^{-1} 150 bar, 2 mL min^{-1}, 35 °C	Screening method development	[56]
	23 commercial + 23 proprietary compounds	MeOH or EtOH or 2-PrOH (+0.1% DEA or TEA as needed) Gradient: 10–55% over 1.5 min at 30% min^{-1}, 55% kept for 1 min 120 bar, 5 mL min^{-1}, 40 °C	Comparison of chiral columns by screening	[54]
	72 commercially available racemates + 44 Amgen compounds	MeOH or EtOH or 2-PrOH, all with 0.2% DEA MeOH + DCM + DEA (80/20/0.2) MeOH + THF + DEA (80/20/0.2) MeOH + DCM + THF + DEA (80/10/10/0.2) Gradient: 5–55% over 8 min 100 bar, 5 mL min^{-1}	Evaluation of unusual modifiers; 13 commercial and 5 Amgen samples separated by screening	[133]
Lux cellulose-1	Fluoro-oxindole derivatives	Various amounts of EtOH or MeOH 150 bar, 3 mL min^{-1}, 25 °C	Effect of modifier and temperature	[38]
	Heterocyclic α-enamido phosphine compounds	Various amounts of MeOH or EtOH 150 bar, 3 mL min^{-1}, 30 °C	Method development, effect of temperature	[46]
	Phosphine containing α-amino acid esters	5% EtOH 150 bar, 3 mL min^{-1}, 30 °C	Effect of modifier and temperature	[45]
	Propranolol	25% (MeOH + 0.1% NH_4OH) 120 bar, 1.5 mL min^{-1}, 40 °C	Ammonium hydroxide as mobile phase additive	[136]
	Trans-stilbene oxide	MeOH + 0.1% NH_4OH Gradient: 5–60% in 1.8 min, hold for 0.6 min, 105 bar, 4 mL min^{-1}, 40 °C	CSP comparison by screening	[173]
	23 commercial + 23 proprietary compounds	MeOH or EtOH or 2-PrOH (+0.1% DEA or TEA as needed) Gradient: 10–55% over 1.5 min at 30% min^{-1}, 55% kept for 1 min 120 bar, 5 mL min^{-1}, 40 °C	Comparison of chiral columns by screening	[54]
	47 basic, neutral, amphoteric and 12 acidic compounds	Various amounts of MeOH + IPA or TFA or both 150 bar, 3 mL minmL min^{-1}, 30 °C	Screening and evaluation of mobile phase additives	[134]
	57 pharmaceutical compounds	Various amounts of 2-PrOH or MeOH, all with IPA or TFA or both 150 bar, 3 mL min^{-1}, 30 °C	Chiral screening approach	[172]
	59 pharmaceutical compounds (basic, amphoteric, neutral, acidic)	10% MeOH (+0.5% IPA or TFA or both) 150 bar, 3 mL min^{-1}, 30 °C	Comparison of polysaccharide columns	[39]
Sepapak-5	57 pharmaceutical compounds	Various amounts of 2-PrOH or MeOH, all with IPA or TFA or both 150 bar, 3 mL min^{-1}, 30 °C	Chiral screening approach	[172]
	59 pharmaceutical compounds (basic, amphoteric, neutral, acidic)	10% MeOH (+0.5% IPA or TFA or both) 150 bar, 3 mL min^{-1}, 30 °C	Comparison of polysaccharide columns	[39]

Table 1 (*Continued*)

CSP/column	Analyte	Separation conditions	Note	References
Kromasil CelluCoat	Ketamin, trichlormethiazide Metoprolol + 9 structure analogues	20% (EtOH + 0.5% DEA) 210 bar, 4 mL min^{-1}, 40 °C 20% MeOH or EtOH or 2-PrOH, all with 0.5% DEA 20% [MeOH + ACN + DEA (50/50/0.5)] 210 bar, 4 mL min^{-1}, 40 °C	Comparison of cellulose-based columns	[185]
	48 compounds	MeOH + 25 mM IBA Gradient: 4% for 4 min, ramp to 40% at 4% min^{-1}, hold for 2 min 200 bar, 2 mL min^{-1}, 35 °C	Screening approach for CSP evaluation; Illustrative separation in Fig. 1	[171]
Epitomize 1C	Trans-stilbene oxide	MeOH + 0.1% NH_4OH Gradient: 5–60% in 1 min, hold for 0.4 min, 105 bar, 1.5 mL min^{-1}, 40 °C	CSP comparison by screening	[173]
RegisCell	Atenolol, metoprolol, propranolol Trans-stilbene oxide, warfarin	50% (MeOH + 0.1% TEA) 120 bar, 5 mL min^{-1}, 30 °C 30% (MeOH + 0.1% TEA) 120 bar, 5 mL min^{-1}, 30 °C	Comparison of Regis columns	[174]
	48 compounds	MeOH + 25 mM IBA Gradient: 4% for 4 min, ramp to 40% at 4% min^{-1}, hold for 2 min 200 bar, 2 mL min^{-1}, 35 °C	Screening approach for CSP evaluation; Illustrative separation in Fig. 1	[171]
	130 compounds screened	10% MeOH 150 bar, 3 mL min^{-1}, 25 °C	Comparison of Regis columns; 56% compounds separand	[178]
Reprosil OM	Ketamin, trichlormethiazide Metoprolol + 9 structure analogues	20% (EtOH + 0.5% DEA) 210 bar, 4 mL min^{-1}, 40 °C 20% MeOH or EtOH or 2-PrOH, all with 0.5% DEA 20% [MeOH + ACN + DEA (50/50/0.5)] 210 bar, 4 mL min^{-1}, 40 °C	Comparison of cellulose-based columns	[185]
Cellulose tris(3-chloro-4-methylphenylcarbamate)				
Chiralcel OZ-H	57 pharmaceutical compounds	Various amounts of 2-PrOH or MeOH, all with TFA or IPA or both 150 bar, 3 mL min^{-1}, 30 °C	Chiral screening approach	[172]
	59 pharmaceutical compounds (basic, amphoteric, neutral, acidic)	10% MeOH (+0.5% IPA or TFA or both) 150 bar, 3 mL min^{-1}, 30 °C	Comparison of polysaccharide columns	[39]
Lux cellulose-2	(4*S*-Trans)-4-(ethylamino)-4-(N-acetamide)-5,6-dihydro-(6*S*)-methyl-4H-thieno-[2,3-b]thiopyran-7,7-dioxide	30% EtOH 200 bar, 2 mL min^{-1}, 35 °C	Method development, effect of temperature	[41]
	Fluoro-oxindole derivatives	Various amounts of EtOH or MeOH 150 bar, 3 mL min^{-1}, 25 °C	Effect of modifier and temperature	[38]
	Phosphine containing α-amino acid esters	5% EtOH 150 bar, 3 mL min^{-1}, 30 °C	Method development, effect of temperature	[45]
	23 commercial + 23 proprietary compounds	MeOH or EtOH or 2-PrOH (+0.1% DEA or TEA as needed) Gradient: 10–55% over 1.5 min at 30% min^{-1}, 55% kept for 1 min 120 bar, 5 mL min^{-1}, 40 °C	Comparison of chiral columns by screening	[54]
	47 basic, neutral, amphoteric and 12 acidic compounds	Various amounts of MeOH + IPA or TFA or both 150 bar, 3 mL min^{-1}, 30 °C	Screening and evaluation of mobile phase additives	[134]
	57 pharmaceutical compounds	Various amounts of 2-PrOH or MeOH, all with TFA or IPA or both 150 bar, 3 mL min^{-1}, 30 °C	Chiral screening approach	[172]
	59 pharmaceutical compounds (basic, amphoteric, neutral, acidic)	10% MeOH (+0.5% IPA or TFA or both) 150 bar, 3 mL min^{-1}, 30 °C	Comparison of polysaccharide columns	[39]

Table 1 (*Continued*)

CSP/column	Analyte	Separation conditions	Note	References
Sepapak-2	48 compounds	MeOH + 25 mM IBA Gradient: 4% for 4 min, ramp to 40% at 4% min^{-1}, hold for 2 min 200 bar, 2 mL min^{-1}, 35 °C	Screening approach for CSP evaluation; Illustrative separation in Fig. 1	[171]
Cellulose tris(4-methylbenzoate)				
Chiralcel OJ	Binaphthol	10% 2-PrOH 16 MPa, 2 mL min^{-1}, 40 °C	Development of method suitable for SMB	[77]
	Ketoprofen Methadone Tetramisol	5% 2-PrOH 5% MeOH 10% MeOH	Comparison of SFC, POSC, NPLC	[160]
	Trans-stilbene oxide	MeOH + 0.1% NH_4OH Gradient: 10–55% over 1.5 min 120 bar, 5 mL min^{-1}, 40 °C	Ammonium hydroxide as mobile phase additive	[136]
	Nutlin-3	35% [EtOH + ACN (1:1)] 100 bar, 2 mL min^{-1}, 30 °C	Development of purification method	[184]
	20 pharmaceutical racemates	EtOH + 0.1% IPA Gradient: t_0 = 20%, $t_{(5\ min)}$ = 20%, $t_{(10\ min)}$ = 35%, $t_{(15\ min)}$ = 35% 18 MPa, 2.5 mL min^{-1}	Evaluation of new SFC/MS experimental arrangement	[163]
	20 compounds (10 amines and their CBZ-derivatives)	20% or 40% of ACN or MeOH or EtOH or 2-PrOH or 2-BuOH (+0.1% DEA) 100 bar, 2.5 mL min^{-1}, 40 °C	CBZ derivatization	[170]
	20 compounds (10 commercial + 10 Pfizer)	MeOH + 0.1% IPA or TFA or both EtOH + 0.1% ESA Gradient: 5–40% at 2.5% min^{-1} 150 bar, 2 mL min^{-1}, 35 °C	Screening method development	[56]
	23 commercial + 23 proprietary compounds	MeOH or EtOH or 2-PrOH (+0.1% DEA or TEA as needed) Gradient: 10–55% over 1.5 min at 30% min^{-1}, 55% kept for 1 min 120 bar, 5 mL min^{-1}, 40 °C	Comparison of chiral columns by screening	[54]
	40 chiral drugs	Various amounts of MeOH or EtOH or 2-PrOH, all with 0.5% IPA or TFA 100 or 200 bar, 1.5 or 3 mL min^{-1}, 30 °C	Screening method development	[57]
	50 compounds	EtOH Gradient: 0–50% at 3.3% min^{-1}, hold for 5 min 3000 psi, 2 mL min^{-1}, 40 °C	Comparison of HPLC, SFC, CE	[59]
	72 commercially available racemates + 44 Amgen compounds	MeOH or EtOH or 2-PrOH, all with 0.2% DEA Gradient: 5–55% over 8 min 100 bar, 5 mL min^{-1}	Evaluation of unusual modifiers; 11 commercial samples separated	[133]
Chiralcel OJ-H	4-Chloro-indole, 6-chloro-indole	20% ACN 100 bar, 3 mL min^{-1}, 40 °C	Achiral and chiral column coupling	[114]
	β-Methylphenylalanine-N-benzylcarbamate methyl ester	20% MeOH 100 bar, 2 mL min^{-1}, 35 °C	Method development	[164]
	Piperidine derivative, warfarin	Various amounts of MeOH + 10 mM NH_4OAc 100 bar, 2 mL min^{-1}	Inteligent 4 column screening	[53]
	Sotolon	1.5% ACN 12 MPa, 1.5 mL min^{-1}, 30 °C	Method development, effect of pressure and temperature	[121]
	Six 3-substitued-4-arylquinolinones	Various amounts of MeOH or EtOH or 2-PrOH 150 bar, 2 mL min^{-1}, 35 °C	Screening for preparative method development	[167]
	8 pyrazinones	10% MeOH or EtOH or 2-PrOH 150 bar, 2 mL min^{-1}, 35 °C	Preclinical testing; 6 compounds separated, 2 baseline	[169]
	9 amides	MeOH (+0.1% IPA) MeOH + ACN + FA + IPA (75/25/0.3/0.3) Gradient: 5–50% at 6.5% min^{-1}, hold for 1 min 150 bar, 4 mL min^{-1}, 40 °C	Effect of additives and column temperature; 4 amides separated, 1 baseline	[48]

Table 1 (*Continued*)

CSP/column	Analyte	Separation conditions	Note	References
	12 compounds, including 4 alcohol sensitive compounds	20% or 40% of MeOH or EtOH or 2-PrOH or ACN or TFE 100 bar, 3 mL min^{-1}, 40 °C	TFE as mobile phase modifier	[88]
	40 commercial compounds + 100 proprietary compounds	MeOH (+0.2% DEA as needed) Gradient: 7% held for 2 min, increased to 50% at 7% min^{-1}, held for 2 min 100 bar, 4 mL min^{-1}, 35 °C	Comparison to P-CAP column by screening; 57% neutral, non-nitrogen containing compounds, 50% acidic compounds and 60% basic and neutral compounds separated	[97]
	57 pharmaceutical compounds	Various amounts of 2-PrOH or MeOH, all with TFA or IPA or both 150 bar, 3 mL min^{-1}, 30 °C	Chiral screening approach	[172]
	59 pharmaceutical compounds (basic, amphoteric, neutral, acidic)	10% MeOH (+0.5% IPA or TFA or both) 150 bar, 3 mL min^{-1}, 30 °C	Comparison of polysaccharide columns	[39]
Lux cellulose-3	23 commercial + 23 proprietary compounds	MeOH or EtOH or 2-PrOH (+0.1% DEA or TEA as needed) Gradient: 10–55% over 1.5 min at 30% min^{-1}, 55% kept for 1 min 120 bar, 5 mL min^{-1}, 40 °C	Comparison of chiral columns by screening	[54]
	57 pharmaceutical compounds	Various amounts of 2-PrOH or MeOH, all with TFA or IPA or both 150 bar, 3 mL min^{-1}, 30 °C	Chiral screening approach	[172]
	59 pharmaceutical compounds (basic, amphoteric, neutral, acidic)	10% MeOH (+0.5% IPA or TFA or both) 150 bar, 3 mL min^{-1}, 30 °C	Comparison of polysaccharide columns	[39]
Sino-Chiral OJ	Polychlorinated biphenyls	100% CO_2 or addition of modifiers (EtOH or MeOH or 2-PrOH) 150 bar, 2 mL min^{-1}, 36 °C	Method development, effect of temperature	[49]
Cellulose tris(3,5-dichlorophenylcarbamate)				
Chiralpak IC	4-(1-Cyclopropylethyl)-6-(6-(difluoromethoxy)-2,5-dimethylpyridin-3-ylamino)-5-oxo-4,5-dihydropyrazine-2-carbonitrile	10% MeOH or EtOH or 2-PrOH 150 bar, 2 mL min^{-1}, 35 °C	Preclinical testing	[169]
	Acebutolol HCl	EtOH + 0.1% NH_4OH Gradient: 5–60% in 1 min, hold for 0.4 min, 105 bar, 1.5 mL min^{-1}, 40 °C	CSP comparison by screening	[173]
	Nicardipine	20% (MeOH + 1% DEA)	Guideline for mobile phase selection	[51]
	Omeprazole	30% (THF + 1% DEA)		
	9 amides	MeOH (+IPA) MeOH + ACN + FA + IPA (75/25/0.3/0.3) MeOH + ACN + TFA + IPA (75/25/0.1/0.1) Gradient: 5–50% at 6.5% min^{-1}, hold for 1 min 150 bar, 4 mL min^{-1}, 40 °C	Effect of additives and column temperature; 8 compounds baseline separated	[48]
	12 compounds, including 4 alcohol sensitive compounds	Various amounts of DCM or ethyl acetate or THF or TFE (+0.1% DEA) 100 bar, 3 mL min^{-1}, 40 °C	TFE as mobile phase modifier	[88]
	23 commercial + 23 proprietary compounds	MeOH or EtOH or 2-PrOH (+0.1% DEA or TEA as needed) Gradient: 10–55% over 1.5 min at 30% min^{-1}, 55% kept for 1 min 120 bar, 5 mL min^{-1}, 40 °C	Comparison of chiral columns by screening	[54]
	48 compounds	MeOH + 25 mM IBA Gradient: 4% for 4 min, ramp to 40% at 4% min^{-1}, hold for 2 min 200 bar, 2 mL min^{-1}, 35 °C	Screening approach for CSP evaluation; Illustrative separation in Fig. 1	[171]

Table 1 (*Continued*)

CSP/column	Analyte	Separation conditions	Note	References
	72 commercially available racemates + 44 Amgen compounds	MeOH or EtOH or 2-PrOH, all with 0.2% DEA MeOH + DCM + DEA (80/20/0.2) MeOH + THF + DEA (80/20/0.2) MeOH + DCM + THF + DEA (80/10/10/0.2) Gradient: 5–55% over 8 min 100 bar, 5 mL min^{-1}	Evaluation of unusual modifiers; 10 commercial and 27 Amgen samples separated by screening	[133]
Cellulose tris(4-chlorophenylcarbamate)				
Chiralcel OF	(−)-(*R*)-2-*tert*-Butyltetrahydro imidazolidin-4-one	MeOH Gradient: 4% for 4 min, ramp to 40% at 2% min^{-1}, hold for 3 min 200 bar, 1.5 mL min^{-1}, 35 °C	Enantiomeric composition	[188]
	Diltiazem hydrochloride	13% (2-PrOH + 0.5% DEA) 180 kg cm^{-2}, 2 mL min^{-1}, 50 °C	Comparison of SFC and HPLC	[183]
	Diltiazem hydrochloride + 3-hydroxy diltiazem hydrochloride	22.5% (2-PrOH + 0.1% DEA) 180 kg cm^{-2}, 2 mL min^{-1}, 60 °C		
Cellulose tris(phenylcarbamate)				
Chiralcel OC	Diltiazem hydrochloride	13% (2-PrOH + 0.5% DEA) 180 kg cm^{-2}, 2 mL min^{-1}, 50 °C	Comparison of SFC and HPLC	[183]
Cellulose tribenzoate				
Chiralcel OB-H	Ibuprofen	2% 2-PrOH 160 bar, 2 mL min^{-1}, 40 °C	Development of method suitable for SMB	[158]
	Sotolon	1.5% MeOH 12 MPa, 1.5 mL min^{-1}, 30 °C	Method development, effect of pressure and temperature	[121]
Cellulose tris(4-chloro-3-methylphenylcarbamate)				
Lux cellulose-4	23 commercial + 23 proprietary compounds	MeOH or EtOH or 2-PrOH (+0.1% DEA or TEA as needed) Gradient: 10–55% over 1.5 min at 30% min^{-1}, 55% kept for 1 min 120 bar, 5 mL min^{-1}, 40 °C	Comparison of chiral columns by screening	[54]
	47 basic, neutral, amphoteric and 12 acidic compounds	Various amounts of MeOH + IPA or TFA or both 150 bar, 3 mL min^{-1}, 30 °C	Screening and evaluation of mobile phase additives	[134]
	57 pharmaceutical compounds	Various amounts of 2-PrOH or MeOH, all with TFA or IPA or both 150 bar, 3 mL min^{-1}, 30 °C	Chiral screening approach	[172]
	59 pharmaceutical compounds (basic, amphoteric, neutral, acidic)	10% MeOH (+0.5% IPA or TFA or both) 150 bar, 3 mL min^{-1}, 30 °C	Comparison of polysaccharide columns	[39]
Sepapak-4	48 compounds	MeOH + 25 mM IBA Gradient: 4% for 4 min, ramp to 40% at 4% min^{-1}, hold for 2 min 200 bar, 2 mL min^{-1}, 35 °C	Screening approach for CSP evaluation; Illustrative separation in Fig. 1	[171]
ChromegaChiral CC4	Norphenylephrine HCl	EtOH + 0.1% NH_4OH Gradient: 5–60% in 1 min, hold for 0.4 min, 105 bar, 1.5 mL min^{-1}, 40 °C	CSP comparison by screening	[173]
Mono-6-O-pentenyl-β-cyclodextrin				
β-Cyclose-6-OH	Aminoglutethimide	30% (MeOH + 0.2% DEA) Δp = 15 bar, 3 mL min^{-1}, 30 °C	CSP synthesis and evaluation	[63]
β-Cyclose-6-OH-T	Aminoglutethimide	30% (MeOH + 0.2% DEA) Δp = 15 bar, 3 mL min^{-1}, 30 °C		

Table 1 (*Continued*)

CSP/column	Analyte	Separation conditions	Note	References
Mono-2-O-pentenyl-β-cyclodextrin				
β-Cyclose-2-OH	Aminoglutethimide	30% (MeOH + 0.2% DEA) Δp = 15 bar, 3 mL min^{-1}, 30 °C	CSP synthesis and evaluation	[63]
β-Cyclose-2-OH-T	Aminoglutethimide	30% (MeOH + 0.2% DEA) Δp = 15 bar, 3 mL min^{-1}, 30 °C	CSP synthesis and evaluation	[63]
Heptakis(2,3,6-tri-O-methyl)-β-cyclodextrin)				
Sumichiral OA-7500	α-Tetralol	2% 2-PrOH 9.8 MPa, 5 mL min^{-1}, 25 °C	Method development, comparison of polysaccharide and CD CSP	[64]
Cationic β-cyclodextrin derivatives				
Mono-6-(3-methyl imidazolium)-6-deoxyperphenyl carbamoyl-β-CD chloride	10 aryl alcohols	3% 2-PrOH 17 MPa, 3 mL min^{-1}, 40 °C	Effect of chiral selector loading on enantioseparation	[65]
Mono-6-(3-octylimidazolium)-6-deoxyperphenylcarbamoyl-β-CD chloride	14 phenyl alcohols	3% 2-PrOH 17 MPa, 3 mL min^{-1}, 40 °C	CSP synthesis and evaluation	[66]
4-Vinylpyridine-β-CD	Bendroflumethiazide, trichlormethiazide	10% MeOH 15 MPa, 1 mL min^{-1}, 40 °C	CSP synthesis and evaluation	[67]
N-allyl-N-methyl amine-β-CD	Bendroflumethiazide	10% MeOH 15 MPa, 1 mL min^{-1}, 40 °C		
6^{A}-(3-vinylimidazolium)-6-deoxyper phenylcarbamate-β-CD chloride	14 Racemates (flavanones, thiazides, DNS-amino acids)	1–40% 2-PrOH 15 MPa, 1 mL min^{-1}, 40 °C	CSP synthesis and evaluation	[68]
β-Cyclodextrin-polysiloxane				
	1-Phenylalcohol, 2-phenyl-1-cyclohexanol	100% CO_2 152 bar, 40 °C	Effect of pore size	[189]
Permethyl-β-cyclodextrin polymethylsiloxane OTC				
	1-Naphthyl-1-ethanol, 1-phenyl-1-propanol, 2-phenyl-trans-cyclohexanol, α-phenylethanol, pantoyllactone, trans-1,2-cyclohexanediol	100% CO_2 130–160 atm, 30 °C	Effects of restrictor	[108]
***R*-naphthylethyl carbamate-cyclofructan 6**				
Lahric RN-CF6	Althiazide	MeOH + EtOH + 2-PrOH (1:1:1) + 0.2% DEA Gradient: 5% 0–0.6 min, 5–60% 0.6–4.3 min, 60% 4.3–6.3 min 4 mL min^{-1}	CSP synthesis and evaluation	[69]
Dimethylphenyl carbamate cyclofructan 7				
Larihc DMP-CF7	8 binaphthol derivatives	5% MeOH (+TFA as needed) or 2-PrOH 120 bar, 4 mL min^{-1}, 40 °C	Comparison of HPLC and SFC, LFER	[70]
	Bendroflumethiazide, BP 34	20% (2-PrOH + 0.5% TFA) 120 bar, 4 mL min^{-1}, 40 °C		
	Butizide, $TTNH_2$	Various amounts of 2-PrOH 120 bar, 4 mL min^{-1}, 40 °C		
O-9(*tert*-Butylcarbamoyl) quinine				
Chiralpak QN-AX	13 β-ketosulfonic acids	25% (MeOH + 200 mM HOAc + 100 mM NH_3) 150 bar, 4 mL min^{-1}, 40 °C	Method development; 11 separations, 8 baseline	[190]
	20 chiral acidic compounds	Various amounts of MeOH + 0.4% FA + 0.35 mM ammonium formate 150 bar, 3 mL min^{-1}, 40 °C	Method development	[72]
	31 chiral acidic analytes	25% (MeOH + 200 mM HOAc + 100 mM NH_3) 150 bar, 4 mL min^{-1}, 40 °C	Method development, effect of temperature; 25 separations, 22 baseline	[74]

Table 1 (*Continued*)

CSP/column	Analyte	Separation conditions	Note	References
O-9(*tert*-Butylcarbamoyl) quinidine				
Chiralpak QD-AX	13 β-ketosulfonic acids	25% (MeOH + 200 mM HOAc + 100 mM NH_3) 150 bar, 4 mL min^{-1}, 40 °C	Method development; 13 separations, 12 baseline	[190]
	31 chiral acidic analytes	25% (MeOH + 200 mM HOAc + 100 mM NH_3) 150 bar, 4 mL min^{-1}, 40 °C	Method development effect of temperature; 27 separations, 21 baseline	[74]
Trans-(*R*,*R*)-2-amino cyclohexane sulfonic acid				
	14 amines	25% (MeOH + 100 mM FA + 50 mM ammonium formate) 25% MeOH + 50 mM NH_3 or TEA 150 bar, 40 °C	CSP synthesis and evaluation	[75]
Teicoplanin				
Chirobiotic T	Binaphthol	10% 2-PrOH 16 MPa, 2 mL min^{-1}, 40 °C	Development of method suitable for SMB	[77]
	Indapamide, N-CBZ-phenylalanine, propranolol	15% MeOH + 0.5% IPA 15 MPa, 2 mL min^{-1}, 30 °C	Evaluation of standard reference material	[159]
	Ibuprofen	5% 2-PrOH 160 bar, 2 mL min^{-1}, 40 °C	Development of method suitable for SMB	[158]
	Nutlin-3	40% MeOH 100 bar, 2 mL min^{-1}, 30 °C	Development of purification method	[184]
	24 dihydrofurocoumarin derivatives	Various amounts of MeOH 100 bar, 3 mL min^{-1}, 31 °C	Method development	[79]
	50 compounds	EtOH + 0.1% HOAc + 0.1% TEA Gradient: 0–50% at 3.3% min^{-1}, hold for 5 min 3000 psi, 2 mL min^{-1}, 40 °C	Comparison of HPLC, SFC, CE	[59]
	111 chiral compounds (heterocyclic compounds, propionic acid derivatives, β-blockers, sulfoxides, derivatized and underivatized amino acids)	Various amounts of MeOH (+TEA or TFA or H_2O or glycerol as needed) 100 bar, 4 mL min^{-1}, 31 °C	Comparison of macrocyclic antibiotics-based columns; 92% compounds separated	[78]
Teicoplanin aglykone				
Chirobiotic TAG	24 dihydrofurocoumarin derivatives	Various amounts of MeOH 100 bar, 3 mL min^{-1}, 31 °C	Method development	[79]
	111 chiral compounds (heterocyclic compounds, propionic acid derivatives, β-blockers, sulfoxides, derivatized and underivatized amino acids)	Various amounts of MeOH (+TEA or TFA or H_2O or glycerol as needed) 100 bar, 4 mL min^{-1}, 31 °C	Comparison of macrocyclic antibiotics-based columns; 92% compounds separated	[78]
Ristocetin A				
Chirobiotic R	3a,4,5,6-Tetrahydrosuccin, 5-(4-hydroxyphenyl)-5-phenyl-hydantoin, 5-methyl-5-phenylhydantoin, efavirenz, imido[3,4-b]acenaphthen-10-one, thalidomide, warfarin	Various amounts of MeOH or EtOH (+0.5% H_2O or HOAc as needed) 210 bar, 2 mL min^{-1}, 30 °C	Effect of modifier, flow rate and temperature	[80]
	Coumachlor, thalidomide, warfarin	25% (MeOH + 10 mM TFA) 150 bar, 1 mL min^{-1}, 40 °C	Comparison of commercial and in-house immobilized CSP	[107]
	24 dihydrofurocoumarin derivatives	Various amounts of MeOH 100 bar, 3 mL min^{-1}, 31 °C	Method development	[79]
	111 chiral compounds (heterocyclic compounds, propionic acid derivatives, β-blockers, sulfoxides, derivatized and underivatized amino acids)	Various amounts of MeOH (+TEA or TFA or H_2O or glycerol as needed) 100 bar, 4 mL min^{-1}, 31 °C	Comparison of macrocyclic antibiotics-based columns; 60% compounds separated	[78]
Ristocetin OTC	Dichlorprop, ketoprofen, warfarin	30% (MeOH + 0.7% TEA) 250 bar, 1 mL min^{-1}, 25 °C	Comparison of commercial and in-house immobilized CSP	[107]

Table 1 (*Continued*)

CSP/column	Analyte	Separation conditions	Note	References
Vancomycin				
Chirobiotic V	Indapamide, propranolol, warfarin	15% (MeOH + 0.5% IPA) 15 MPa, 2 mL min^{-1}, 30 °C	Evaluation of standard reference material	[159]
	12 chiral drugs	15% MeOH (+0–1% IPA or TEA) 20 MPa, 2 mL min^{-1}, 30 °C	Comparison of TEA and IPA additives	[81]
	20 compounds (10 commercial + 10 Pfizer)	MeOH + 0.1% IPA or TFA or both EtOH + 0.1% ESA Gradient: 5–40% at 2.5% min^{-1} 150 bar, 2 mL min^{-1}, 35 °C	Screening method development	[56]
Vancomycin OTC	Dichlorprop, ketoprofen, mepivacaine, metoprolol, thalidomide, verapamil	30% (MeOH + 1% TEA) 250 bar, 35 °C	Comparison of SFC, RPLC, NPLC, POPLC	[191]
(*R*)-1-Naphthyl glycine and 3,5-dinitro benzoic acid				
Chirex 3005	Ketoprofen	55% MeOH 5 mL min^{-1}	Determination in plasma sample	[84]
	Ketoprofen, warfarin	15% (MeOH + 0.5% IPA) 15 MPa, 2 mL min^{-1}, 30 °C	Evaluation of standard reference material	[159]
	20 compounds (10 commercial + 10 proprietary)	EtOH (+TFA or NH_4TFA or NH_4OAc) MeOH (+0.1% IPA) gradient 5–40%	Comparison of Pirkle CSPs	[83]
(*S*)-Indoline-2-carboxylic acid and (*R*)-1-(α-napthyl)ethylamine				
Chirex 3022	Indapamide	20% (MeOH + 0.5% IPA) 15 MPa, 2 mL min^{-1}, 30 °C	Evaluation of standard reference material	[159]
	20 compounds (10 commercial + 10 proprietary)	EtOH (+TFA or NH_4TFA or NH_4OAc) MeOH (+0.1% IPA) gradient 5–40%	Comparison of Pirkle CSPs	[83]
(*S*)-*tert*-Leucine and (*R*)-1-(α-naphthyl)ethylamine				
Chirex 3020	20 compounds (10 commercial + 10 proprietary)	EtOH (+TFA or NH_4TFA or NH_4OAc) MeOH (+0.1% IPA) gradient 5–40%	Comparison of Pirkle CSPs	[83]
(*S*)-Proline and (*R*)-1-(α-naphthyl)ethylamine				
Chirex 3018	20 compounds (10 commercial + 10 proprietary)	EtOH (+TFA or NH_4TFA or NH_4OAc) MeOH (+0.1% IPA) gradient 5–40%	Comparison of Pirkle CSPs	[83]
(*R*)-Phenylglycine and 3,5-dinitrobenzoic acid				
Chirex 3001	20 compounds (10 commercial + 10 proprietary)	EtOH (+TFA or NH_4TFA or NH_4OAc) MeOH (+0.1% IPA) gradient 5–40%	Comparison of Pirkle CSPs	[83]
(*R*)-Phenylglycine and 3,5-dinitroaniline				
Chirex 3012	20 compounds (10 commercial + 10 proprietary)	EtOH (+TFA or NH_4TFA or NH_4OAc) MeOH (+0.1% IPA) gradient 5–40%	Comparison of Pirkle CSPs	[83]
4-(3,5-Dinitrobenzamido) tetrahydrophenanthrene				
(*S*,*S*)-Whelk-O1	2-Methyl-1-indanone	5% 2-PrOH 1500 psi, 2 mL min^{-1}, 50 °C	Simulated moving columns (two tandem Whelk columns)	[87]
	12 compounds, including 4 alcohol sensitive compounds	20% or 40% of MeOH or EtOH or 2-PrOH or ACN or TFE Various amounts of DCM or ethyl acetate or THF or TFE (+0.1% DEA) 100 bar, 3 mL min^{-1}, 40 °C	TFE as mobile phase modifier	[88]
	20 compounds (10 amines and their CBZ-derivatives)	20% or 40% ACN or MeOH or EtOH or 2-PrOH or 2-BuOH (+0.1% DEA) 100 bar, 2.5 mL min^{-1}, 40 °C	CBZ derivatization	[170]
	20 compounds (10 commercial + 10 proprietary)	MeOH (+0.1% IPA or HOAc) gradient 5–40%	Comparison of Pirkle CSP	[83]

Table 1 (*Continued*)

CSP/column	Analyte	Separation conditions	Note	References
(*R*,*R*)-Whelk-Ol	N-(p-Methoxybenzyl)-1,3,2-benzo dithiazol-1-oxide	20% MeOH 200 bar, 2 mL min^{-1}, 50 °C	Enantiomerization energy barrier	[86,85]
	Lorazepam, oxazepam, temazepam	12.5% (MeOH + 0.5% DEA) 200 bar, 2 mL min^{-1}, 40 °C	Enantiomerization energy barrier	[85]
Whelk-O1	Chlormezanone, devrinol, indapamide, Troger's base	Various amounts of 2-PrOH or MeOH or EtOH or THF or ACN	Effect of varying cosolvents	[89]
	20 compounds (10 commercial + 10 Pfizer)	MeOH + 0.1% IPA or TFA or both EtOH + 0.1% ESA Gradient: 5–40% at 2.5% min^{-1} 150 bar, 2 mL min^{-1}, 35 °C	Screening method development	[56]
3,5-Dinitrobenzoyl derivative of diphenylethylenediamine				
(*S*,*S*)-Ulmo	20 compounds (10 commercial + 10 proprietary)	MeOH (+0.1% IPA or HOAc) Gradient 5–40%	Comparison of Pirkle CSPs	[83]
N-3,5-Dinitrobenzoyl-3-amino-3-phenyl-2-(1,1-dimethylethyl)-propanoate				
(*S*,*S*)-β-Gem	20 compounds (10 commercial + 10 proprietary)	MeOH (+0.1% IPA or HOAc) Gradient 5–40%	Comparison of Pirkle CSPs	[83]
Dimethyl N-3,5-dinitro-benzoyl-amino-2,2-dimethyl-4-pentenyl phosphonate				
(*S*,*S*)-α-Burke	20 compounds (10 commercial + 10 proprietary)	MeOH (+0.1% IPA or HOAc) Gradient 5–40%	Comparison of Pirkle CSPs	[83]
3,5-Dinitrobenzoyl leucine				
L-Leucine	20 compounds (10 commercial + 10 proprietary)	MeOH (+0.1% IPA) Gradient 5–40%	Comparison of Pirkle CSPs	[83]
3,5-Dinitrobenzoyl derivative of 1,2-diaminocyclohexane				
(*S*,*S*)-DACH	20 compounds (10 commercial + 10 proprietary)	MeOH (+0.1% IPA or HOAc) Gradient 5–40%	Comparison of Pirkle CSPs	[83]
O,O′-Bis(4-*tert*-butylbenzoyl)-N,N'-diallyl-L-tartar diamide				
Kromasil CHI-TBB	Binaphthol	5% 2-PrOH 16 MPa, 2 mL min^{-1}, 40 °C	Development of method suitable for SMB	[77]
	Ibuprofen	4–7% 2-PrOH 160 bar, 2 mL min^{-1}, 40 °C	Development of method suitable for SMB	[158]
		4% 2-PrOH 13 MPa, 1 mL min^{-1}, 311.15 K	Method development, effect of pressure and temperature	[92]
		5% 2-PrOH 15.6 MPa, 313 K	Comparison of SMB and batch processes	[192]
		2-PrOH 100 bar, 1 mL min^{-1}, 311.15 K	Method development, effect of pressure and temperature	[95]
		5% 2-PrOH 15.6 MPa, 313 K	Adsorption isotherm	[193]
	Mitotane	14% MeOH 160 bar, 5 mL min^{-1}, 303.15 K	Method development, effect of temperature	[91]
	Naproxen	11% 2-PrOH 9.4 MPa, 293 K	Method development, effect of pressure and temperature	[93]
O,O′-Bis(3,5-dimethyl benzoylbenzoyl)-N,N'-diallyl-L-tartar diamide				
Kromasil CHI-DMB	Binaphthol	10% ethyl acetate 16 MPa, 2 mL min^{-1}, 40 °C	Development of method suitable for SMB	[77]
	Ibuprofen	100% CO_2 160 bar, 2 mL min^{-1}, 40 °C	Development of method suitable for SMB	[158]
Trans-(1*S*,2*S*)-cyclohexanedicarboxylic acid bis-4-vinylphenylamide				
	57 compounds	Various amounts of MeOH + TFA 4 mL min^{-1}, 32 °C	CSP comparison in SFC and HPLC mode; 26 separations, 9 baseline	[96]

Table 1 (*Continued*)

CSP/column	Analyte	Separation conditions	Note	References
Trans-N,N′-(1*R*,2*R*)-cyclohexanediyl-bis-4-ethenylbenzamide				
	57 compounds	Various amounts of MeOH + TFA 4 mL min^{-1}, 32 °C	CSP comparison in SFC and HPLC mode; 24 separations, 4 baseline	[96]
N,N′-(*S*,*S*)-1,2-cyclohexanediyl-bis-2-propenamide				
P-CAP	40 commercial compounds and 100 proprietary	MeOH (+0.2% DEA as needed) Gradient: 7% held for 2 min, increased to 50% at 7% min^{-1}, held for 2 min 100 bar, 4 mL min^{-1}, 35 °C	Comparison of P-CAP to polysaccharide columns; 50% of neutral, non-nitrogen containing compounds and 40% of basic and neutral compounds separand	[97]
	88 compounds	10–40% MeOH or EtOH or 2-PrOH, all with 0.2% TFA 100 bar, 4 mL min^{-1}, 31 °C	Comparison of synthetic polymeric CSPs; 49 separations, 12 baseline	[98]
N,N′-(1,2-diphenyl-1,2-ethanediyl)bis-2-propenamide				
(*R*,*R*)-P-CAP-DP	88 compounds	10–40% MeOH or EtOH or 2-PrOH, all with 0.2% TFA 100 bar, 4 mL min^{-1}, 31 °C	Comparison of synthetic polymeric CSPs; 49 separations, 14 baseline	[98]
(*S*,*S*)-P-CAP-DP	100 compounds	Various amounts of MeOH or EtOH, all with 0.1% TFA 2 mL min^{-1}, 40 °C	CSP synthesis and evaluation; 44 separations, 8 baseline	[99]
(1*S*,2*S*)-1,2-Bis(2,4,6-trimethylphenyl)ethylenediamine				
3Me-P-CAP-DP	100 compounds	Various amounts of MeOH or EtOH, all with 0.1% TFA 2 mL min^{-1}, 40 °C	CSP synthesis and evaluation; 43 separations, 13 baseline	[99]
(1*S*,2*S*)-1,2-Di-1-naphthyl ethylenediamine				
Naph-P-CAP-DP	100 compounds	Various amounts of MeOH or EtOH, all with 0.1% TFA 2 mL min^{-1}, 40 °C	CSP synthesis and evaluation; 57 separations, 9 baseline	[99]
(1*S*,2*S*)-1,2-Bis(2-chlorophenyl)ethylenediamine				
Cl-P-CAP-DP	100 compounds	Various amounts of MeOH or EtOH, all with 0.1% TFA 2 mL min^{-1}, 40 °C	CSP synthesis and evaluation; 47 separations, 5 baseline	[99]
Trans-9,10-dihydro-9,10-ethanoanthracene-(11*S*,12*S*)-11,12-dicarboxylic acid bis-4-vinylphenylamide				
	88 compounds	10–40% MeOH or EtOH or 2-PrOH, all with 0.2% TFA 100 bar, 4 mL min^{-1}, 31 °C	Comparison of synthetic polymeric CSPs; 49 separations, 20 baseline	[98]
N,N′-[(1*R*,2*R*)-1,2-Diphenyl-1,2-ethanediyl]bis[4-vinylbenzamide]				
	88 compounds	10–40% MeOH or EtOH or 2-PrOH, all with 0.2% TFA 100 bar, 4 mL min^{-1}, 31 °C	Comparison of synthetic polymeric CSPs; 18 separations, 1 baseline	[98]
Molecularly imprinted polymers				
(−)-Ephedrine	Ephedrine	30% [MeOH + IPA + H_2O (93:5:2)] 200 bar, 2 mL min^{-1}, 60 °C	Comparison of different imprinted polymers	[103]
Racemic propranolol	Metoprolol (succinate), propranolol, propranolol HCl	Various amounts of MeOH + HOAc 150 bar, 2 mL min^{-1}, 50 °C	Method development for MIP CSP	[104]
L-Phenylalanine anilide	Phenylalanine anilide	40% [MeOH + HOAc (95:5)] 150 bar, 2 mL min^{-1}, 50 °C		
Boromycin				
	DL-Methionine-β-naphthylamide, DL-Tryptophan benzyl ester, DL-Tryptophan methyl ester HCL	30% (MeOH + 20 mM tetramethylammonium nitrate) 100 bar, 4 mL min^{-1}, 40 °C	Determination of structural characteristics and separation mechanism	[105]
Nickel(II)-bis[(3-hepta fluorobutanoyl)-10-methylene-(1*R*)-camphorate]				
Chirasil-Nickel	2-(29-Methylphenyl)-2,3-dihydro-4H-pyron	60 °C	Complexation SFC in capillary columns	[106]
	5-Isopropyl-l-methyl-5-propyl-(1H,3H,5H)-pyrimidin-2,4,6-trione	50 °C, gradient		

Table 1 (*Continued*)

CSP/column	Analyte	Separation conditions	Note	References
	5-ethyl-l-methyl-5-propyl-(1H,3H,5H)-pyrimidin-2,4,6-trione	50 °C, gradient		
	dimethyl-1,19-binaphthyl-2,29-dicarboxylate	100 °C		
	endosulfanlactone	60 °C		
	mecoprop methyl ester	110 °C		
	MTH-proline	27 °C, 35 MPa		
Zinc(II)-bis[(3-heptafluorobutanoyl)-10-methylene-(1*R*)-camphorate]				
Chirasil-Zinc	2-Naphthyloxirane	40 °C, gradient	Complexation SFC in capillary columns	[106]
	Ibuprofen	80 °C, gradient		

2-BuOH, 2-butanol; 2-PrOH, 2-propanol; ACN, acetonitrile; CBZ, carboxybenzyl; CD, cyclodextrin; DCM, dichloromethane; DEA, diethylamine; DMEA – dimethylethylamine; DMOA, N,N-dimethyloctylamine; DNS, dansylchloride; ESA, ethanesulfonic acid; EtOH, ethanol; FA, formic acid; HOAc, acetic acid; IBA, isobutylamine; IPA, isopropylamine; MeOH, methanol; NH_4OAc, ammonium acetate; NH_4TFA, ammonium trifluoroacetate; NPLC, normal phase liquid chromatography; POPLC, polar organic phase liquid chromatography; POSC, polar organic solvent chromatography; TEA, triethylamine; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol; THF, tetrahydrofuran,

found to significantly deteriorate whereas the thermally initiated propranolol MIP revealed only subtle changes of separation performance after a long period of operation. Extremely broad peak in combination with retention dependency on the sample load remain a general problem with the application of MIPs in SFC [104]. Twelve years later, (−)-ephedrine-molecularly imprinted polymers differing in amount and type of functional monomer, crosslinker and concentration of (−)-ephedrine were prepared and used as CSP [103]. The authors used and compared 21 (−)-ephedrine-imprinted CSPs prepared. The optimized separation conditions were: the mobile phase CO_2/modifier 70/30 (v/v), where the modifier comprised MeOH/IPA/H_2O 93/5/2 (v/v/v), the column temperature 60 °C, the pressure 200 bar and the flow rate 2.0 mL min^{-1}. These CSPs could find applications in preparative SFC.

2.8. Miscellaneous CSPs

Boromycin is a macrodiolide antibiotic that contains a stereogenic borate moiety. D-Valine ester of boromycin was covalently bonded to silica gel through a urea linkage forming CSP [105]. High selectivity for enantiomers of primary amine containing compounds was observed. Enantioselective retention mechanism was ascribed to charge–charge interactions, hydrogen bonding with the cleft oxygens, and steric repulsion.

2.9. Packed capillary CSPs

Packed capillaries were utilized formerly in chiral SFC where packed columns dominate nowadays. Nickel (II)- and zinc (II)-*bis*[(3-heptafluorobutanoyl)-10-methylene-(1*R*)-camphorate] were chemically bonded to poly(dimethylsiloxane) (Chirasil–nickel and Chirasil–zinc, 50 μm i.d. packed capillaries) to form chiral Lewis acid selectors [106]. These CSs were successfully used in complexation SFC for enantioseparation of Lewis base solutes (selectands). Supercritical CO_2, a potential complexation agent, was compatible with complexation SFC and did not appear to block coordination sites at the Lewis acid selector in competition with Lewis base selectands. Complexation SFC seems to be a useful tool for separation of thermally labile, configurationally labile and less-volatile selectands. Ristocetin A CSP in a packed capillary was prepared and compared with commercial ristocetin A CSP (Chirobiotic R^{TM}) in terms of enantioselectivity [107]. The commercial ristocetin A CSP gave similar results for enantioseparation of warfarin, coumachlor and thalidomide. Interestingly, differences were observed between CSPs for enantiomers of dichlorprop and ketoprofen, which were separated on the prepared CSP and could not be separated on the commercial phase. Differences between these CSPs were observed due to different immobilization of the CS. Capillaries packed with cyclodextrin CSs were also utilized in SFC. 12–15 cm capillary columns packed with 5 μm porous (300 Å) silica particles deactivated with 3-cyanopropyldimethylchlorosilane and encapsulated with CD-substituted polymethylsiloxane were prepared [108]. Most separations were carried out in less than 1 min using these columns. The effect of pore size on a speed of enantioseparation was tested using β-CD polysiloxane-encapsulated 10 μm silica particles (80, 300 and 1400 Å pore sizes) packed in capillary columns [109]. The highest column efficiency was achieved using 1400 Å pore particles, indicating that convective mass transfer was generated by a very small portion of mobile phase flowing through the larger pores. No significant difference between 80 and 1400 Å pore particles in terms of resolution per unit time was observed.

2.10. Tandem-column coupling and two-dimensional SFC

Low-pressure drops generated in SFC enable tandem coupling of columns [110]. Separation selectivity and/or efficiency can be altered by coupling different or identical columns in series, respectively. Whatever is the nature of the stationary phase used, the retention time on coupled columns is lower than the sum of the retention times on individual columns [2]. This was explained by the lower retention time in the first column because of the greater internal pressure due to the serially coupled column. It must be noticed that with SFC, the different back-pressures experienced by the columns in the two A-B or B-A arrangements could possibly lead to subtle differences in chromatographic results under isocratic elution [82]. With gradient elution, the order of the columns in a tandem arrangement can have a more profound influence on chromatographic behavior, with the greatest difference when the retention on the two individual columns is significantly different. KR100-5CHI-TBB (25 cm) and Chiralpak AD (5 cm) or Chiralcel OD (5 cm) columns connected in series were used for control of drug enantiomeric purity [111] – see Table 1. A tandem-column method using Chiralpak AD-H and Chiralcel OD-H columns was used to achieve baseline separation of a mixture of four stereoisomers [112]. This mixture could not be baseline separated with individual Chiralpak AD-H and Chiralcel OD-H columns. All four stereoisomers were baseline separated with tandem-columns in mobile phase composed of 90% CO_2 and 10% 2-PrOH/EtOH (50/50 (v/v)) within 14 min.

Two dimensional separation systems are used mainly if achiral separation followed by an enantioselective one are performed. A directly coupled achiral/chiral SFC/MS method was developed for the profiling of a three-step stereoselective synthesis of cinnamonitrile and hydrocinnamonitrile intermediates [113]. The most effective separation was observed with Phenomenex Luna Silica

column coupled with Chiralcel OD-H column in 15% MeOH as mobile phase, and flow rate of 1 mL min^{-1}. The baseline separation of all components (enantiomers and diastereomers) was obtained within 9 min.

An analytical two-dimensional SFC/SFC/MS system was designed and implemented to streamline enantiomeric analysis of complex mixtures of pharmaceutical racemate samples [114]. The first dimension chromatography was performed on an achiral (pyridine-based) column to separate a desired racemate from impurities and the second dimension chromatography was conducted on a chiral column (Chiralcel OJ-H) to resolve the pair of enantiomers.

2.11. Preparative SFC

Chromatographic enantioseparation in preparative scale is routinely used in pharmaceutical R&D to generate individual enantiomers. SFC has many advantages over HPLC for these separations, *i.e.* rapid screening of separation conditions at the analytical scale, rapid preparative separations, higher purification throughputs, lower solvent consumption and waste generation and higher product concentrations post separation [115–117]. A review focused mainly on the latest examples of pharmaceutical separations on CSPs in SFC for efficient analyses and preparative-scale purifications was prepared by Wang et al. [118]. The advantages of the use of preparative SFC instrumentation with tandem UV and polarimetric detection for confirming enantioseparation and for determining optimum preparative column injections were presented [119]. Polarimetric confirmation of enantioseparation was carried out for racemic mixtures of propranolol HCl, thioridazine HCl, tramadol HCl, and flurbiprofen using Chiralpak AD-H column using CO_2/MeOH in various volume ratios as mobile phases. An evaluation of injection conditions, *i.e.* mixed stream *vs.* modifier stream injection, for preparative SFC under isocratic conditions was performed by Miller and Sebastian [120]. Mixed stream injection introduces sample solution just prior to the column after carbon dioxide and the modifier solvent are mixed. Modifier stream injection introduces sample solution into the modifier flow stream prior to mixing with carbon dioxide. For the majority of compounds evaluated, modifier stream injection gave better resolution.

Many papers dealing with preparative SFC indicate great application potential of this method [*e.g.* 122–124]. The specific applications are summarized in Table 2.

2.12. Supercritical fluid simulated moving bed chromatography

The first enantioseparation in supercritical fluid simulated moving bed chromatography (SF-SMB) unit was reported in 2001 [124]. The main objective in SMB is to overcome the fixed bed operation of the single column chromatography and to implement a configuration, in which the stationary and the mobile phases move in countercurrent directions [15]. Triangle theory is one of the best known approaches for designing the SMB [15,125]. The triangle theory is based on the equilibrium theory of chromatography, in which mass transfer resistances are neglected, *i.e.* it is assumed that the efficiency of the columns is infinite. This theory allows an easy graphical description of the internal flow rates and the switch time which are determining the flow rate ratios. The triangle is determined through the adsorption isotherm and Henry constants. In SMB the solid beds are fixed and the continuous movement of the solid is simulated by periodically switching the inlet and outlet ports of the unit in the same direction of the mobile phase flow. Each section of the SMB unit is divided into a number of subsections so as to closely mimic the counter-current movement of the solid phase. Each subsection consists of a chromatographic column, equipped with a sufficient number of valves for connecting it to all the outlets and to all the inlets of the process. After mixing with or withdrawal of an external stream the resulting stream is fed to the following chromatographic column [126]. The productivity of the SMB SFC process depends on a large number of parameters, such as the property of stationary phase, column length, number of columns in each zone, temperature, pressure gradient, modifier type, and modifier content. Changing only one of these parameters influences the separation behavior of the system and an empirical prediction for a suitable combination of parameters is not possible [77]. An increase in availability and robustness of SFC–SMB will potentially boost the exploitation of enantioselective chromatography into drug development and production [127]. SMB or other multicolumn setup are predominating at large scale production (>20-kg scale) [128]. Adsorption isotherms of ibuprofen enantiomers were used for simulation of the chromatographic separation of the enantiomers in SMB SFC [94]. Rajendran et al. demonstrated that the triangle theory is well suited for the design of SF-SMB units [129]. Nonlinear isotherms measured on a Chiralcel OD column were used in combination with the triangle theory for simulated moving bed design to select operating conditions for the supercritical fluid SMB [129].

2.13. Mobile phases in SFC

Mobile phases in SFC consist of carbon dioxide combined with an organic modifier for affecting polarity of the mobile phases and thus the interaction/elution behavior [130]. Without any organic modifier the analytes mostly do not elute. Mobile phase additives also improve enantioseparations and peak shapes. The modifiers mostly used are alcohol-type solvents, such as methanol, ethanol, 2-propanol or acetonitrile. The use of basic and acidic additives, *i.e.* triethylamine, trifluoroacetic acid (TFA) must also be considered/evaluated. Various aliphatic and cyclic amines were used as mobile phase additives for improvement of enantioseparation of amine compounds on Chiralpak AD and AD-H columns [131]. All enantiomers of amphetamine and metamphetamine were baseline resolved in 5 min using 10% 2-PrOH with 0.5% cyclohexylamine as a mobile phase on Chiralpak AD-H column. Stringham used ethanesulfonic acid as an additive for successful separation of basic compounds that were not separated in SFC previously [132]. This strong acid acts as a counter-ion to a wide range of amines. Byrne et al. used 2,2,2-trifluoroethanol as an alternative modifier in the analysis and purification of alcohol-sensitive chiral compounds [88]. Other non-traditional modifiers, *i.e.* dichloromethane and tetrahydrofuran with methanol were also used [133]. It was found out that the use of non-traditional solvents could result in drastic changes, both positive and negative, in analytical enantioselectivity. These modifiers are utilized as a second tier approach when adequate selectivity is not obtained with common modifiers or when low methanol solubility results in poor preparative separations. The work by De Klerck et al. was focused on the simultaneous use of the acidic additive TFA and the basic additive isopropylamine (IPA) for enantioseparations [134]. The results showed that combining TFA and IPA in the mobile phase can substantially increase enantioselectivity of the chromatographic system, compared to the individual use of these additives. Non-aqueous ammonia [135] and ammonium hydroxide [136] were successfully used as mobile phase additives instead of diethylamine. The clear advantage of these additives over more commonly used basic modifiers is their high volatility that makes them easy to remove in order to simplify the post-purification. Reversal of elution order on Chiralpak AD column with the change of alcohol modifiers, methanol for 2-propanol, was observed [137].

Table 2
Summary of chiral separation system compositions and separation conditions used for preparative or semipreparative purposes. Main mobile phase component was CO_2.

CSP/column	Analyte	Separation conditions	References
Amylose tris(3,5-dimethylphenylcarbamate)			
Chiralpak AD	Lansoprazole	20% MeOH 20 MPa, 8 mL min^{-1}, 35 °C	[194]
	Pantoprazole	25% 2-PrOH 20 MPa, 8 mL min^{-1}, 35 °C	
	Rabeprazole	25% MeOH 20 MPa, 8 mL min^{-1}, 35 °C	
	Albendazole sulfoxide	30% 2-PrOH 200 bar, 8 mL min^{-1}, 35 °C	[195]
	Omeprazole	25% EtOH 20 MPa, 8 mL min^{-1}, 35 °C	[196]
	Omeprazole	50% [MeOH + 2-PrOH + TEA (90/10/0.2)] 210 bar, 76 g min^{-1}, 40 °C	[156]
	Sotolon	2.5% MeOH 8 MPa, 8 mL min^{-1}, 28 °C	[121]
	Warfarin	30% (MeOH + 0.2% DEA) 100 bar, 80 mL min^{-1}	[133]
Chiralpak AD-H	1-(4-Chlorobenzylhydryl) piperazine, 4-benzoloxy-2-azetidinone, disopyramide, pantothenol, sulconazole, warfarin	25% (MeOH + 0.2% DEA) 120 bar, 126 mL min^{-1}, 40 °C	[120]
	1,5-Dimethyl-4-phenyl-2-imidazolidinone	15% (MeOH + 0.2% DEA) 120 bar, 126 mL min^{-1}, 40 °C	
	α-(2,4-Dichlorophenyl)-1H-imidazole-1-ethanol	35% (MeOH + 0.2% DEA) 120 bar, 126 mL min^{-1}, 40 °C	
	Benzylmandelate, propranolol	20% (MeOH + 0.2% DEA) 120 bar, 126 mL min^{-1}, 40 °C	
	Fenoterol	30% (MeOH + 0.2% DEA) 120 bar, 126 mL min^{-1}, 40 °C	
	2-Phenylglutaric anhydride	15% TFE 100 bar, 10 mL min^{-1}, 40 °C	[88]
	3,5-Difluoromandelic acid	3% (EtOH + 0.5% TFA) 100 bar, 2.4 mL min^{-1}	[57]
	Flurbiprofen	15% MeOH 147 bar, 72 g min^{-1}, 30 °C	[197]
	β-Methylphenylalanine-N-benzylcarbamate methyl ester	15% [MeOH + EtOH (50/50)] 100 bar, 50 mL min^{-1}, 35 °C	[164]
	Warfarin	30% MeOH 100 bar, 30–70 mL min^{-1}, 40 °C	[114]
Chiralpak IA	Warfarin	30% [MeOH + DCM + DEA (50/50/0.2)] 100 bar, 80 mL min^{-1}	[133]
RegisPack	Naringenin	25% MeOH 4 mL min^{-1}, 30 °C	[122]
Amylose tris((*S*)-α-methylbenzylcarbamate)			
Chiralpak AS	Binaphthol	18% MeOH 100 bar, 70 mL min^{-1}, 60 °C	[97]
	Substituted piperazine	12% (MeOH + 25 mM IBA) 100 bar, 50 mL min^{-1}, 35 °C	[198]
Cellulose tris(3,5-dimethylphenylcarbamate)			
Chiralcel OD	1-Phenyl-1-propanol	2.55% MeOH 18 MPa, 30 g min^{-1}, 30 °C	[129]
	Nutlin-3	35% MeOH 100 bar, 300 mL min^{-1}, 30 °C	[184]
	Alprenolol, arginin(pmc), atenolol, corticosterone, coumarin, erythromycin, FMOC-alanin-COOH, FMOC-threonin-(t-Bu)-COOH, imipramine, metoprolol, promethazine, tolbutamide, verapamil, warfarin	MeOH Gradient: 10–60% in 2.5 min 2000 psi, 15 mL min^{-1}, 40 °C	[199]
Chiralcel OD-H	Atenolol	20% (MeOH + 20 mM NH_3 or 0.2% DEA) 80 g min^{-1}	[135]
Cellulose tris(3,5-dichlorophenylcarbamate)			
Chiralpak IC	Mandelamide	10% MeOH 100 bar, 70 mL min^{-1}, 40 °C	[54]
Cellulose tris(3-chloro-4-methylphenylcarbamate)			
Lux Cellulose-2	Fluoro-oxindole derivatives	10% MeOH 150 bar, 3 mL min^{-1}, 30 °C	[38]

Table 2 (*Continued*)

CSP/column	Analyte	Separation conditions	References
Cellulose tris(4-chloro-3-methylphenylcarbamate)			
Lux Cellulose-4	Bendroflumethiazide	25% MeOH 100 bar, 70 mL min^{-1}, 40 °C	[54]
4-(3,5-Dinitrobenzamido)tetrahydrophenanthrene			
Whelk-O1 (R,R)	CBZ-N-benzyl-α-methyl benzylamine	43% 2-PrOH 100 bar, 350 mL min^{-1}	[170]
N,N′-(S,S)-1,2-Cyclohexanediyl-bis-2-propenamide			
P-CAP	Binaphtol	30% MeOH 100 bar, 70 mL min^{-1}, 40 °C	[97]

See Table 1 footnotes for abbreviations.

As chiral environment in SFC can be also created with chiral mobile phase (and an achiral stationary phase) various chiral selectors were used as a mobile phase additives. However, this way of creating enantioseparation conditions is less popular than the use of CSPs. An ion-pairing agent, Z-(L)-arginine was used as chiral counter ion with Hypercarb column for enantioseparation of substituted dihydropyridines [138]. The kinetics of adsorption and desorption of dimethylated-β-cyclodextrin mixtures (MeCD) as mobile phase additives was tested on Hypercarb column [139]. The proposed chiral separation system had a short equilibration time and showed high reproducibility. MeCD as chiral selector in the mobile phase with Hypercarb column were used for enantioseparation of different chiral compounds [140]. The adsorbed quantity of MeCD onto the Hypercarb column was measured for various chiral selector concentrations using the breakthrough method. Authors found out that dominant mechanism for the chiral discrimination was the diastereomeric complexation in the mobile phase. Gyllenhaal and Karlsson used L-(+) tartaric acid as a mobile phase additive to methanol modified CO_2 with DMOA and Hypercarb column for separations of various enantiomers [141]. Good selectivity was obtained for tertiary amino alcohols. Retention and selectivity increased with increasing concentration of the chiral selector.

2.14. Temperature and pressure

Mobile phase density that partially determines the solvent strength is dependent on temperature and pressure [3]. Generally, mobile phase density increases with increasing pressure and decreases with increasing temperature [1,142]. Selectivity of chiral separations decreases with temperature until enantiomers coelute at the isoeluotropic temperature [143]. Above this temperature, the elution order reverses and selectivity increases with temperature. The isoelution temperature of a racemic mixture is not only dependent on the analyte but also on the mobile phase composition [110,143]. Although these effects are well known, temperature and pressure are often chosen empirically [110]. The reversal of elution order on Chiralpak AD column was achieved only by change of temperature [137].

3. Applications

Analytical and preparative scale SFC applications, including CSPs, separation conditions and aim of each study, are summarized in Tables 1 and 2, respectively. This transparent way of presentation can offer an easy orientation in the separation systems used in SFC for enantioselective analyses.

Chiral mobile phase additives which represent another, seldom used possibility of creation of the chiral separation system in SFC are discussed in the previous section, in Section 2.13.

4. Conclusions

Supercritical fluid chromatography seems to be a separation technique of future because it offers fast and efficient analyses. The growing interest in the field of SFC can be seen from the increasing number of papers dealing with this separation technique. It is not surprising that SFC found its use also for separation of chiral compounds. Enantioselective separation environment in SFC is mostly created with CSPs, chiral mobile phase additives are used rarely. This fact reflects also the results of our literature search where separation systems with CSPs predominate.

This review article gives an overview of chiral separation systems that were used in theoretical studies and/or applications in SFC in recent years. It shows the possibilities of SFC in enantioselective separations and serves as an aid for easier choice of the proper chiral separation system in SFC.

Acknowledgements

The authors gratefully acknowledge financial support of the KONTAKT AM 2010 project LH11018 and the Grant Agency of the Czech Republic, Grant no. 14-19278P.

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5 Výsledky a diskuze

5.1 Publikace II - Charakterizace chirálních stacionárních fází na bázi derivatizovaných cyklofruktanů pomocí modelu LFER

Jak již bylo zmíněno dříve, derivatizace nativního cyklofruktanu zásadním způsobem změní jeho retenční a enantioselektivní vlastnosti. Zatímco zavedení alifatické derivatizační skupiny zvyšuje enantioselektivitu cyklofruktanových CSP pro aminy, aromatické derivatizační skupiny rozšiřují enantiodiskriminační interakce o π-π interakce a zvyšují tak enantioselektivitu cyklofruktanových CSP pro celou řadu dalších látek. **Publikace II** se zabývá charakterizací a vzájemným porovnáním interakčních mechanizmů tří komerčně dostupných cyklofruktanových CSP pomocí modelu LFER v podmínkách NP HPLC. Jedná se o dimethylfenyl karbamát cyklofruktanu 7 CSP (DMP-CF7 CSP), *R*-naftylethyl karbamát cyklofruktanu 6 CSP (RN-CF6 CSP) a izopropyl karbamát cyklofruktanu 6 CSP (IP-CF6 CSP). Porovnání CSP bylo prováděno ve dvou mobilních fázích tvořených hexanem (hex) a propan-2-olem (IPA), příp. kyselinou trifluorooctovou (TFA) o složení hex/IPA 80/20 (*v/v*) a hex/IPA/TFA 80/20/0,5 (*v/v/v*) za použití optimálních LFER modelů, které uvažují pouze statisticky významné interakce. Regresní koeficienty byly získány multidimenzionální lineární regresí retenčních dat sady 44 strukturně odlišných analytů se známými deskriptory. Korelační koeficient pro všechny uvedené regresní závislosti byl stejný nebo vyšší než 0,95, což ukazuje velmi dobrou korelaci s experimentálními daty.

Na všech zkoumaných CSP byly jako statisticky významné určeny regresní koeficient *b* (popisující rozdíl mezi stacionární a mobilní fází v působení jako donor vodíku pro tvorbu vodíkových vazeb), koeficient *s* (popisující rozdíl mezi stacionární a mobilní fází v dipolaritě/polarizibilitě) a koeficient *v* (popisující rozdíl v disperzních interakcích). První dva koeficienty dosahují ve všech zkoumaných systémech kladných hodnot, tedy odpovídající interakce jsou preferované se stacionární fází a přispívají ke zvýšení retence. Třetí koeficient byl ve všech testovaných systémech záporný, což je typické pro podmínky NP HPLC, ve kterém je stacionární fáze polárnější než fáze mobilní.

Významnost koeficientu *b* vyplývá ze struktury CSP, které poskytují mnohem více interakčních míst pro tvorbu vodíkových vazeb v porovnání s použitými mobilními fázemi. Nejvyšší hodnota koeficientu *b* byla zjištěna pro DMP-CF7 CSP, což se jeví jako důsledek struktury základního makrocyklu této CSP, který obsahuje sedm fruktofuranosových jednotek a poskytuje tedy více interakčních míst oproti CSP, které obsahují šest fruktofuranosových jednotek. Hodnoty koeficientů *b* pro IP-CF6 CSP a RN-CF6 CSP jsou srovnatelné. Okyselením mobilní fáze hodnoty koeficientů *b* všech zkoumaných CSP mírně poklesly, neboť přítomnost kyseliny v mobilní fázi zvýší možnost tvorby vodíkové interakce v této fázi.

Na základě hodnot koeficientů *v*, reflektujících disperzní interakce, se jeví nejméně polární DMP-CF7 CSP, následovaná IP-CF6 CSP a nejpolárnější RN-CF6 CSP. Stejný trend byl pozorován i v okyselené mobilní fázi. Možným vysvětlením mohou být sterické vlivy, které se uplatňují při derivatizaci jednotlivých CS.

Koeficient *s* reprezentující rozdíl mezi fázemi v dipolaritě/polarizibilitě nabývá kladných hodnot pro všechny zkoumané systémy. Okyselení mobilní fáze zapříčinilo nárůst hodnot koeficientů *s* pro IP-CF6 CSP a DMP-CF7 CSP, zatímco pro RN-CF6 CSP hodnota tohoto koeficientu klesla. Výsledky modelu LFER naznačují, že se TFA adsorbuje na jednotlivé CSP v různé míře. Stericky objemný naftylethylový substituent brání sorpci TFA na CSP oproti ostatním CSP s menšími substituenty, což reflektuje pokles koeficientu *s*.

Model LFER poskytl základní náhled do retenčních a interakčních mechanizmů cyklofruktanových CSP a umožnil jejich prvotní srovnání v podmínkách NP HPLC.

Publikace II

Characterization of Cyclofructan-based Chiral Stationary Phases by Linear Free Energy Relationship

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Journal of Separation Science **2011**, 34, 2639-2644

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Received May 25, 2011
Revised July 11, 2011
Accepted July 11, 2011

Research Article

Characterization of cyclofructan-based chiral stationary phases by linear free energy relationship

Cyclofructans (CFs), a new class of chiral selectors, have been recently introduced for application in liquid chromatography and capillary electrophoresis. So far, derivatized CFs have performed interesting separation possibilities for a variety of compounds. The current work is focused on characterization of three different CF-based chiral stationary phases (CF-based CSPs), i.e. isopropyl carbamate cyclofructan 6 (IP-CF6), *R*-naphthylethyl carbamate cyclofructan 6 (RN-CF6) and dimethylphenyl carbamate cyclofructan 7 (DMP-CF7). The linear free energy relationship (LFER) model was used to reveal the dominant interactions participating in the complex retention mechanism. A set of 44 different test solutes, with known solvation parameters, was used to determine the regression coefficients of the LFER equation under two mobile-phase compositions in normal separation mode. The LFER results showed that hydrogen bond acidity, hydrophobicity and dipolarity/polarizibility mostly affect the retention and separation process on the CF-based columns in the studied separation systems.

Keywords: Cyclofructan-based chiral stationary phases / HPLC / LFER / Normal-phase mode
DOI 10.1002/jssc.201100462

1 Introduction

Interest in the field of the enantiomeric separation and development of chiral separation media (chiral selectors, chiral stationary phase (CSPs)) has increased greatly in the past few decades due to the demand of pharmaceutical, agrochemical and food analysis. HPLC with CSPs has become the most powerful method for separating racemic samples at analytical and preparative scales and/or determining enantiomeric purity. A variety of CSPs with complex interaction mechanisms have been reported by many research groups, which dominate the works of Davankov, Pirkle, Okamoto, Blaschke, Allenmark, Hermansson, Armstrong, Gasparrini, and Lindner [1–5]. Numerous applications have been reported that involve different chiral selectors chemically bonded to silica gel (or polymeric support) such as polysaccharides [6, 7], proteins [8], macrocyclic antibiotics [9, 10], crown ethers [11] and cyclodextrins [12].

Despite the applicability and broad selectivity of many existing CSPs the research effort for the development of new or improved chiral selectors continues. In 2009 a novel class of CSPs based on cyclofructans (CFs) was introduced by Armstrong [13]. This group of chiral selectors was shown to have potential both for HPLC [13–17] and CZE [18]. CFs are macrocyclic oligosaccharides as cyclodextrins. However, CFs are quite different in both their structure and behavior. They consist of six or more β-(2→1) linked D-fructofuranose units [19, 20]. Their abbreviations CF6, CF7, CF8, etc. indicate the number of fructofuranose units in the macrocyclic ring. Each fructofuranose unit contains four stereogenic centers and three hydroxyl groups, which can be utilized for derivatization. While native CFs have rather limited enantioselectivity in HPLC [13] their derivatized forms show improved and unique chiral recognition abilities for a wide range of analytes [13, 14, 16, 21]. Aliphatic or aromatic functionalization of a native chiral selector is a common strategy used to develop new CSPs and improve the enantioseparation performance. Figure 1 shows the molecular structure of cyclofructans CF6 and CF7 and the derivatization groups studied in this work. These CSPs can be operated in all common separation modes (normal, reversed-phase and polar organic) but mostly higher selectivity was obtained in the normal-phase mode.

A comprehesive method for characterization and comparison of separation systems is the model of linear free energy relationship (LFER) [22–26], which can

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Abbreviation: CSP, chiral stationary phase; **DMP-CF7**, dimethylphenyl carbamate cyclofructan 7; **hex**, *n*-hexane; **IPA**, propane-2-ol; **IP-CF6**, isopropyl carbamate cyclofructan 6; **LFER**, linear free energy relationship; **RN-CF6**, *R*-naphthylethyl carbamate cyclofructan 6

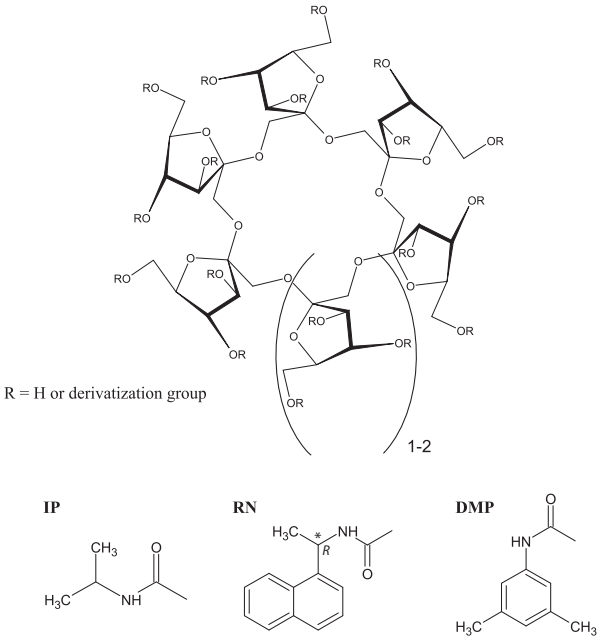

Figure 1. Molecular structure of CF CF6 and CF7 and the derivatization groups studied in this work.

independently describe the contributions of individual interactions to the retention process. The LFER equation expresses relationship between the retention characteristic (i.e. retention factor k) determined for a representative set of analytes in a given separation system and the solute fundamental properties described by its descriptors [27]:

$$\log k = c + eE + sS + aA + bB + vV \quad (1)$$

The independent variables in Eq. (1) are solute descriptors, where E is the solute excess molar refraction modelling the solute ability to interact via n- and/or π-electron pairs, S is the solute dipolarity/polarizibility, A is the effective or overall hydrogen bond acidity, B is the effective or overall hydrogen bond basicity and V is the McGowan's characteristic molecular volume reflecting hydrophobicity [28–30]. The selection of a representative set of structurally diverse analytes is essential for aquiring reliable results [31]. Multivariate regression analysis is applied for the determination of the coefficients in Eq. (1) that reflect the different types of molecular interactions in the studied system. In HPLC, the regression coefficients relate to the differences in the properties of the stationary and the mobile phases. The c intercept in the LFER equation is characteristic of the given system but it does not reflect any interaction [32]. The coefficient e reflects the difference in disposition of the stationary and the mobile phases to interact with n- and π-electron pairs of the solutes, s is equal to the difference in dipolarity/polarizibility, a reflects the difference in hydrogen bond basicity, b refers to the difference in hydrogen bond acidity and the coefficient v represents the difference in hydrophobicity between the two phases.

This work is focused on characterization and comparison of interaction abilities of three different CF-based CSPs in normal-phase separation mode using the LFER model. These CSPs differ in the substituents and/or the CF core size. Effect of acidification of *n*-hexane/propane-2-ol mobile phase is also examined. Description of the interactions revealed by LFER can serve as a tool for prediction of analytes' retention. Although the application of the LFER model to chiral separations is not explicit, because no chiral term is involved in the equation, this approach can be useful for the estimation of the interactions participating in the enantiorecognition process.

2 Materials and methods

2.1 Chemicals and materials

Organic solvents of HPLC grade, *n*-hexane (hex), propane-2-ol (isopropanol, IPA) and methanol, were purchased from Sigma-Aldrich (Steinheim, Germany). Trifluoroacetic acid (TFA; 99.8% purity) was a product of Merck (Darmstadt, Germany). The test solutes for LFER were of analytical grade purity and were purchased from Sigma-Aldrich. The solutes with their descriptors are listed in Table 1.

Chromatographic columns (Larihc CF6-P, Larihc CF6-RN and Larihc CF7-DMP, AZYP, Arlington, TX, USA) which contain IP-CF6, RN-CF6 and DMP-CF7 with silica gel as a support were used in this study. The chiral selectors were bonded to the silica gel surface. IP-CF6 utilizes isopropyl carbamate CF6 as the chiral selector, RN-CF6 is *R*-naphthylethyl carbamate modified CF6 and DMP-CF7 represents 3,5-dimethylphenyl carbamate functionalized CF7. The dimensions of these columns were 250 mm × 4.6 mm id; particle size 5 µm. The synthesis procedure has been described previously [13].

The concentrations of stock solutions of solid and liquid samples were 1 mg/mL and 20 µL/mL, respectively. Methanol was used for preparation of all sample solutions.

2.2 HPLC method

All chromatographic measurements were carried out on two HPLC systems (Waters, Milford, USA): (i) Waters HPLC Breeze System consisting of HPLC Gradient Pump 1525, an autosampler 717Plus, a column heater Jetstream 2 Plus and a UV–Vis dual absorbance detector 2487, handled by Breeze software; (ii) Waters Alliance System with Waters 2695 Separation Module, Waters 2996 Photodiode Array Detector, an autosampler 717Plus, Waters Alliance Series column heater, controlled by the Empower software.

The temperature of the columns and samples was kept at 25°C. The injection volume was 10 µL and the flow rate was 1 mL/min. The detection was performed at 254 nm. Normal separation mode was chosen for the whole study due to higher selectivity reported in the literature [13, 14]. Mobile phases were composed of *n*-hexane and propane-2-ol 80:20 v/v, small addition of TFA was also tested. System peaks obtained by injection of *n*-hexane to the studied separation systems served for determination of the dead time.

Table 1. Set of test solutes, their solvation parameters and obtained retention data

Analyte	Solvation parameters					log *k*					
	E	*S*	*A*	*B*	*V*	Hex/IPA/TFA 80:20:0.0 v/v/v			Hex/IPA/TFA 80:20:0.5 v/v/v		
						RN-CF6	IP-CF6	DMP-CF7	RN-CF6	IP-CF6	DMP-CF7
Benzamide	0.99	1.50	0.49	0.67	0.973	0.550	0.634	0.674	0.533	0.609	0.665
2-Naphthol	1.52	1.08	0.61	0.40	1.144	−0.210	−0.123	−0.278	−0.176	−0.139	−0.253
Resorcinol	0.98	1.00	1.10	0.58	0.834	0.139	0.261	0.030	0.176	0.265	0.070
Benzophenone	1.45	1.50	0.00	0.50	1.481	−0.336	−0.348	−0.266	−0.330	−0.359	−0.286
Hydroquinone	1.00	1.00	1.16	0.60	0.834	0.245	0.362	0.143	0.277	0.392	0.185
1,2-Cresol	0.84	0.86	0.52	0.31	0.916	−0.379	−0.232	−0.485	−0.341	−0.246	−0.430
Benzonitrile	0.74	1.11	0.00	0.33	0.871	−0.199	−0.177	−0.150	−0.199	−0.198	−0.124
1,3-Cresol	0.82	0.88	0.57	0.34	0.916	−0.345	−0.203	−0.426	−0.311	−0.207	−0.378
Benzylalcohol	0.80	0.87	0.33	0.56	0.916	−0.147	−0.033	−0.093	−0.128	−0.038	−0.094
Benzene	0.61	0.52	0.00	0.14	0.716	−1.159	−0.728	−1.038	−1.022	−0.737	−1.004
Naphthalene	1.34	0.92	0.00	0.20	1.085	−0.889	−0.677	−0.804	−0.821	−0.699	−0.764
Pyrocatechol	0.97	1.07	0.85	0.52	0.834	−0.006	0.104	−0.080	0.030	0.121	−0.020
Dibenzothiophene	1.96	1.31	0.00	0.18	1.379	−0.670	−0.617	−0.499	−0.625	−0.617	−0.568
Ethylbenzene	0.61	0.51	0.00	0.15	0.998	−1.447	−0.822	−0.987	−1.229	−0.823	−1.262
Benzaldehyde	0.82	1.00	0.00	0.39	0.873	−0.284	−0.252	−0.212	−0.276	−0.260	−0.198
Toluene	0.60	0.52	0.00	0.14	0.857	−1.304	−0.785	−0.916	−1.137	−0.788	−1.103
1,2-Toluidine	0.97	0.92	0.23	0.45	0.957	0.910	0.918	1.041	1.562	[a]	1.629
Biphenyl	1.36	0.99	0.00	0.22	1.324	−0.925	−0.725	−0.667	−0.900	−0.726	−0.796
Phenanthrene	2.06	1.29	0.00	0.26	1.454	−0.666	−0.621	−0.553	−0.625	−0.623	−0.529
1,2,3-Trichlorobenzene	1.03	0.86	0.00	0.00	1.084	−0.775	−0.624	−0.682	−0.731	−0.633	−0.661
3-Nitrotoluene	0.87	1.10	0.00	0.25	1.032	−0.398	−0.380	−0.313	−0.379	−0.387	−0.360
1,2-Xylene	0.66	0.56	0.00	0.16	0.998	−1.377	−0.802	−1.196	−0.893	−0.812	−1.311
Bromobenzene	0.88	0.73	0.00	0.09	0.891	−0.971	−0.692	−0.872	−0.879	−0.691	−0.937
2-Nitrotoluene	0.87	1.11	0.00	0.27	1.032	−0.394	−0.366	−0.325	−0.376	−0.378	−0.341
1,3-Xylene	0.62	0.52	0.00	0.16	0.998	−1.482	−0.822	−1.300	−1.229	−0.841	−1.411
Chlorobenzene	0.72	0.65	0.00	0.07	0.839	−1.000	−0.697	−0.916	−0.910	−0.703	−0.966
1,4-Xylene	0.61	0.52	0.00	0.16	0.998	−1.495	−0.849	−1.334	−1.276	−0.838	−1.240
2-Chlorophenol	0.85	0.88	0.32	0.31	0.898	−0.355	−0.233	−0.464	−0.328	−0.233	−0.425
3-Chlorophenol	0.91	1.06	0.69	0.15	0.898	−0.335	−0.197	−0.465	−0.309	−0.204	−0.427
4-Chlorophenol	0.92	1.08	0.67	0.21	0.898	−0.294	−0.163	−0.418	−0.271	−0.172	−0.383
2-Nitrophenol	1.02	1.05	0.05	0.37	0.949	−0.358	−0.327	−0.202	−0.340	−0.329	−0.309
4-Nitrophenol	1.07	1.72	0.82	0.26	0.949	−0.038	0.025	−0.104	−0.026	0.034	−0.085
3-Hydroxybenzaldehyde	0.99	1.38	0.74	0.40	0.932	−0.032	0.004	−0.100	−0.052	0.020	−0.072
Acetone	0.18	0.70	0.04	0.49	0.547	−0.031	0.098	0.125	−0.024	0.090	0.094
Aniline	0.96	0.96	0.26	0.41	0.816	0.171	0.250	0.288	0.709	0.902	[a]
Anthracene	2.29	1.34	0.00	0.26	1.454	−0.670	−0.633	−0.571	−0.642	−0.636	−0.615
Tetrachlorobenzene	1.18	0.92	0.00	0.00	1.206	−0.824	−0.702	−0.585	−0.788	−0.709	−0.802
Pyrene	2.81	1.71	0.00	0.29	1.585	−0.567	−0.577	−0.458	−0.541	−0.581	−0.463
Caffeine	1.50	1.60	0.00	1.33	1.364	1.737	1.679	1.951	1.688	1.635	1.889
1,4-Toluidine	0.92	0.95	0.23	0.45	0.957	0.172	0.252	0.304	0.790	1.022	−0.570
Theophylline	1.50	1.60	0.54	1.34	1.222	1.130	1.125	1.351	1.131	1.121	1.309
Thymine	0.80	1.00	0.44	1.83	0.893	0.841	0.927	0.920	0.855	0.946	0.990
Ethylacetate	0.11	0.62	0.00	0.45	0.747	−0.815	−0.671	−0.850	−0.849	−0.692	−0.863
Uracil	0.81	1.00	0.44	1.00	0.752	1.027	1.181	1.222	1.038	1.191	1.235
Phenol	0.81	0.89	0.60	0.30	0.775	−0.304	−0.179	−0.391	−0.268	−0.177	−0.343

a) No elution within 300 min.

2.3 LFER procedure

The regression coefficients of the LFER equation were obtained from a series of measurements of the retention times of the set of 44 solutes with known solvation parameters [27, 28, 31] that are summarized in Table 1. The retention times were measured in triplicates and from these data retention factors were calculated. The resulting regression coefficients were obtained for each separation system by multiple linear regression analysis of log *k* against solutes' descriptors using the NCSS software (Kaysville, USA) [33].

3 Results and discussion

The LFER model is often used for characterization and comparison of separation systems. This approach can describe individual molecular interactions participating in the retention and separation process. In this work, the LFER method was applied to the systems with three different CF-based CSPs under two mobile-phase compositions, i.e. six separation systems were investigated. A set of 44 analytes of different properties, i.e. with different descriptors, was used for the measurements. The retention data ($\log k$) are displayed in Table 1.

Table 2 summarizes the LFER data calculated for the investigated separation systems. Plots of the experimental values of $\log k$ against the calculated/predicted $\log k$ values of the test solutes show linear dependencies with correlation coefficients higher than 0.95 in all cases. That indicates strong correlation of the LFER model with the experimental data. The optimal model was chosen for the comparison of the individual separation systems (see Fig. 2) because it includes only significant interactions while the complete model involves all the interactions no matter what their statistical significance is.

The dominant contribution to retention is described by the coefficient *b*, which reaches the highest values in all the systems investigated. This coefficient represents the difference between the stationary and the mobile phases in hydrogen bond-donating properties. Positive values

Table 2. Regression coefficients of the LFER equation and correlation coefficient *R*

Column	Mobile phase	Model	*v*	*a*	*b*	*s*	*e*	*c*	*R*
RN CF6	Hex/IPA/TFA	C.M.	−1.623	−0.035	1.669	0.995	0.278	−0.659	0.957
	80:20:0.0	±95% CI	0.704	0.247	0.312	0.364	0.328	0.475	
	v/v/v	*p*	0.000	0.776	0.000	0.000	0.092	0.008	
		O.M.	**−1.169**	**x**	**1.596**	**1.128**	**x**	**−0.944**	**0.953**
		±95% CI	0.382		0.302	0.298		0.307	
		p	0.000		0.000	0.000		0.000	
IP CF6	Hex/IPA/TFA	C.M.	−0.849	0.040	1.571	0.492	0.001	−0.423	0.963
	80:20:0.0	±95% CI	0.530	0.194	0.219	0.284	0.253	0.358	
	v/v/v	*p*	0.003	0.675	0.000	0.001	0.991	0.022	
		O.M.	**−0.887**	**x**	**1.573**	**0.518**	**x**	**−0.401**	**0.963**
		±95% CI	0.282		0.211	0.228		0.232	
		p	0.000		0.000	0.000		0.001	
DMP CF7	Hex/IPA/TFA	C.M.	−1.252	−0.222	1.781	0.835	0.211	−0.710	0.954
	80:20:0.0	±95% CI	0.737	0.263	0.289	0.368	0.329	0.499	
	v/v/v	*p*	0.002	0.096	0.000	0.000	0.203	0.007	
		O.M.	**−0.703**	**x**	**1.729**	**0.803**	**x**	**−1.048**	**0.950**
		±95% CI	0.391		0.287	0.305		0.315	
		p	0.001		0.000	0.000		0.000	
RN CF6	Hex/IPA/TFA	C.M.	−1.019	0.092	1.556	0.759	0.130	−0.823	0.967
	80:20:0.5	±95% CI	0.623	0.216	0.237	0.299	0.275	0.421	
	v/v/v	*p*	0.002	0.395	0.000	0.000	0.342	0.000	
		O.M.	**−0.918**	**x**	**1.535**	**0.891**	**x**	**−0.892**	**0.965**
		±95% CI	0.316		0.231	0.244		0.254	
		p	0.000		0.000	0.000		0.000	
IP CF6	Hex/IPA/TFA	C.M.	−0.651	0.178	1.480	0.467	0.014	−0.654	0.961
	80:20:0.5	±95% CI	0.600	0.212	0.228	0.293	0.266	0.411	
	v/v/v	*p*	0.034	0.097	0.000	0.003	0.916	0.003	
		O.M.	**−0.821**	**x**	**1.486**	**0.594**	**x**	**−0.553**	**0.957**
		±95% CI	0.311		0.226	0.242		0.254	
		p	0.000		0.000	0.000		0.000	
DMP CF7	Hex/IPA/TFA	C.M.	−1.100	−0.099	1.654	0.910	0.153	−0.900	0.958
	80:20:0.5	±95% CI	0.731	0.259	0.278	0.358	0.325	0.500	
	v/v/v	*p*	0.004	0.445	0.000	0.000	0.347	0.001	
		O.M.	**−0.765**	**x**	**1.622**	**0.927**	**x**	**−1.110**	**0.956**
		±95% CI	0.368		0.267	0.287		0.300	
		p	0.000		0.000	0.000		0.000	

CI represents ±95% confidence interval; x, insignificant interaction; C.M., complete model of the LFER equation; O.M., optimal model of the LFER equation; *p*, statistical *p*-value. The *p*-values express probability of the error that the individual coefficient does not contribute to the model, i.e., *p*-values represent the significance of the individual coefficients.

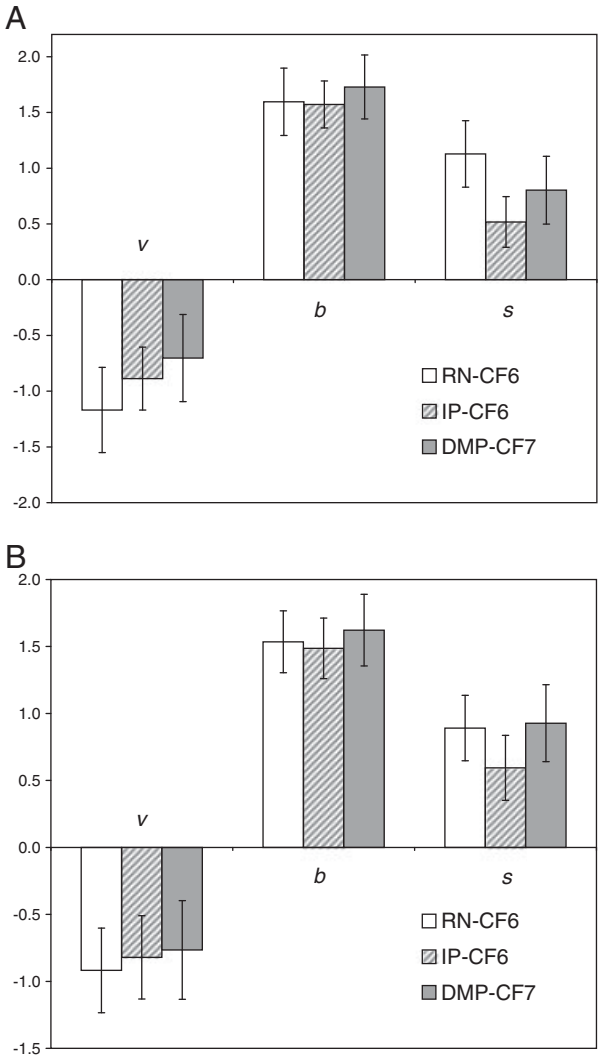

Figure 2. Comparison of the regression coefficient values (with their standard errors) obtained from the optimal LFER models for the three CF-based CSPs in the mobile phases: (A) hex/IPA/TFA 80:20:0.0 v/v/v; (B) hex/IPA/TFA 80:20:0.5 v/v/v.

of the coefficient *b* denote that this type of interaction is preferred in the stationary phase. While hydrogen donating groups are available on the stationary phases they are not present in the mobile phase composed of hex/IPA. Addition of a low amount of TFA to the mobile phase causes small decrease of the *b*-values in the separation systems with any of the CF-based CSPs because the acidified mobile phase can contribute to the H-donating interactions. Comparison of the acidity of the individual columns evidenced the sequence IP-CF6 $\leq$ RN-CF6 $<$ DMP-CF7. The difference in the H-donating properties between the two CF6 columns (RN-CF6 and IP-CF6) is very small, the stationary phases under the concerned conditions are comparable from the point of their acidity. Owing to its larger basic structure (core), DMP-CF7 offers more derivatization (hydrogen donating) groups, and so its ability to interact via H-bonds is the highest.

The regression coefficient *a* is statistically insignificant in all the studied systems. It means that the hydrogen bond basicity (ability to accept protons) of the stationary and the mobile phases is comparable.

The regression coefficient *v*, reflecting the difference in hydrophobicity between the stationary and the mobile phases, is negative in all cases. This is consistent with the applied normal separation mode where, in principle, the mobile phase is less polar than the stationary phase. The lowest absolute values of coefficient *v* were obtained for the systems with DMP-CF7 column. Based on these results this stationary phase can be considered the most non-polar from the three tested columns. Comparing the hydrophobicity of the two CF6 columns that have the same core size, much smaller isopropyl derivatization group has better accessibility to the OH groups of CF while the derivatization is more difficult with the bigger naphthylethyl substituent. Owing to steric reasons more underivatized OH groups remain on the CF in the latter case and increase the polarity of the RN-CF6 stationary phase. Therefore, the biggest difference in hydrophobicity between the stationary and the mobile phases (i.e. the highest absolute value of coefficient *v*) was observed for the systems with RN-CF6 CSP. By addition of TFA the mobile phase becomes more polar, so the difference in hydrophobicity between the stationary and the mobile phases is reduced and the absolute values of coefficient *v* can decrease. This fact is significant mainly for the RN-CF6 column. The absolute value of coefficient *v* for the system with DMP-CF7 slightly increases after the addition of TFA. The properties of the stationary phase can be affected by sorption of the components of the mobile phase [34].

The difference between the stationary and the mobile phase dipolarity/polarizibility is described by the coefficient *s*, which is positive for all the investigated separation systems. Polarizibility of the attached derivatization groups clearly increases in the sequence: isopropyl- $<$ dimethylphenyl- $<$ naphthylethyl-. In a more rigorous approach, the core size of CF must also be considered. Addition of TFA to the mobile phase affects the *s* values of IP-CF6 and DMP-CF7, and RN-CF6 in a different way. While the dipolarity/polarizibility coefficients increased for the two former columns lower *s* value was obtained for the latter CSP. The interaction of the acid with the stationary phase (CF) takes place most easily if CF is substituted with the biggest naphthylethyl group, which is sterically less convenient to reach the CF basic structure.

The *e* coefficient describing the difference in the propensity of the stationary and the mobile phases to interact with *n*- and π-electron pairs of the solute is statistically insignificant for all studied separation systems. This denotes that the ability of the stationary and the mobile phases to participate in this type of interactions is comparable.

The results of LFER indicate that significant interactions affecting retention in all separation systems studied in this work are the same, namely H-donating interactions and dipolarity/polarizibility (with positive regression coefficient values) and hydrophobicity (with negative values).

The LFER approach characterizes the prevailing interactions in the separation system but it does not relate to information on enantioselective behavior of analytes. Based on the LFER results retention of analytes can be estimated or even predicted if molecular descriptors are known. Concerning enantioseparation the LFER results indicate that analytes should offer H-accepting groups and polarizable moieties near the stereogenic center and low hydrophobicity.

4 Concluding remarks

The LFER model was used to describe interactions participating in the retention and separation process on the newly developed CF-based CSPs RN-CF6, IP-CF6 and DMP-CF7 in normal separation mode. Although LFER does not take into account chirality and spatial arrangement of analytes, it proved which forces take part in the interaction mechanism. The same types of interactions in a different extent were shown to be preferred by all three stationary phases, i.e. hydrogen bond acidity and dipolarity/polarizibility. Also the effect of hydrophobicity as the retention reducing factor plays a role with all tested CF-based CSPs. Hydrogen bond basicity and interactions with *n*- and π-electron pairs seemed to be insignificant. Some differences of the concerned stationary phases due to different CF core size and/or the substituents were shown by the LFER model.

The Grant Agency of the Charles University in Prague, projects no. 356411 and SVV 261204, the Ministry of Education, Youth and Sports of the Czech Republic, projects Kontakt LH11018, Centrum 1M06011, RP 14/63 and the long-term project MSM0021620857 are gratefully acknowledged for the financial support.

The authors have declared no conflict of interest.

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5.2 Publikace III - Srovnání enantioselektivního potenciálu cyklofruktanových a cyklodextrinových chirálních stacionárních fází

Model LFER poskytuje cenné informace o interakcích nabízených stacionárními fázemi. Nicméně ve své základní podobě neobsahuje člen, který by popisoval sterické vlivy. Základní objasnění vlivu prostorového uspořádání cyklofruktanových CSP bylo provedeno v **Publikaci III** srovnáním se známějšími CSP na bázi derivatizovaných cyklodextrinů v podmínkách NP HPLC. Celkem byly vzájemně porovnávány čtyři komerčně dostupné CSP. DMP-CF7 CSP tvořená sedmi fruktofuranosovými jednotkami derivatizovanými dimethylfenyl karbámátovými skupinami byla srovnána s DMP-CD CSP, jejímž základem je cyklodextrin tvořený sedmi glukopyranosovými jednotkami se stejnou derivatizační skupinou. Druhým srovnávaným párem byly RN-CF6 CSP a RN-CD CSP, které mají stejnou derivatizační skupinu, *R*-naftylethyl karbamát, ale liší se počtem sacharidových jednotek. Cyklofruktanová CSP je v tomto páru tvořena šesti fruktofuranosovými jednotkami, zatímco cyklodextrinovou CSP tvoří sedm glukopyranosových jednotek. Sadu srovnávacích analytů tvořily binaftol a jeho deriváty, které vykazují axiální chiralitu, a dále tři páry strukturně odlišných sloučenin s centrální chiralitou. Na základě změny retenčního/enantioseparačního chování látek v páru lze vyvodit roli dané funkční skupiny v retenčním/enantiodiskriminačním mechanizmu.

Význam odlišného oligosacharidového makrocyklu lze jasně demonstrovat na separacích atropizomerů derivatizovaných binaftolů. Na DMP-CF7 CSP bylo dosaženo úplného (hodnota rozlišení $Rs > 1{,}5$) či částečného chirálního rozdělení pro pět z osmi binaftolových derivátů. Při použití DMP-CD CSP došlo k separaci dvou párů atropizomerů, z toho jednoho částečně a druhého na základní linii. Z výsledků jasně vyplývá, že sacharidový skelet není pouze pasivním nosičem derivatizačních skupin, ale významným způsobem se podílí na enantioselektivitě.

Retenční chování párů vybraných analytů potvrdily LFER výsledky, které objasnily příspěvky jednotlivých funkčních skupin analytů k retenci. Příkladem může být pár oxprenolol/alprenolol na DMP-CF7 CSP, i přes skutečnost, že nedošlo k enantioseparaci zmíněných analytů na této CSP. Oxprenolol vykazoval násobně vyšší retenci oproti alprenololu. Příčinou je přítomnost další etherové skupiny

v molekule oxprenololu oproti alprenololu. Tato skupina působí jako akceptor vodíku při tvorbě vodíkových vazeb (statisticky významná interakce viz podkapitola 5.1), tedy významným způsobem zvyšuje retenci. Etherová skupina je však vzdálena chirálnímu centru, a proto odpovídající interakce nemá enantiodiskriminační charakter. Jiným příkladem může být dvojice analytů BP34/BP766. Molekula BP34 má volnou amino skupinu lokalizovanou blízko chirálního centra, zatímco v molekule BP766 je tato amino skupina blokována trifluoroacetylovou skupinou. Amino skupina slouží jako donor i akceptor vodíku při tvorbě vodíkových vazeb. Dle výsledků LFER je však pro retenci na cyklofruktanových CSP významnější tento substituent v roli H-akceptoru. BP34 vykazoval ve všech testovaných systémech velmi vysoké retence. Přídavkem TFA do separačního systému byly interakce amino skupiny omezeny, nicméně si zachovaly enantiodiskriminační charakter, a proto došlo k částečnému rozdělení enantiomerů BP34, zatímco enantiomery BP766 nebyly na DMP-CF7 CSP rozděleny.

Srovnání RN-CF6 CSP a RN-CD CSP také prokázalo mimořádnou selektivitu cyklofruktanových CSP pro separace derivátů binaftolu. Přestože tyto analyty dosahovaly vyšších retencí na cyklodextrinové CSP, bylo na této fázi dosaženo pouze jedné částečné separace, zatímco na RN-CF6 CSP bylo separováno (částečně nebo na základní linii) pět z osmi atropizomerních párů. Stejné trendy pro dvojice analytů oxprenolol/alprenolol a BP34/BP766 získané na DMP-CF7 CSP byly pozorovány i v separačním systému s RN-CF6 CSP. Enantiomery analytu BP34 byly separovány na základní linii. Pro enantiomery oxprenololu bylo dosaženo v použitých mobilních fázích částečné separace.

Souhrnem lze konstatovat, že cyklofruktanové fáze prokázaly obecně vyšší separační potenciál pro binaftolové deriváty než cyklodextrinové CSP. Separace na porovnávaných CSP na bázi cyklodextrinů a cyklofruktanů vykazovaly určitou komplementaritu.

Publikace III

Chiral HPLC Separation on Derivatized Cyclofructan *versus* Cyclodextrin Stationary Phases

Vozka J., Kalíková K., Janečková L., Armstrong D. W., Tesařová E.

Analytical Letters **2012**, 45, 2344–2358

Analytical Letters, 45: 2344–2358, 2012

ISSN: 0003-2719 print/1532-236X online
DOI: 10.1080/00032719.2012.686128

Chromatography

CHIRAL HPLC SEPARATION ON DERIVATIZED CYCLOFRUCTAN VERSUS CYCLODEXTRIN STATIONARY PHASES

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Cyclodextrins (CDs) and cyclofructans (CFs) are chiral cyclic oligosaccharides. While β-CD is composed of seven glucopyranose units forming rigid cavity, hydrophobic inside, CF6 and CF7, contain six and seven fructofuranose units, respectively, creating a polar crown ether core. These basic structures can be easily derivatized to form even more potential chiral selectors that enable enantioselective separation of various chiral compounds. Chiral stationary phases (CSPs) based on CFs and CDs that were derivatized with the same derivatization group, either dimethylphenyl or R-naphthylethyl, were compared. A set of analytes with different interaction possibilities was used for characterization of retention and enantioseparation abilities of these CSPs in normal separation mode of HPLC. The results showed that both cyclic oligosaccharide structure and derivatization group influenced the retentionlseparation behavior of analytes. Complementary enantioseparations were obtained for some analytes.

Keywords: Chiral separation; Cyclodextrin; Cyclofructan; HPLC

INTRODUCTION

Ignorance of different biological activities of individual enantiomeric forms has caused many serious problems in the past. Rapid development of scientific

Received 20 January 2012; accepted 12 April 2012.

The Ministry of Education, Youth and Sports of the Czech Republic, Project Kontakt AM 2010 LH11018; the Grant Agency of the Charles University in Prague, Project No. 356411; and the long-term project MSM0021620857 are gratefully acknowledged for the financial support.

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knowledge of diverse metabolic paths of enantiomers caused an increased demand for enantioseparation methods, applicable especially in drug, agrochemical, and food industries.

High performance liquid chromatography (HPLC), utilizing chiral stationary phases (CSPs), is one of the most powerful and widely used techniques for separation of enantiomers at both the analytical and preparative scales. Many different CSPs have been designed during the years (Lämmerhofer 2010; Cavazzini et al. 2011). However, researchers still continue to develop new CSPs that would be more versatile.

In 1984 Armstrong and DeMond introduced cyclodextrin (CD) CSPs in HPLC; 25 years later, cyclofructan (CF)-based chiral stationary phases were used by the same research group for the first time (Sun et al. 2009). Both CDs and CFs are macrocyclic oligosaccharides. While CDs commonly used in separation science consist of six, seven, or eight α-(1,4) linked D-glucopyranose units (abbreviations: α-CD, β-CD, and γ-CD, respectively), molecules of CF consist preferentially of six, seven, or eight D-fructofuranose units (abbreviations: CF6, CF7, and CF8, respectively) connected by β-(2,1) linkage (Sawada et al. 1991; Immel, Schmitt, and Lichtenthaler 1998; Sun et al. 2009). Until now, only CF6 and CF7 and their derivatives have found application in separation procedures according to the literature data (Sun and Armstrong 2010; Sun et al. 2011; Cavazzini et al. 2011). Different saccharide units, along with their bounding chemistry, result in different spatial arrangements that can yield diverse enantioseparation behavior. CDs possess hydrophobic cavities with inner diameters increasing from 0.57 nm for α-CD to 0.95 nm for γ-CD (Beesly and Scott 1998). CFs have much smaller cavity with inner diameters ranging from 0.23 nm for CF6 to 0.47 nm for CF8 (Sun et al. 2009). Additionally, fructofuranose units of CF form crown-ether skeletons. Therefore, a CF cavity is not hydrophobic due to the presence of core crown oxygen is folded almost inside the molecule (Sun et al. 2009).

Basic separation principles on CD-CSPs and application in enantioseparation have been shown and discussed in many reviews and research articles (e.g., Cavazzini et al. 2011; Lämmerhofer 2010; Qiu, Liang, et al. 2011; Zhou et al. 2010; Tang and Ng 2008; Remsburg et al. 2008; Muderawan, Ong, and Ng 2006; Han 1997). On the other hand CF-CSPs have been introduced quite recently, in 2009 (Sun et al. 2009). Therefore, only a few papers have been published dealing with their use in enantioselective separation (e.g., Cavazzini et al. 2011; Kalíková, Riesová, and Tesařová 2011).

The CF-CSPs, like CD-CSPs, were proved to be multimodal, that is, they can be used under normal-phase mode (NP), reversed-phase mode (RP), and polar-organic mode (PO). However, better separations with CF-CSPs can be achieved in NP or PO mode. Characterization of interactions participating in separation process on CF-CSPs was performed by Tesařová and colleagues. Comparison of three CF-based CSPs, namely isopropyl carbamate CF6, *R*-naphtylethyl carbamate CF6, and dimethylphenyl carbamate CF7 CSPs was based on a linear free energy relationship model (Janečková et al. 2011). Furthermore, the same model was used to evaluate the differences and/or similarities between *R*-naphtylethyl carbamate CF6-CSP and *R*-naphtylethyl carbamate β–CD-CSP (Kalíková, Janečková, et al. 2011). Evaluation of CF-CSPs in terms of enantioselectivity was carried out for aromatic-derivatized CFs (*R*-naphtylethyl carbamate CF6-CSP,

dimethylphenyl carbamate CF7) (Sun et al. 2011) and for aliphatic-derivatized CF (isopropyl carbamate CF6) (Sun and Armstrong 2010; Aranyi et al. 2011).

Recently, underivatized and sulfonated CFs have demonstrated interesting separation properties in hydrophilic interaction liquid chromatography (HILIC) for achiral separation of polar compounds (Qiu, Loukotková, et al. 2011; Padivitage and Armstrong 2011).

The aim of the present work is to investigate and compare retention and enantioselective separation potential of CF-CSPs vs. CD-CSPs in HPLC under normal-phase conditions. Two pairs of CSPs were chosen for comparison: (1) Dimethylphenyl carbamate cyclofructan 7 (DMP-CF7) and dimethylphenyl carbamate β-cyclodextrin (DMP-CD), both containing the same derivatization group and the same number of saccharide units; and (2) *R*-naphtylethyl carbamate cyclofructan 6 (RN-CF6) and *R*-naphtylethyl carbamate β-cyclodextrin (RN-CD), both CSPs containing the same derivatization group but CD having an additional saccharide unit. Figure 1 shows the structures of chiral selectors studied in this work. These CSPs are evaluated by injection of structurally different chiral compounds of practical use with various functional groups, that is, binaphthyl derivates used as catalysts for asymmetric synthesis and chiral compounds from diverse groups of pharmaceuticals. Retention and enantiorecognition processes were considered, selectivity and resolution potential of the tested columns were compared.

EXPERIMENTAL

Chemicals and Materials

Organic solvents of HPLC grade, *n*-hexane (hex), propane-2-ol (isopropanol, IPA) and methanol were purchased from Sigma-Aldrich (Steinheim, Germany).

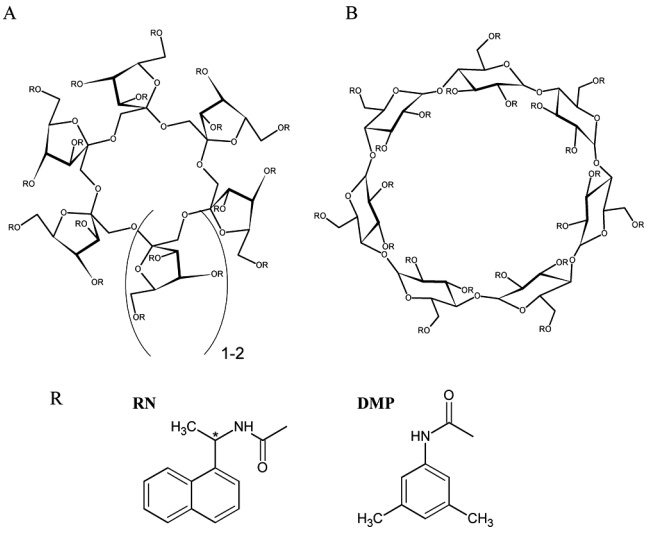

Figure 1. The molecular structures of the cyclofructan skeleton (A), β-cyclodextrin skeleton (B), and derivatization groups (R) studied in this work. RN means *R*-naphtylethyl carbamate and DMP stands for dimethylphenyl carbamate.

Trifluoroacetic acid (TFA) of 99.8% purity was product of Merck (Darmstadt, Germany). Binaphthyl derivatives were synthesized as racemates at the Department of Organic and Nuclear Chemistry, Faculty of Science, Charles University in Prague, according to previous research (Vyskočil et al. 2002; Kočovský, Vyskočil, and Smrčina 2003; Loukotková et al. 2008). Oxprenolol was purchased from EDQM (Strasbourg, France). BP 766 (Fluridil) and its hydrolytic decomposition product BP 34 were synthesized at the University of California, Radiology Research (San Diego, USA) (Sovák et al. 2002). Other tested chiral compounds, all of p.a. purity, were products of Sigma-Aldrich (Steinheim, Germany).

Chromatographic columns based on derivatized cyclofructans Larihc CF6-RN, Larihc CF7-DMP that contained RN-CF6 and DMP-CF7 CSs, respectively, and immobilized on silica gel support were obtained from AZYP (Arlington, TX, USA). Cyclobond I 2000 DMP and Cyclobond I 2000 RN with DMP-CD and RN-CD CSs, respectively, were products of Astec (Whippany, NJ, USA). The dimensions of all these columns were 250 mm × 4.6 mm i.d.; particle size 5 μm. The concentrations of stock solutions of the samples were 1 mg/mL. Methanol was used as sample solvent.

HPLC Method

All chromatographic measurements were carried out on HPLC system Waters Alliance System with Waters 2695 Separation Module, Waters 2996 Photodiode Array Detector, an autosampler 717 Plus, Waters Alliance Series column heater, controlled by Empower software.

Temperature of the columns and samples was kept at 25°C. The injection volume was 10 μL and flow rate was 1 mL/min. The detection was performed at 254 nm in most cases. The wavelengths of the absorption maxima were used for detection of certain analytes. System peaks obtained by injection of *n*-hexane to the studied separation systems served for determination of the void volume.

RESULTS AND DISCUSSION

Four CSPs (DMP-CF7, DMP-CD, RN-CF6, and RN-CD CSPs) were tested and compared in terms of retention and enantioselectivity. As better selectivity has been reported for CF-based CSPs in NP mode than in RP or PO modes, NP mode was chosen in this study (Sun et al. 2009; Sun and Armstrong 2010). Mobile phases were composed of *n*-hexane and propane-2-ol in various volume ratios (*v*/*v*), that is, 60/40 and 80/20; small additions of TFA were also tested. The separation performance of CF-CSPs as well as that of CD-CSPs is dependent on the nature and spatial arrangement of the oligosaccharide molecules and on the type and degree of their substitution (Sun et al. 2009). These features are crucial in the enantiorecognition mechanism. Pairs (groups) of compounds with closely related structures were chosen as analytes because subtle difference in the structure of an analyte can cause substantial difference of retention/separation behavior. Each analyte possesses one or more chemical groups (e.g., amino group, hydroxyl group), which we consider to be suitable for various interactions with chiral selectors. Our consideration is based on our previous studies of interaction mechanisms of these CSPs (Kalíková, Janečková,

et al. 2011; Janečková et al. 2011). Regarding the structure of the CSs, a set of binaphthyl derivatives (see Fig. 2) was also chosen. These analytes appear to be well suited for interactions with the aromatic moieties of the chiral selectors. Despite the similar basic axial chirality of the binaphthyl derivatives, the type and positions of the substituents can substantially affect their properties and chromatographic behavior (Han et al. 2007; Loukotková et al. 2008; Loukotková et al. 2010). Other compounds tested were pharmaceuticals BP 766 and its hydrolytic decomposition product BP 34; β-blockers, namely oxprenolol and alprenolol; and amlodipine with nitrendipine (see Fig. 3). All couples show certain differences in their structures.

Comparison of DMP-CF7 CSP and DMP-CD CSP

DMP-CF7 and DMP-CD CSPs contain the same derivatization groups except the oligosaccharide structures, and therefore the spatial arrangement of these CSs is different. Large molecules with a number of functional groups (i.e., binaphtyl derivatives) can serve as sensitive markers of the impact of the different spatial arrangement of these CSs on retention and resolution. The chromatographic data values obtained in the mobile phases composed of hex/IPA 80/20 (v/v) and hex/IPA/TFA 80/20/0.5 ($v/v/v$) are summarized for binaphthyl derivatives in Table 1.

DMP-CF7 CSP retained almost all the analyzed binaphthyls more strongly than DMP-CD CSP in all mobile phases used. Additionally, the derivatized CF molecule seems to be more sensitive to the structural differences among binaphthyl atropoisomers than the derivatized CD molecule under the NP mode conditions (see Table 1). Therefore, DMP-CF7 CSP showed higher selectivity for the individual

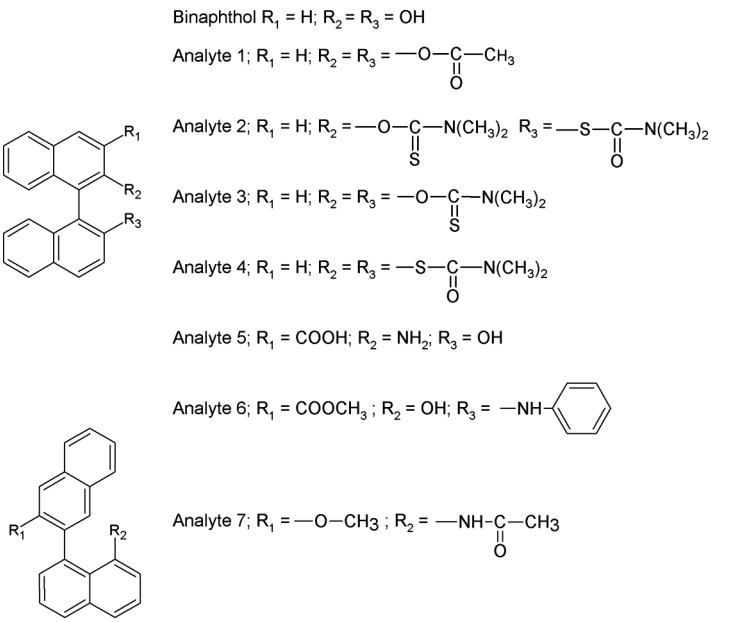

Figure 2. The structures of binaphthyl derivates.

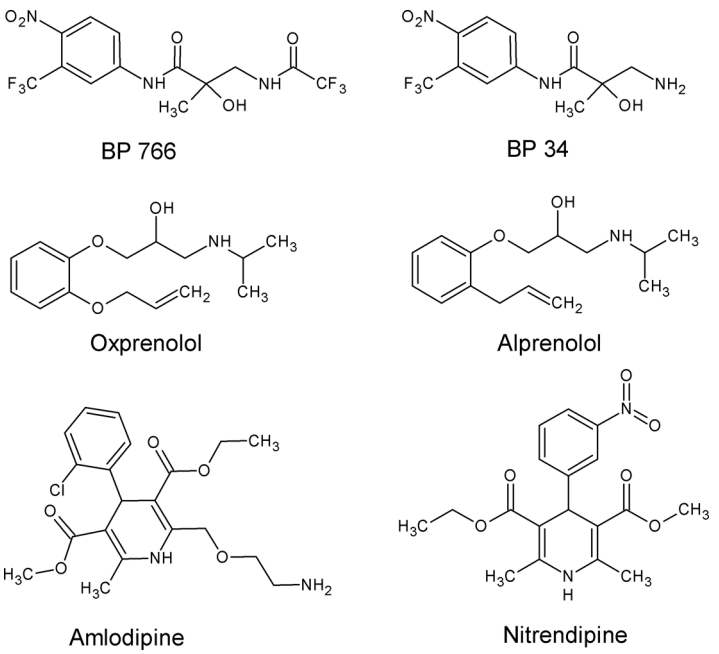

Figure 3. The structures of the other chiral compounds studied in this work.

Table 1. Chromatographic data of the binaphthyl derivates separated on the DMP-CF7 CSP and DMP-CD CSP

	Mobile phase:					
	hex/IPA/TFA 80/20/0.0 (*v*/*v*/*v*)			hex/IPA/TFA 80/20/0.5 (*v*/*v*/*v*)		
	k_1	α	*R*	k_1	α	*R*
DMP-CF7 CSP Analyte						
Binaphthol	1.47	1.43	3.44	1.69	1.35	3.59
1	1.14	1.04	0.50	1.11	1.05	0.62
2	2.46	1.27	2.95	2.34	1.30	3.11
3	1.45	2.65	10.73	1.50	2.51	9.49
4	7.40	1.00	0.00	5.65	1.00	0.00
5	12.35	1.00	0.00	1.87	1.00	0.00
6	1.22	1.03	0.39	1.16	1.10	1.14
7	4.83	1.00	0.00	4.13	1.00	0.00
DMP-CD CSP Analyte						
Binaphthol	1.72	1.09	0.71	1.71	1.09	0.76
1	0.81	1.00	0.00	0.70	1.00	0.00
2	2.13	1.00	0.00	1.94	1.00	0.00
3	1.31	1.00	0.00	1.18	1.00	0.00
4	5.74	1.00	0.00	4.82	1.00	0.00
5	11.23	1.00	0.00	1.68	1.00	0.00
6	1.06	1.00	0.00	0.96	1.00	0.00
7	4.21	1.29	1.57	3.63	1.27	1.86

Note. k_1, retention factor of the first eluted atropoisomer; α, selectivity; *R*, resolution.

atropoisomeric pairs. Baseline and/or partial chiral separations of binaphthol, analyte 1, analyte 2, analyte 3, and analyte 6 were achieved on DMP-CF7 CSP in all mobile phases tested. However, on DMP-CD CSP, only analyte 7 was baseline resolved and binaphthol was partly separated in all mobile phases. Analyte 6 was partly resolved only on DMP-CF7 CSP in mobile phases composed of hex/IPA 60/40 (*v*/*v*) or hex/IPA/TFA 60/40/0.5 (*v*/*v*/*v*). The results indicate that DMP-CF7 CSP is more suitable for chiral separation of binaphthyl derivatives than DMP-CD CSP. An interesting trend in retention and separation can be observed for analytes 2, 3, and 4. The retention decreases in the same sequence, that is, analyte 4> analyte 2> analyte 3, on both DMP-CF7 and DMP-CD CSPs. However, resolution of the atropoisomers that can be achieved only on the CF-based CSP has just the opposite trend. The resolution decreases in the sequence: analyte 3> analyte 2> analyte 4; thus, higher retention does not yield better chiral separation. This result confirms the known fact that it is not the absolute strength of the interaction, but the difference of the interaction forces that is important for chiral resolution. All the aforementioned analytes (analytes 2, 3, and 4) have hydrogen atom in the R1 position and similar derivatization groups in R2 and/or R3 positions on the binaphthyl skeleton (see Fig. 2). The carbonyl group in the close vicinity to secondary amino group of the analytes (analyte 4 has two carbonyl groups and analyte 2 has one carbonyl group) strongly contributes to retention but does not have positive impact on resolution. The importance of the secondary amino group for retention confirms analyte 1 does not have this group and exhibits the lowest retention on both CSPs in the corresponding mobile phases.

Binaphthol, possessing two OH groups (in R2 and R3 positions), is a good indicator of H-bonding interactions with the stationary phases. DMP-CF7 CSP resolves atropoisomers of binaphthol easily; baseline separation of this analyte was achieved in all mobile phases studied. On the other hand, only partial separation of binaphthol was obtained on DMP-CD CSP in hex/IPA 80/20 (*v*/*v*) and hex/IPA/TFA 80/20/0.5 (*v*/*v*/*v*) mobile phases (see Table 1). Analyte 5 was highly retained by both columns in the mobile phases without TFA. However, addition of TFA caused a dramatic decrease of its retention because TFA, as an ion-pairing agent, hampers the H-bonding interactions between the analyte with accessible ionizable groups (carboxylic group, amino group) and the chiral selector or residual silanol groups of the silica gel surface. No enantioseparation of analyte 5 was observed on DMP-CF7 or on DMP-CD CSPs.

Despite quite similar retention of analyte 6 on both CSPs in the corresponding mobile phases, DMP CF7 CSP was able to resolve, at least partly, atropoisomers of analyte 6 while the CD-based column was not. Opposite results were obtained for analyte 7, which was separated only on DMP CD CSP (see Table 1).

In general, DMP-CF7 CSP performed good separation capabilities and yielded some excellent chiral resolutions of binaphthyl derivatives, while DMP-CD CSP showed rather limited ability for separation of this type of analytes, with exception of analyte 7. DMP-CF7 CSP can be considered suitable for the chiral separation of binaphthyl derivates because their molecules seem to be compatible with the spatial arrangement of the derivatized CF. Overall, addition of TFA to the mobile phase had almost negligible effect on retention of the majority of analytes on the both columns; slightly reduced retention was mostly accompanied by somewhat increased

resolution in the acidified mobile phase. Only the retention of analyte 5 (with ionizable groups) was substantially influenced by the addition of TFA.

Pairs of compounds that can reveal the role of H-bonding interactions in the retention mechanism of the compared CSPs are pharmaceuticals BP 34 and BP 766. They differ in the presence of an underivatized amino group in the molecule of BP 34; whereas, BP 766 contains a trifluoracetylated secondary amino group (see Fig. 3). The compared CSPs show similar retention behavior but different/opposite selectivity with regard to these analytes (see Table 2). DMP CF7 CSP showed extremely high affinity for analyte BP 34, no elution was achieved in mobile phases without TFA within three hours. Partial separation was observed only in mobile phases containing the acid, that is, hex/IPA/TFA 60/40/0.5 and 80/20/0.5 ($v/v/v$),

DMP-CD CSP retarded BP 34 less than DMP-CF7 CSP, but the retention was still high. The free amino group of BP 34 contributes to retention on these CSPs but has rather limited enantioselective potential.

Retention of BP 766 is substantially reduced, as compared to BP 34, on the both tested CSPs. No enantioseparation of BP 766 was obtained on DMP-CF7 CSP but, surprisingly, DMP-CD CSP baseline separated enantiomers of analyte BP 766 in all mobile phases was used. The best value of enantioresolution, $R = 3.79$, was achieved in the mobile phase composed of hex/IPA/TFA 80/20/0.5 ($v/v/v$). Illustrative chromatograms obtained for BP 766 on the compared columns are depicted in Figure 4. Low retention, symmetrical peak, and no separation of enantiomers appeared on DMP-CF7 CSP while higher retention, good enantioseparation, but worse peak shape resulted from the DMP-CD CSP. The interactions that replaced H-bonding between the DMP-CD CSP and the primary amino group

Table 2. Chromatographic data of chiral compounds separated on the DMP-CF7 CSP and DMP-CD CSP

	Mobile phase:					
	hex/IPA/TFA 60/40/0.0 ($v/v/v$)			hex/IPA/TFA 60/40/0.5 ($v/v/v$)		
	k_1	α	R	k_1	α	R
DMP-CF7 CSP Analyte						
BP 766	0.58	1.00	0.00	0.63	1.00	0.00
BP 34	n.e.	–	–	7.25	1.26	0.88
Alprenolol	5.45	1.00	0.00	3.65	1.00	0.00
Oxprenolol	11.98	1.00	0.00	9.25	1.00	0.00
Amlodipine	19.41	1.00	0.00	16.49	1.00	0.00
Nitrendipine	0.64	1.00	0.00	0.67	1.00	0.00
DMP-CD CSP Analyte						
BP 766	1.22	1.74	2.19	1.15	1.81	2.19
BP 34	8.00	1.00	0.00	2.86	1.00	0.00
Alprenolol	2.56	1.11	0.56	1.06	1.14	1.02
Oxprenolol	6.49	1.00	0.00	1.93	1.12	0.52
Amlodipine	10.82	1.05	0.32	3.67	1.10	0.46
Nitrendipine	0.70	1.00	0.00	0.65	1.00	0.00

Note. k_1, retention factor of the first eluted enantiomer; α, enantioselectivity; R, enantioresolution; n.e., no elution within three hours.

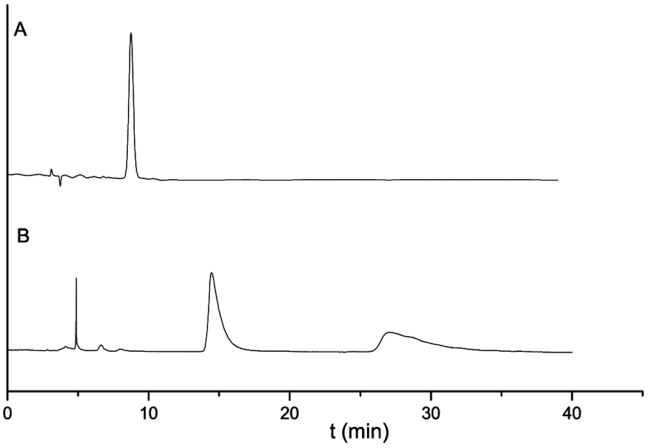

Figure 4. Chromatograms of the enantioseparation of BP 766 on DMP-CF7 CSP (A) and DMP-CD CSP (B). Mobile phase: hex/IPA/TFA 80/20/0.5 (*v*/*v*/*v*); temperature: 25°C; flow rate: 1 mL/min; UV detection: 280 nm.

of BP 34 do not contribute much to the retention of BP 766 but affect enantioselectivity. Despite the fact that inclusion of analytes to the CD cavity is not a dominating enantioseparation mechanism in NP separation mode, the results indicate that some competition between the mobile phase constituents and analytes (hydrophobic parts of their molecules) for the CD cavity can be involved (Armstrong et al. 1990). The inclusion can increase retention but mainly improves enantioseparation.

The couples of analytes BP 34 and BP 766 demonstrate the complementarity of DMP-CF7 and DMP-CD CSPs (see Table 2).

The structures of oxprenolol and alprenolol are very similar (see Fig. 3). Both contain secondary amino group, hydroxyl group, aromatic ring, and ether group but oxprenolol has an additional ether group. As can be seen in Table 2, oxprenolol exhibited higher affinity to both DMP-derivatized CSPs. This result can be attributed to the additional ether group available for interaction. DMP-CF7 CSP was not suitable for enantioseparation of these β-blockers; no partial resolution was observed. DMP-CD CSP showed higher enantioselectivity: Partial separation of alprenolol in all mobile phases tested and partial separation of oxprenolol in mobile phases containing TFA were achieved. Resolution of alprenolol was always higher than resolution of oxprenolol. Alprenolol appears to get closer to the interaction sites of DMP-CD and thus yield better enantioseparation. However, the additional oxygen atom in the molecule of oxprenolol is far away from its stereogenic center; therefore, it can contribute to retention but has limited, if any, enantioselective recognition potential. Hence, DMP-CD allows better resolution of oxprenol and alprenolol even at lower retention of these analytes due to higher rigidity of the CD and lower steric hindrance.

The molecules of amlodipine and nitrendipine differ in types and positions of two substituents while the basic skeleton is the same. Amlodipine possesses – CH_2-O-CH_2-CH_2-NH_2 group on its pyridine ring and chlorine atom in *ortho* position on the aromatic ring, while the structure of nitrendipine contains a methyl group

connected to the pyridine ring and a nitro group in the *meta* position on the benzene ring. Despite the similarity of the analytes, they show considerably different retention behavior (see Table 2). Amlodipine is strongly retarded (like analyte BP 34) by both stationary phases in the mobile phase without TFA. Addition of TFA to the mobile phase reduces its retention significantly more on DMF-CD CSP than on the CF-based CSP. Higher retention of amlodipine on DMP-CF7 CSP can be ascribed to the interaction of amino group of the analyte with crown ether skeleton of CF7. However, the amino group is far away from the chiral center and does not improve enantioseparation. Partial separation of amlodipine enantiomers was achieved only on DMP-CD CSP. The best resolution, $R = 0.71$, was obtained in mobile phase composed of hex/IPA/TFA 80/20/0.5 (*v*/*v*/*v*). Very low retention of nitrendipine on the both columns (see Table 2) could not result in any enantioseparation.

Comparison of RN-CF6 and RN-CD CSPs

The second pair of CSPs studied, RN-CF6 CSP *vs.* RN-CD CSP, contained the same derivatization groups, naphthyl ethyl-, but the central skeleton was composed of different cyclic oligosaccharides with a different number of saccharide units. RN-CF6 has 6 fructofuranose units while RN-CD is composed of 7 glucopyranose units. Moreover, the RN derivatization group contains an additional stereogenic center, which can contribute to enantioseparation.

Chromatographic data obtained for binaphthyls are shown in Table 3. All binaphthyl derivatives displayed much higher retention on RN-CD CSP. This can be either a consequence of a higher number of saccharide units in the cycle (higher number of derivatization groups available for interactions) or influence of the dimensions of CD cavity better compatible with the size and structure of these analytes. Despite the higher retention, RN-CD CSP was not suitable for chiral separation of binaphthyls. RN-CF6 CSP served well for separation of these atropoisomers. Binaphthol, analyte 1, analyte 2, analyte 3, and analyte 5 were resolved (at least partly) in all mobile phases tested. Interestingly, the same retention trend as on DMP-derivatized CSPs was observed for analyte 2, analyte 3, and analyte 4 on both RN-substituted CSPs, that is, the retention decreased in the sequence of analyte 4> analyte 2> analyte 3 in all mobile phases. And, similarly, resolution on RN-CF6 CSP decreased in the opposite way: analyte 3> analyte 2 and no chiral separation of analyte 4 could be achieved. Figure 5 illustrates the different behavior of the RN-derivatized columns considering retention and separation of atropoisomers of analytes 3 and 4.

The addition of TFA to mobile phase caused some reduction of retention but had just negligible impact on chiral resolution except for analyte 5 (with ionizable carboxyl and amino groups), for which acidification of the mobile phase resulted in substantial improvement of separation.

The pair of analytes BP 34 and BP 766 exhibited again very different retention and enantioseparation patterns (see Table 4) as on DMP-CSPs.

Analyte BP 34 again showed high retention on both compared CSPs. The contribution of its free amino group to H-bonding was obvious (Kalíková, Janečková, et al. 2011). Baseline enantioseparation was achieved on RN-CF6 CSP in the mobile phases: hex/IPA/TFA 60/40/0.5 (*v*/*v*/*v*) and 80/20/0.5 (*v*/*v*/*v*); partial

Table 3. Chromatographic data of binaphthyl derivates separated on RN-CF6 CSP and RN-CD CSP

	Mobile phase:					
	hex/IPA/TFA 80/20/0.0 (*v*/*v*/*v*)			hex/IPA/TFA 80/20/0.5 (*v*/*v*/*v*)		
	k_1	α	*R*	k_1	α	*R*
RN-CF6 CSP Analyte						
Binaphthol	1.38	1.08	1.08	1.47	1.08	1.04
1	0.94	1.10	1.32	0.89	1.10	1.44
2	2.17	1.07	0.98	2.02	1.07	0.95
3	1.35	1.16	1.95	1.29	1.17	1.87
4	6.34	1.00	0.00	5.38	1.00	0.00
5	2.17	1.05	0.14	1.76	1.10	1.46
6	0.89	1.00	0.00	0.88	1.00	0.00
7	3.19	1.00	0.00	2.76	1.00	0.00
RN-CD CSP Analyte						
Binaphthol	7.67	1.00	0.00	3.11	1.00	0.00[a]
1	1.56	1.00	0.00	1.26	1.00	0.00
2	4.02	1.00	0.00	3.04	1.00	0.00
3	3.09	1.00	0.00	2.42	1.00	0.00
4	8.26	1.00	0.00	5.83	1.00	0.00
5	8.55	1.00	0.00	3.44	1.05	0.31
6	2.17	1.00	0.00	1.61	1.00	0.00
7	5.52	1.00	0.00	4.23	1.00	0.00

Note. k_1, retention factor of the first eluted atropoisomer; α, selectivity; *R*, resolution.
[a]Slight indication of chiral separation.

enantioseparation was observed in the mobile phase composed of hex/IPA 60/40 (*v*/*v*). RN-CD CSP was not suitable for enantioseparation of analyte BP 34. Retention of BP 766 was much lower than that of BP 34 on both RN-CSPs. Higher

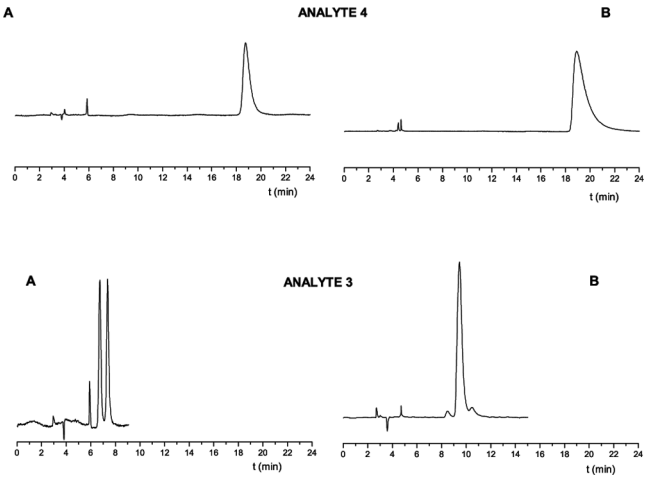

Figure 5. Comparison of the chromatographic behavior of atropoisomers of analytes 3 (bottom) and 4 (top) on the RN-CF6 CSP (A) and RN-CD CSP (B). Mobile phase: hex/IPA/TFA 80/20/0.5 (*v*/*v*/*v*); temperature: 25°C; flow rate: 1 mL/min; UV detection: 254 nm.

Table 4. Chromatographic data of chiral compounds separated on RN-CF6 CSP and RN-CD CSP

	Mobile phase:					
	hex/IPA/TFA 60/40/0.0 (*v*/*v*/*v*)			hex/IPA/TFA 60/40/0.5 (*v*/*v*/*v*)		
	k_1	α	*R*	k_1	α	*R*
RN-CF6 CSP Analyte						
BP 766	0.86	1.00	0.00	0.88	1.00	0.00
BP 34	13.96	1.27	0.97	10.16	1.33	2.25
Alprenolol	2.26	1.00	0.00	1.71	1.00	0.00
Oxprenolol	3.96	1.04	0.36	3.15	1.05	0.38
Amlodipine	51.70	1.00	0.00	15.57	1.09	0.71
Nitrendipine	0.84	1.00	0.00	0.85	1.00	0.00
RN-CD CSP Analyte						
BP 766	2.04	1.00	0.00	1.87	1.00	0.00
BP 34	13.63	1.00	0.00	4.38	1.00	0.00
Alprenolol	3.49	1.40	0.71	1.50	1.11	0.40
Oxprenolol	6.49	1.25	0.49	2.58	1.09	0.37
Amlodipine	22.27	1.00	0.00	5.20	1.06	0.30
Nitrendipine	1.16	1.00	0.00	1.10	1.00	0.00

Note. k_1, retention factor of the first eluted enantiomer; α, enantioselectivity; *R*, enantioresolution.

retention of BP 766 was offered by RN-CD CSP but no partial enantioseparation could be achieved on any of these CSPs.

Concerning the interaction possibilities, the structures of oxprenolol and alprenolol are more alike than those of the previous pair of analytes (BP 34 and BP 766); therefore, the difference of their retention is correspondingly smaller (see Table 4). Both RN-derivatized CSPs had a higher affinity for oxprenolol than for alprenolol in all studied mobile phases. Higher retention of oxprenolol on RN-CF6 CSP was accompanied by partial enantioresolution that could not be achieved for alprenolol. On the CD-based column, less retained alprenolol could be somewhat better resolved than oxprenolol, which retention factor value was about twice that of alprenolol. While almost no effect of addition of TFA to mobile phase on retention and resolution values was observed on CF6-based column for these analytes, acidification of the mobile phase caused decrease of both retention and resolution on CD-based CSP. Both CSPs show similar enantiodiscrimination ability for oxprenolol while RN-CD CSP is more appropriate for enantioselective interaction with alprenolol.

Comparison of retention of amlodipine and nitrendipine shows similar trend as that observed on the DMP-derivatized CSPs (compare data in Table 2 and Table 4). Amlodipine exhibited high retention on both RN-derivatized CSPs and even higher affinity to RN-CF6 CSP (see Table 4). Crown ether core of CF that enables the interaction with the amino group of amlodipine and spatial arrangement of the RN-substituted CF6 that better correlates with the structure of amlodipine, are responsible for these results. Slightly retained nitrendipine was more retarded on RN-CD CSP than on RN-CF6 CSP. This means that the higher was the ability of the given CSP to interact with amlodipine the lower was the affinity of nitrendipine to this CSP. Partial enantioresolution of amlodipine was obtained on both RN-derivatized CSPs in mobile phases with TFA. The main interaction of the free

amino group of amlodipine was reduced at addition of TFA but this interaction was not enantioselective potential.

Concerning only the retention data (k_1 values) of the two groups of analytes, that is, binaphthyl derivatives (see Tables 1 and 3) and various drugs (see Tables 2 and 4), interesting consequences were found. While both the DMP-derivatized CSPs showed the same retention order of the binaphthyl derivatives, their retention sequence differed on the RN-derivatized CSPs (compare the values in Tables 1 and 3). For the other analytes (drugs), the retention trends were just the opposite. Their retention order was the same for both RN-derivatized CSPs (Table 4), whereas it was different on the DMP-CF7 CSP and DMP-CD CSP (Table 2). Thus, the influence of the DMP and RN substituents is different for diverse classes of compounds.

CONCLUSION

CSPs based on derivatized cyclofructans performed interesting separation capabilities for binaphthyl derivatives and other chiral compounds. Their enantioseparation potential in normal-phase mode was discussed and compared with that of their cyclodextrins analogues. Two pairs of CSPs were tested, namely, DMP-CF7 CSP and DMP-CD CSP, containing the same number of saccharide units (fructofuranose and glucopyranose, respectively) in the basic skeleton, and RN-CF6 CSP and RN-CD CSP, where the cyclodextrin based CS contained one additional saccharide unit. Despite the similarities between the derivatized cyclofructans and derivatized cyclodextrins, the CSPs performed significantly different retention and resolution characteristics. Overall, 36 chiral separations having a resolution over 1 were achieved.

The results showed that the basic cyclic oligosaccharides, cyclodextrin vs. cyclofructan, cannot be considered simply carriers of the derivatization groups but also affect the interaction/separation of analytes. This effect was obvious even in an NP separation system where inclusion of analytes to the hydrophobic cavity of CD did not play an important role. Although the interaction with the CD cavity was reduced or almost eliminated in the NP mode, rigidity of CD determines the spatial arrangement of substituents and, therefore, it affects the retention and enantiodiscrimination properties of the CSs. The crown ether core of CFs is less rigid but enables H-bonding interaction.

This comparison can contribute to the better understanding of enantioseparation mechanisms on these columns and help in the optimization of a chromatographic system suitable for separation of required enantiomers.

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5.3 Publikace IV - Charakterizace DMP-CF7 CSP v podmínkách SFC - základní porovnání s HPLC systémem

SFC je považovaná za vhodnou alternativu k HPLC. Mezi hlavní výhody této separační techniky patří nižší náklady, obvykle nižší časy potřebné k analýze a nezanedbatelným faktorem jsou také menší ekologické nároky používaných mobilních fází. Z tohoto důvodu byla provedena v rámci **Publikace IV** základní charakteristika DMP-CF7 CSP pomocí modelu LFER v podmínkách SFC. Získané zastoupení jednotlivých retenčních interakcí bylo srovnáno a následně diskutováno v souvislosti s analogickými systémy NP HPLC. V rámci srovnání obou separačních technik byly také diskutovány enantioselektivní separace sady chirálních látek odlišné struktury.

Srovnávané mobilní fáze byly CO_2/IPA 80/20 (*v/v*), CO_2/IPA/TFA 80/20/0,5 (*v/v/v*) pro SFC a hex/IPA 80/20 (*v/v*), hex/IPA/TFA 80/20/0,5 (*v/v/v*) pro HPLC. Dále byly charakterizovány dva další SFC systémy, CO_2/MeOH 95/5 (*v/v*) a CO_2/MeOH/TFA 95/5/0,5 (*v/v/v*), neboť je známo, že v SFC systémech s mobilními fázemi s velmi malým přídavkem organického modifikátoru dochází ve zvýšené míře k adsorpci složek mobilní fáze na fázi stacionární, čímž jsou významně ovlivněny retenční/enantiodiskriminační mechanizmy. Pro posledně jmenované mobilní fáze nebyla prováděna analogická měření LFER v HPLC z důvodu neúměrně vysokých retencí testovacích analytů.

Základním rozdílem mezi porovnávanými HPLC a SFC systémy byl statisticky významný koeficient *e* pro všechny SFC systémy, zatímco pro analogické HPLC systémy byl nevýznamný. Tento koeficient vyjadřuje rozdíl mezi stacionární a mobilní fází v možnosti interakce prostřednictvím n-/π- elektronových párů. Možným vysvětlením je reakce oxidu uhličitého s volnými hydroxylovými skupinami CS za tvorby karboxylové kyseliny. Jiné vysvětlení může být založeno na adsorpci alkoholového modifikátoru na CSP. Takové interakce jsou v HPLC limitovány, zatímco SFC nabízí širší škálu interakčních možností. Jiným významným rozdílem bylo ovlivnění dipolarity/polarizibility (koeficient *s*) přídavkem TFA. Pro HPLC se tento koeficient po acidifikaci mobilní fáze zvýšil, pro SFC byl efekt minimální.

Koeficient *b* (schopnost fáze poskytnout vodík pro tvorbu vodíkových interakcí) je významný pro všechny zkoumané systémy. Okyselení mobilní fáze

zapříčinilo mírný pokles toho koeficientu v obou separačních systémech. Koeficient *a* (akceptor vodíku pro tvorbu vodíkových interakcí) je statisticky nevýznamný pro všechny zkoumané systémy.

Koeficient *v* vykazoval ve všech systémech záporné hodnoty, což odpovídá charakterům mobilních a stacionárních fází. Vyšší absolutní hodnoty pro SFC systémy naznačují, že je v těchto systémech větší rozdíl mezi stacionární a mobilní fází ve schopnosti interagovat disperzními interakcemi oproti HPLC.

Modely LFER pro SFC systémy s mobilními fázemi s nízkým obsahem organického modifikátoru prokázaly, že všech pět typů interakcí se uplatňuje v retenčním mechanizmu.

Jako výchozí mobilní fáze pro chirální separace derivátů binaftolu byla zvolena CO_2/IPA/TFA 80/20/0,5 (*v/v/v*). V porovnání s HPLC separacemi v analogickém systému poskytla DMP-CF7 CSP v SFC vyšší retenční faktory, nižší hodnoty enantioselektivit a obecně horší hodnoty rozlišení. Nejvýznamnější rozdíl byl zaznamenán pro binaftol. Atropizomery tohoto analytu byly v HPLC rozděleny až na základní linii, zatímco v podmínkách SFC nebyla pozorována ani částečná separace. Částečnou optimalizací separačních podmínek (změna typu a množství organického modifikátoru, změna množství TFA) bylo dosaženo celkem šesti separací (úplných/částečných) v podmínkách SFC, zatímco v HPLC bylo dosaženo pěti separací.

DMP-CF7 CSP v obou systémech silně zadržuje látky s volnou amino skupinou. Přídavek TFA do separačního systému významným způsobem zkrátí retenci těchto látek. Pozorované výsledky částečně potvrzené modely LFER ukazují, že se mechanizmus působení TFA liší v závislosti na obsahu alkoholového modifikátoru. Zatímco v mobilních fázích s vyšším obsahem modifikátoru dochází přednostně k ovlivnění amino skupiny analytu, při nižších koncentracích modifikátoru se TFA adsorbuje na povrch CSP, což způsobí mírné zvýšení retence analytů s volnou amino skupinou [15,16].

Využitím sady vhodně zvolených analytů byla pro SFC systém, stejně jako pro HPLC systém, demonstrována role amino skupiny v retenčním chování, resp. enantiodiskriminaci. V případě, že je tato skupina lokalizována v molekule analytu

daleko od prvku chirality, přispívá zejména k retenci. Je-li lokalizována v blízkosti prvku chirality, má silný enantiodiskriminační charakter.

Publikace IV

An Insight into the Use of Dimethylphenyl Carbamate Cyclofructan 7 Chiral Stationary Phase in Supercritical Fluid Chromatography: The Basic Comparison with HPLC

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Journal of Separation Science **2013**, 36, 1711–1719

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Received December 20, 2012
Revised February 25, 2013
Accepted February 27, 2013

Research Article

An insight into the use of dimethylphenyl carbamate cyclofructan 7 chiral stationary phase in supercritical fluid chromatography: The basic comparison with HPLC

Cyclofructan-based chiral stationary phases were previously shown as a promising possibility for separation of chiral compounds in high performance liquid chromatography. In this work retention and enantiodiscrimination properties of the 3,5-dimethylphenyl carbamate cyclofructan 7 chiral stationary phase are described in supercritical fluid chromatography. The results obtained in both of the separation methods were compared. A set of compounds with axial or central chirality was used as analytes. The effect of mobile phase composition, that is, addition of different alcohol modifiers and/or trifluoroacetic acid to carbon dioxide, was examined in the supercritical system. Similarly, mobile phases composed of hexane modified with propan-2-ol and/or trifluoracetic acid were used in liquid chromatography. A linear free energy relationship model was utilized for characterization of interactions that are decisive for retention and separation in both techniques. Dispersion interactions showed similar negative values using both methods. The main contribution of hydrogen bond acidity was also comparable for both methods. The propensity to interact with *n*- and/or π-electron pairs of solutes was significant only in the supercritical system.

Keywords: Chiral separation / Cyclofructan / HPLC / Linear free energy relationship / Supercritical fluid chromatography
DOI 10.1002/jssc.201201174

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1 Introduction

Cyclofructans (CFs) belong to a group of cyclic oligosaccharides like cyclodextrins. The structure of CFs is formed by D-fructofuranose units connected by β-2,1 linkage and creating the crown-ether-like skeleton (see Fig. 1) [1–3].

Although derivatized CFs were tested as chiral selectors (CSs) in different separation techniques [4–7], the main use of derivatized CFs has been in high performance liquid chromatography [8–12]. The spatial arrangement of the saccharide units and the derivatization groups makes derivatized CFs a unique group of chiral selectors, which behaves complementary to better-known cyclodextrins in some cases [13]. Chiral stationary phases based on derivatized CFs (CF-CSPs) are considered to be multimodal, that is, they can be used in normal phase, reversed phase and also polar-organic modes. Recently, CF-CSPs were successfully used also in hydrophilic interaction liquid chromatography (HILIC) [14–16].

The main interaction forces responsible for retention on CF-CSPs in normal phase HPLC (NP-HPLC) and in HILIC have been already described by our group [15, 17, 18]. We have also compared CF-CSPs with analogous CSPs based on derivatized cyclodextrin [13]. Therefore, the logical continuance of our work is to study the chromatographic behavior of CF-CSPs using supercritical fluid chromatography (SFC), which utilizes carbon dioxide (CO_2) in the mobile phase. SFC is generally considered a normal phase separation method due to the nonpolar nature of CO_2. However, mixtures of CO_2 with polar modifiers can sometimes be a substitute for some highly efficient separations obtained in reversed phase HPLC [19,20]. SFC usually allows separations with short analysis time, high separation efficiency, and low solvent consumption under what is considered environmental friendly conditions [19–22]. The first article that introduced CF-CSPs proposed the possibility of employing the derivatized CFs as

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Abbreviations: **CF**, cyclofructan; **CS**, chiral selector; **CSP**, chiral stationary phase; **DMP-CF7**, dimethylphenyl carbamate cyclofructan 7; **EtOH**, ethanol; **hex**, *n*-hexane; **HILIC**, hydrophilic interaction liquid chromatography; **IPA**, propan-2-ol; **LFER**, linear free energy relationship; **MeOH**, methanol; **SFC**, supercritical fluid chromatography

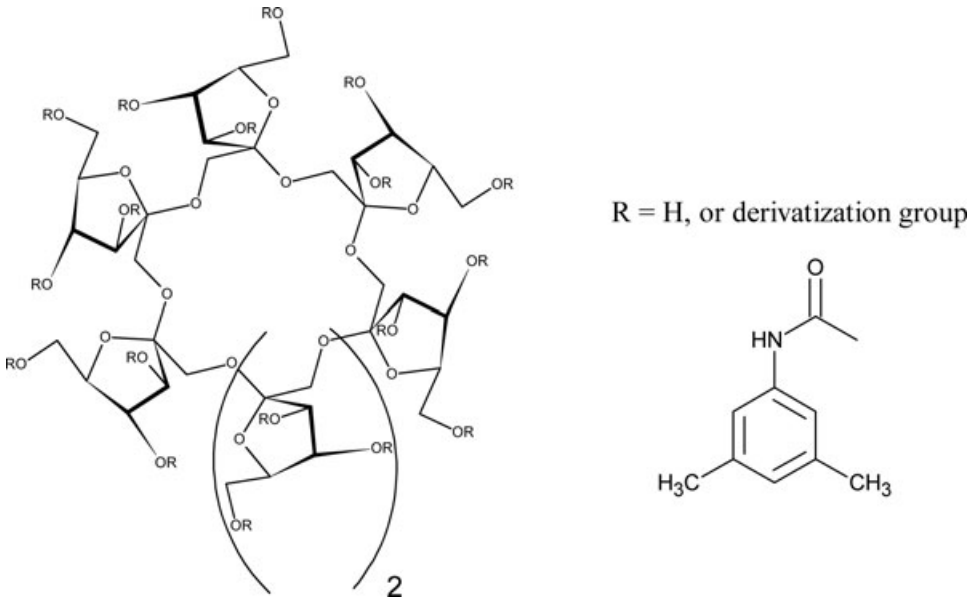

Figure 1. The chemical structure of DMP-CF7 chiral selector, which bonded to silica gel forms the chiral stationary phase.

chiral selectors in SFC [8]. However, a detailed study dealing with interactions taking part on CF-CSPs in SFC has not been published yet.

The aim of this work is to provide two different points of view for the use of one member of the CF-CSPs family, namely 3,5-dimethylphenyl carbamate cyclofructan 7 (Larihc DMP-CF7) CSP in SFC. The work is divided into two parts. The first part is focused on a comparison of the chromatographic behavior of DMP-CF7 CSP in HPLC versus SFC in terms of retention, selectivity, and resolution. A test set containing various compounds with central or axial chirality was chosen for this purpose. The second part of this work deals with interactions that are responsible for the retention process. The linear free energy relationship model (LFER) is an effective tool for qualitative and quantitative characterization of interactions participating in the retention mechanism (e.g. [23–27]). However, it must be understood that not all interactions that lead to retention can lead to chiral recognition. The results are compared with those obtained in previous HPLC studies [13, 18].

2 Materials and methods

2.1 Chemicals and materials

Organic solvents of HPLC grade *n*-hexane (hex), propan-2-ol (isopropanol, IPA), ethanol (EtOH), and methanol (MeOH), were purchased from Sigma-Aldrich (Steinheim, Germany). Trifluoroacetic acid (TFA) of 99.8% purity was product of Merck (Darmstadt, Germany).

The CO_2 used for SFC from Air Liquide (Paris, France) was Alphagaz CO_2 SFC, L50TP, purity : 99.998% with maximum impurities : H_2O <5 ppm, O_2 <2 ppm, CO <5 ppm, H_2 <0.5 ppm, C_nH_m <2 ppm, $NO+NO_x$ <2 ppm, and total sulfur <1 ppm.

Binaphthyl derivatives were synthesized as racemates at the Department of Organic and Nuclear Chemistry, Faculty of Science, Charles University in Prague, Czech Republic [28, 29]. BP 766 (Fluridil) and its hydrolytic decomposition product BP 34 were synthesized at the University of California, Radiology Research (San Diego, CA, USA) [30]. Butizide and bendroflumethiazide were provided by Prof. Martin Schmid from the Department of Pharmaceutical Chemistry, University of Graz, Austria. TTNH2 and TTCH3 were obtained from the Department of Dynamic Stereochemistry and Chirality at Aix Marseille University, Marseille, France. Thalidomide, chlorthalidone and the test solutes for LFER were purchased from Sigma-Aldrich (Steinheim, Germany).

2.2 Equipment and chromatographic conditions

The chromatographic column Larihc CF7-DMP is based on derivatized CF, namely DMP-CF7 CSs immobilized on silica gel support, was obtained from AZYP, LLC (Arlington, TX, USA). The column dimensions were 250 mm × 4.6 mm i.d.; particle size 5 μm. The concentrations of stock solutions of the samples were 1 mg/mL for solid and 20 μL/mL for liquid samples. MeOH was used as sample solvent.

The SFC measurements were achieved on a system SFC-PICLAB Analytic from PIC SOLUTION (Avignon, France). The amount of the co-solvent in the mobile phase was adjusted by a piston pump, the co-solvent was directly added in the CO_2 feeding, and the mixture of co-solvent and CO_2 was pumped by another piston pump at the total flow rate of 4 mL/min. For mobile phases containing TFA, alcohol modifier and TFA were mixed at first in the appropriate volume ratio and then added to the system. The head of this pump was cooled to –7°C by a cryostat. The SFC equipment contained also autosampler, oven, UV DAD detector, and back-pressure

regulator to control the outlet pressure. The outlet tube was heated at 55°C to avoid ice formation during the CO_2 depressurization. Data were recorded with SFC PicLab Analytic Online 3.1.2 and processed with Analytic Offline 3.2.0. Temperature was maintained at 40°C, and back pressure was set at 120 bar in SFC. The injection volume was 20 μL.

All HPLC measurements were carried out on Waters Alliance System with Waters 2695 Separation Module, Waters 2996 Photodiode Array Detector, an autosampler 717 Plus, Waters Alliance Series column heater (Milford, MA, USA), controlled by Empower software. Temperature of the column and samples was kept at 25°C. The injection volume was 10 μL and flow rate was 1 mL/min.

The UV detection was performed at 254 nm in most cases in both methods. The wavelengths of the absorption maxima were used for detection of certain analytes. The void volume was determined using solvent peak in both methods. All retention measurements were carried out in triplicates and from these data retention factors were calculated.

2.3 LFER procedure

Linear free energy relationship model was used to get an insight on the retention mechanism taking part on DMP-CF7 CSP in SFC [31–33].

The main principle of the LFER model is based on the idea that retention (expressed as log*k*) is divided to several different contributions according to the interaction type involved. The basic LFER Eq. (1) has with five independent variables, solute descriptors, describing specific features of analytes [33]. Variable E is the excess molar refraction of a solute and it indicates an ability to interact via *n*- and/or π-electrons, S is the solute dipolarity/polarizibility, A is the effective or overall hydrogen bond acidity, B the effective or overall hydrogen bond basicity, and V is the McGowan's characteristic molecular volume reflecting dispersion interactions [34, 35]. Our probe set contained 37 solutes from different chemical groups with their solute descriptors spanning a wide range of values (see Supporting Information) [36, 37].

$$\log k = eE + sS + aA + bB + vV + c \quad (1)$$

The regression coefficients *e, s, a, b, v* of the LFER equation were obtained from multivariate linear regression analysis (log*k* against solutes' descriptors) using the NCSS software (Kaysville, USA) [38] and describe the differences between the stationary phase and the mobile phase to interact by the corresponding interaction. A positive value of the coefficient indicates that the interaction dominates in the stationary phase, thus increases retention of analytes, whose retention involves this type of interaction. A negative value means that this interaction is stronger in the mobile phase and so decreases retention. Term *c* is the intercept of the LFER equation also obtained from multivariate linear regression analysis, it depends on the experimental system used (nature of the organic modifier used, phase ratio) but it does not describe any specific interaction [39].

3 Results and discussion

3.1 Comparison of chiral separation potential of DMP-CF7 CSP in HPLC and SFC

Retention, selectivity, and resolution were investigated for the chiral compounds listed in Fig. 2.

The first group of analytes examined were binaphthyl derivatives, compounds possessing axial chirality. The initial mobile phase for SFC was composed of CO_2/IPA/TFA 80:20:0.5 v/v/v because it corresponded with the optimized mobile phase composed of hex/IPA/TFA 80:20:0.5 v/v/v, which proved to be the most suitable for chiral separation of binaphthyl derivatives on DMP-CF7 CSP in HPLC [13]. Table 1 illustrates that the DMP-CF7 CSP provided much higher *k* values, lower α values and therefore lower separation potential for binaphthyl derivatives in SFC compared to HPLC. All atropisomers, which were at least partially resolved in HPLC ($Rs < 1.5$), had lower *Rs* values in SFC. The main difference shows binaphthol, its atropisomers were separated with $Rs = 3.59$ in HPLC but no separation was observed in SFC (see Fig. 3A).

The impact of the type and the amount of alcohol modifiers (MeOH, EtOH, and IPA, from 5 to 40 volume%) and the influence of addition of TFA (in the volume range 0.05–0.50%) to the mobile phase on chiral separations of binaphthyl derivatives on DMP-CF7 CSP in SFC were studied. The chromatographic conditions listed in Table 2 represent the optimized mobile phase compositions. Overall, six of eight binaphthyls were at least partially separated on DMP-CF7 CSP in SFC. The only atropisomers that remained unresolved either in SFC or in HPLC on the DMP-CF7 CSP were those of analyte 4. The comparison of the alcohol modifiers revealed that DMP-CF7 CSP best separated the majority of these atropisomers in mobile phases containing MeOH. However, the best chiral separation of analyte 6 and partial separation of analyte 7 were achieved in the mobile phase containing IPA.

Comparison of analytes 2, 3, and 4, with isomerically analogous substituents (see Fig. 2), shows a very interesting relationship between their structure and chromatographic behavior in SFC as well as in HPLC [13] (see Table 1). The results indicate that oxygen or sulfur atoms in close proximity to the compounds axis substantially affect their chiral recognition. DMP-CF7 CSP performed extraordinary separation potential for analyte 3 (see Fig. 3B) with two oxygen atoms in this position. Analyte 2 possessing only one oxygen atom there showed lower resolution, and the atropisomers of analyte 4 containing two sulfur atoms next to binaphthyl skeleton were not separated in any of the mobile phases tested.

DMP-CF7 CSP strongly retains analytes containing free amino groups in both SFC and HPLC (see analyte 5). Addition of TFA to the mobile phase reduces the retention

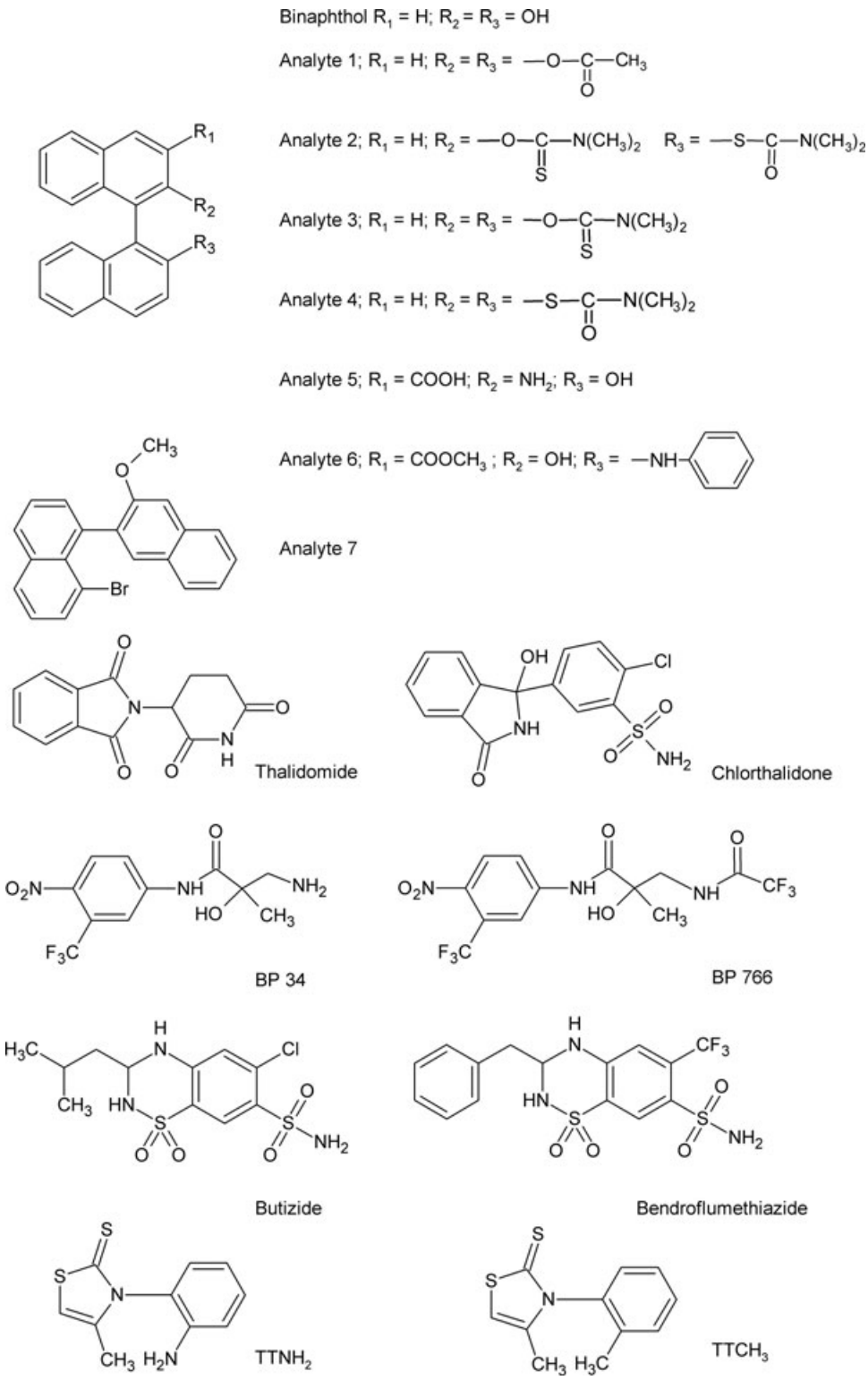

Figure 2. The set of chiral compounds used for measurements in SFC and for the comparison of DMP-CF7 CSP in SFC versus HPLC.

significantly. However, the effect of TFA in SFC seems to differ substantially at higher and lower alcohol contents in the mobile phases. In the former case TFA affects mainly the amino group of analytes and therefore, decreases their retention. In the latter case TFA has higher affinity to stationary phase, modifies its surface, and thus causes minor increase of retention and resolution. These observations were further confirmed by the LFER model in the second part of this work.

In the next step, a set of pairs of analytes that offered certain structural differences was tested.

Table 1. The chromatographic parameters of the binaphthyls using DMP-CF7 CSP and analogous mobile phases in SFC: CO_2/IPA/TFA 80:20:0.5 v/v/v, and in HPLC: hex/IPA/TFA 80:20:0.5 v/v/v. k_1, retention factor of the first eluted atropisomer; α, selectivity; *Rs*, resolution

Compound	Method	k_1	α	*Rs*
Binaphthol	SFC	3.38	1.00	0.00
	HPLC	1.69	1.35	3.59
Analyte 1	SFC	1.62	1.00	0.00
	HPLC	1.11	1.05	0.62
Analyte 2	SFC	5.17	1.14	2.48
	HPLC	2.34	1.30	3.11
Analyte 3	SFC	4.13	1.57	7.97
	HPLC	1.50	2.51	9.49
Analyte 4	SFC	9.45	1.00	0.00
	HPLC	5.65	1.00	0.00
Analyte 5	SFC	3.58	1.00	0.00
	HPLC	1.87	1.00	0.00
Analyte 6	SFC	3.71	1.04	0.21
	HPLC	1.16	1.10	1.14
Analyte 7	SFC	2.66	1.00	0.00
	HPLC	0.59	1.00	0.00

The starting mobile phase contained 20% of alcohol modifiers (MeOH, EtOH, IPA). Figure 4 shows the influence of the alcohol type on the retention and separation of butizide enantiomers in SFC. The resolution values increased in the sequence MeOH < EtOH < IPA. The same trend was observed for all analytes from this set. Table 2 shows the optimized mobile phase compositions for chiral separations in terms of resolution of analytes in SFC and the comparison with HPLC. The comparison of the retention behavior clearly confirms the important role of the free amino group in the retention mechanism on DMP-CF7 CSP. The effect on enantioseparation is obvious only if this group is close to the chiral center or axis, so that the interaction of the amino group with the CSP has an important effect on selectivity.

Retention and resolution values of BP 34 enantiomers (see Fig. 2) are substantially affected by the presence of TFA in the both methods. In mobile phases without TFA, the free amino group contributes to high retention and that was accompanied by lower resolution due to severe peak tailing. Acidification of the mobile phase significantly shortened retention and improved the resolution value (e.g. in SFC – CO_2/IPA 80:20 v/v: $k_1 = 17.48$, $Rs = 0$; CO_2/IPA/TFA 80:20:0.5 v/v/v: $k_1 = 9.78$, $Rs = 1.34$; and in HPLC – hex/IPA 80:20 v/v: no elution within 4 h; hex/IPA/TFA 80:20:0.5 v/v/v: $k_1 = 29.46$, $Rs = 0.72$). A related compound BP 766 has its amino group trifluoracetylated. This negated the "amine interaction" with the CSP and these enantiomers were not separated.

Analytes $TTNH_2$ and $TTCH_3$ (see Fig. 2) possessing axial chirality again confirmed the contribution of the free amino group to retention and chiral resolution on DMP-CF7 CSP. The addition of TFA in SFC caused a decrease of resolution and a slight increase in retention, while decreases in both resolution and retention were observed in HPLC (e.g. SFC – CO_2/IPA 80:20 v/v: $k_1 = 4.70$, $Rs = 0.35$; CO_2/IPA/TFA 80:20:0.5 v/v/v: $k_1 = 5.27$, $Rs = 0.2$; HPLC – hex/IPA 80:20 v/v: $k_1 = 6.14$, $Rs = 1.00$; hex/IPA/TFA 80:20:0.5 v/v/v: $k_1 = 5.88$, $Rs = 0.72$).

DMP-CF7 CSP was not able to separate enantiomers of thalidomide and chlorthalidone in SFC. However,

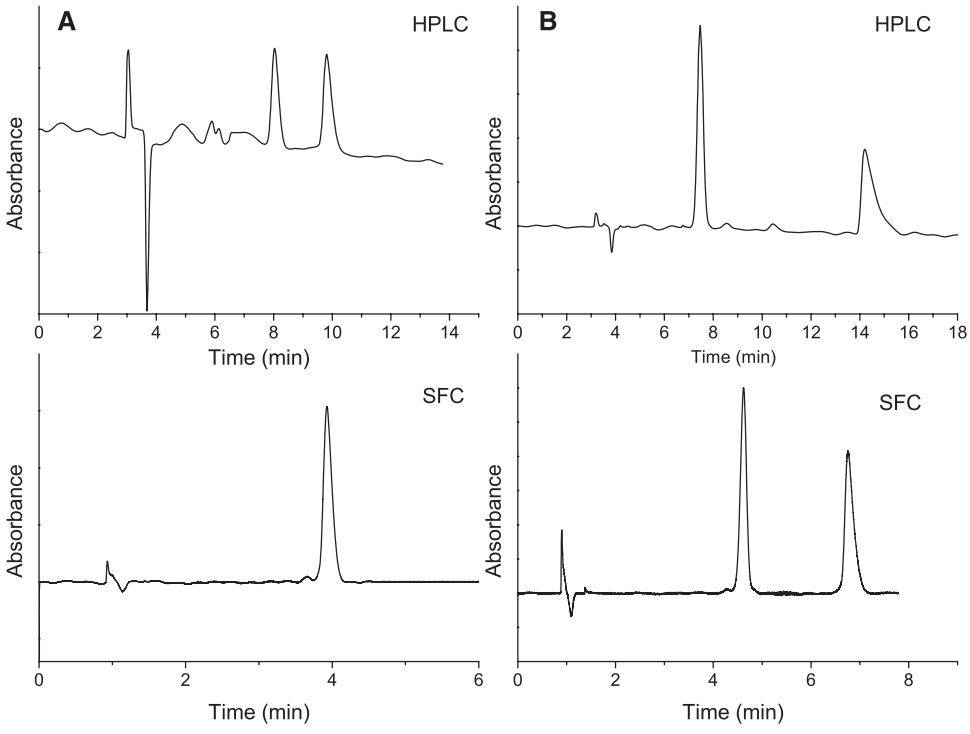

Figure 3. Separation of atropisomers of binaphthol (chromatograms (A)), and analyte 3 (chromatograms (B)) HPLC: mobile phase hex/IPA/TFA 80:20:0.5 v/v/v; SFC: mobile phase CO_2/IPA/TFA 80:20:0.5 v/v/v. For details see Section 2.

Table 2. The optimized mobile phase compositions for chiral separation of binaphthyl atropisomers in SFC and for chiral separation of different analytes in SFC and HPLC

Compound	Method	k_1	α	*Rs*	Mobile phase (v/v/v)
Binaphthol	SFC	17.38	1.00	0.00	CO_2/MeOH/TFA 95:5:0.1
Analyte 1	SFC	4.08	1.07	0.97	CO_2/MeOH/TFA 95:5:0.1
Analyte 2	SFC	27.15	1.25	4.16	CO_2/MeOH/TFA 95:5:0.1
Analyte 3	SFC	19.17	1.87	11.89	CO_2/MeOH/TFA 95:5:0.1
Analyte 4	SFC	48.87	1.00	0.00	CO_2/MeOH/TFA 95:5:0.1
Analyte 5	SFC	41.76	1.05	0.92	CO_2/MeOH/TFA 95:5:0.05
Analyte 6	SFC	20.58	1.06	1.02	CO_2/IPA/TFA 95:5:0
Analyte 7	SFC	8.98	1.03	0.30	CO_2/IPA/TFA 95:5:0
Thalidomide	SFC	3.89	1.00	0.00	CO_2/IPA/TFA 80:20:0.1
	HPLC	23.84	1.05	0.58	hex/IPA/TFA 80:20:0.1
Chlorthalidone	SFC	17.89	1.00	0.00	CO_2/IPA/TFA 80:20:0.1
	HPLC	25.33	1.00	0.00	hex/IPA/TFA 80:20:0.1
BP 34	SFC	9.78	1.27	1.34	CO_2/IPA/TFA 80:20:0.5
	HPLC	7.25	1.26	0.88	hex/IPA/TFA 60:40:0.5
BP 766	SFC	1.17	1.00	0.00	CO_2/IPA/TFA 80:20:0.5
	HPLC	0.63	1.00	0.00	hex/IPA/TFA 60:40:0.5
Butizide	SFC	14.30	1.19	2.72	CO_2/IPA/TFA 80:20:0.5
	SFC	4.93	1.16	2.01	CO_2/IPA/TFA 70:30:0
	HPLC	23.35	1.27	2.59	hex/IPA/TFA 80:20:0.5
	HPLC	6.40	1.24	1.98	hex/IPA/TFA 70:30:0
Bendroflumethiazide	SFC	8.80	1.05	0.62	CO_2/IPA/TFA 80:20:0.5
	HPLC	18.12	1.03	0.35	hex/IPA/TFA 80:20:0.5
$TTNH_2$	SFC	31.59	1.04	0.71	CO_2/IPA/TFA 95:5:0
	HPLC	6.14	1.08	1.00	hex/IPA/TFA 80:20:0
$TTCH_3$	SFC	7.21	1.00	0.00	CO_2/IPA/TFA 95:5:0
	HPLC	2.48	1.00	0.00	hex/IPA/TFA 80:20:0

If no chiral separation was achieved the retention data stand for the pairs of compounds in analogous HPLC and SFC mobile phases. k_1, retention factor of the first eluted atropisomer/enantiomer; α, selectivity; *Rs*, resolution

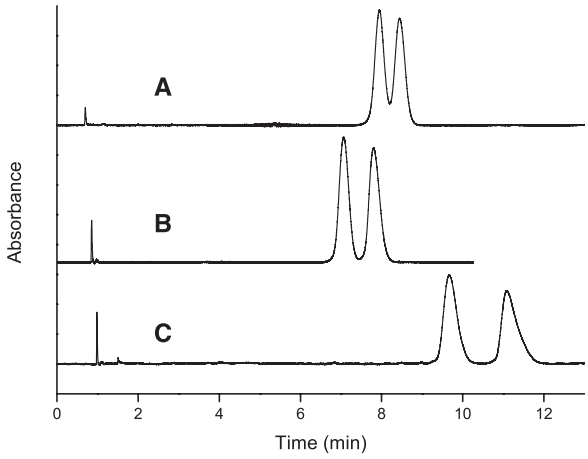

Figure 4. The influence of the different organic modifiers in mobile phase on chiral separation of butizide in SFC. Mobile phase: CO_2/organic modifier 80:20 v/v, A – methanol, B – ethanol, C – propan-2-ol. For details see Section 2.

partial enantioseparation of thalidomide was achieved in HPCL.

The pair butizide/bendroflumethiazide showed the best enantioresolution results among these pairs of analytes. Baseline separation of butizide enantiomers was obtained in reasonable analysis time of 5.46 min at flow rate 4 mL/min in SFC (see Table 2).

3.2 Interactions revealed by LFER model

LFER model can be used as a tool for comparison of the interactions that contribute to the retention processes on DMP-CF7 CSP in SFC and in HPLC.

Two mobile phases composed of CO_2/IPA 80:20 v/v, and CO_2/IPA/TFA 80:20:0.5 v/v/v were chosen for characterization of the DMP-CF7 CSP in SFC. The obtained LFER coefficients were further compared with the LFER results for the HPLC system from previous work [18], where mobile phases with the same proportional composition but with *n*-hexane instead of CO_2 were studied. The HPLC data were partially re-evaluated, using the same probe set in both studies, in order to enable comparison with SFC data. The optimal models, which take into account only statistically significant interactions according to *p*-values ($p < 0.05$), were chosen for further discussion. The results are summarized in Table 3.

Generally, one of the most important differences between retention mechanisms of DMP-CF7 CSP in HPLC and SFC is the statistical significance of *e* term in SFC, while this

Table 3. Regression coefficients of the LFER equation and correlation coefficient *R*

Method	Mobile phase	Model	*e*	*s*	*a*	*b*	*v*	*c*	*R*
HPLC	**Hex/IPA/TFA**	**O.M.**	**x**	**0.789**	**x**	**1.597**	**−0.810**	**−0.881**	**0.95**
	80:20:0.0	± 95% CI		0.277		0.310	0.389	0.330	
	v/v/v	*p*		0.000		0.000	0.000	0.000	
HPLC	**Hex/IPA/TFA**	**O.M.**	**x**	**0.933**	**x**	**1.516**	**−0.917**	**−0.940**	**0.96**
	80:20:0.5	± 95% CI		0.254		0.274	0.348	0.296	
	v/v/v	*p*		0.000		0.000	0.000	0.000	
SFC	**CO_2/IPA/TFA**	**O.M.**	**0.459**	**0.514**	**x**	**0.949**	**−1.011**	**−0.410**	**0.94**
	80:20:0.0	± 95% CI	0.250	0.263		0.251	0.494	0.359	
	v/v/v	*p*	0.001	0.000		0.000	0.000	0.026	
SFC	**CO_2/IPA/TFA**	**O.M.**	**0.537**	**0.472**	**x**	**0.936**	**−1.204**	**−0.227**	**0.94**
	80:20:0.5	± 95% CI	0.253	0.267		0.255	0.501	0.364	
	v/v/v	*p*	0.000	0.001		0.000	0.000	0.212	
SFC	**CO_2/MeOH/TFA**	**O.M.**	**0.557**	**0.495**	**0.891**	**1.020**	**−0.806**	**−0.685**	**0.97**
	95:5:0.0	± 95% CI	0.308	0.351	0.279	0.314	0.735	0.512	
	v/v/v	*p*	0.001	0.007	0.000	0.000	0.033	0.010	
SFC	**CO_2/MeOH/TFA**	**O.M.**	**0.627**	**0.553**	**0.795**	**1.113**	**−1.165**	**−0.422**	**0.93**
	95:5:0.1	± 95% CI	0.446	0.508	0.404	0.455	1.064	0.741	
	v/v/v	*p*	0.007	0.034	0.000	0.000	0.033	0.254	

CI represents ±95% confidence interval, i.e., the values determining the interval, in which a measurement or trial falls corresponding to a given probability. x, insignificant interaction; O.M., optimal model of the LFER equation; *p*, statistical *p*-value. The *p*-values express probability of the error that the individual coefficient does not contribute to the model, i.e., *p*-values represent the significance of the individual coefficients.

term is insignificant for HPLC. The *e* term represents the propensity to interact with solutes *n*- and/or π-electron pairs. As has already been published, the components of mobile phase can interact with CSP in different ways [40,41]. One of the possible interactions in SFC is the reaction of CO_2 with the free hydroxyl groups of CSP and formation of carboxylic group [42–44]. The other possibility is adsorption of the alcoholic component of the mobile phase on CSP. In the given HPLC system such interactions are limited and comparable in stationary and mobile phases.

Another interesting difference shows the term *s* reflecting dipolarity/polarizibility. The acidification of the mobile phase increased term *s* for the studied HPLC system. This effect was almost negligible in SFC just slightly decreased with respect to the confidence interval.

The term *b*, which represents the difference in ability of the stationary and the mobile phases to interact as a hydrogen bond donor, has the highest value among all positive coefficients in both methods. Acidification of the mobile phase caused a minute decrease of *b* values in the both methods. Although it could have certain chemical interpretation, from the statistical point of view the decrease of *b* value cannot be considered relevant with respect to the confidence interval.

The term *a* representing hydrogen bond basicity is insignificant for all systems with similar mobile phase compositions compared in HPLC and SFC, except for two additional mobile phases in SFC, which were also studied and will be discussed further.

The *v* coefficient describing dispersion interactions represents the most important interaction that leads to decrease of retention in both separation systems. Negative values of the term *v* are typical for a normal phase mode [25, 26, 45]. Higher absolute values of the *v* coefficient in SFC indicates that the mobile phase participates in dispersion interactions with the solute to a greater extent in SFC than in HPLC. The addition of TFA to the mobile phase causes similar change in the dispersion interactions in both SFC and HPLC.

In order to get a deeper understanding of the effect of alcohol modifier in mobile phase on interaction mechanisms taking part on DMP-CF7 CSP in SFC two additional mobile phases composed of CO_2/MeOH 95:5 v/v and CO_2/MeOH/TFA 95:5:0.1 v/v/v were tested. These mobile phases were chosen because they produced the best separations for atropisomers of the binaphthyls. (Corresponding mobile phases were not tested in HPLC because the retention of the chiral analytes would have been unacceptably high.) Surprisingly, the LFER model revealed that all five types of interactions that were considered within Eq. (1) are significant and can be involved in the chiral discrimination process (see Table 3).

However, the *p*-values indicate that dispersion interactions (*v* term) in both separation systems and the dipolarity/polarizibility (*s* term) in the system with mobile phase composed of CO_2/MeOH/TFA 95:5:0.1 v/v/v contribute less to the retention process, and also substantially increase the error of the model.

It is believed that the adsorption of mobile phase components is more probable in the mobile phases containing small amounts of organic modifier (≤5%) [19, 20]. Therefore, *b* term increases in the acidified mobile phase with lower MeOH contents (5% MeOH). Taking into account the

hydrogen bond basicity descriptors, B(TFA) = 0.22 and B(MeOH) = 0.62, TFA in the SFC system with CO_2/MeOH/TFA 95:5:0.1 v/v/v contributes to the term *a* (hydrogen bond basicity) less than methanol. On the other hand, TFA and MeOH contribute to hydrogen bond acidity (*b* term) in a similar extent; hydrogen bond acidity descriptors, A(TFA) = 0.99 and A(MeOH) = 0.93, are almost the same.

4 Concluding remarks

The Larihc DMP-CF7 CSP was described with respect to its retentive and enantioselective properties in SFC and compared with its behavior in HPLC. The same set of compounds possessing central and axial chirality was run under SFC and HPLC conditions using the same amounts of alcoholic modifiers and TFA. TFA as a mobile phase additive can influence properties of the CSP surface and in this manner provide additional interaction possibilities. Considering the structure of analytes, the presence of a $-NH_2$ group seems to be essential. Amino groups located far from the stereogenic center or chiral axis influence predominantly retention while those located in a close vicinity of these elements of chirality are important for chiral discrimination and influence chiral resolution.

A LFER model was used to characterize the interactions that participate in retention mechanism on DMP CF7 CSP in SFC. The obtained results were compared with those from a previous HPLC study. The LFER model confirmed that different interactions participated to different degrees in the retention process on DMP-CF7 CSP in SFC and HPLC. The results suggested that the adsorption of some components of the mobile phases is more important in SFC than in HPLC. The lower content of alcoholic modifier in the mobile phase, the higher the adsorption, which significantly changes the characteristics of the separation system in SFC.

The use of the DMP-CF7 CSP in SFC could be considered as a faster and promising alternative to HPLC. However, different interactions between stationary and mobile phases and/or between chromatographic phases and the analyte strongly influence the enantioseparation process and therefore, the transfer of an HPLC method to SFC may not be always straightforward.

The Grant Agency of the Charles University in Prague, project No. 356411, the Ministry of Education, Youth and Sports of the Czech Republic, project Kontakt LH11018, and the long-term project MSM0021620857 are gratefully acknowledged for the financial support. The authors want to express their gratitude to Prof. M. Abraham for providing some molecular descriptors and to Prof. M. Schmid for his generous gift of some enantiomers tested. The international cooperation between Charles University in Prague and Aix-Marseille University was established in the frame of LPP – ERASMUS PROGRAMME.

The authors have declared no conflict of interest.

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5.4 Publikace V - Využití komplexace cyklofruktanového chirálního selektoru s barnatými ionty pro chirální separace v HPLC a CE

Přestože jsou CSP na bázi derivatizovaných cyklofruktanů multimodální, jsou aplikovány zejména v podmínkách NP a POM HPLC. **Publikace V** demonstruje využití cyklofruktanové CSP, konkrétně IP-CF6 CSP, v RP HPLC. Pro chirální separace byla vybrána skupina pěti analytů, derivátů binaftolu s různými derivatizačními skupinami, vykazujících axiální chiralitu.

Zajímavou alternativou jak rozšířit/upravit enantioselektivní potenciál cyklofruktanových CSP je přídavek určitých iontů do mobilní fáze. Vzhledem ke své vysoké komplexační konstantě byl vybrán Ba^{2+} iont [71]. Na rozdíl od *crown*-etherů, u kterých probíhá komplexace iontu uprostřed roviny molekuly, v případě cyklofruktanového selektoru se barnatý kationt komplexuje nad rovinu cyklofruktanového *crown*-etherového skeletu [71]. Smuts a kol. nedávno prokázali, že přídavek Ba^{2+} iontů do mobilní fáze zvyšuje enantioselektivní potenciál cyklofruktanových CSP v POM HPLC pro analyty obsahující zbytek kyseliny fosforečné či sírové [63]. Proto byl studován analogický efekt na enantioselektivitu IP-CF6 CSP v RP HPLC v rámci **Publikace V.** Mobilní fáze byly složeny z různých objemových poměrů MeOH a 20 mM octanového pufru (pH 4,5), bez a s přídavkem octanu barnatého (5 mM). Složky mobilní fáze byly voleny tak, aby mohla být případně použita hmotnostní detekce. Všech pět analytů bylo rozděleno na základní linii při použití mobilní fáze bez přídavku barnatého kationtu, což demonstruje aplikovatelnost IP-CF6 CSP v RP HPLC. Přídavek Ba^{2+} iontů do mobilní fáze způsobill vzrůst retencí pro analyty s fosfátovou skupinou, která pravděpodobně dobře interaguje s barnatým kationtem komplexovaným na cyklofruktanový CS.

Pro analyty bez fosfátové skupiny ve své struktuře došlo k mírnému snížení retence a odpovídajícím poklesům hodnot rozlišení. Lze tedy uzavřít, že interakce mezi Ba^{2+} iontem komplexovaným v derivatizovaném cyklofruktanovém CS zlepší enantioseparaci pouze těch analytů, které jsou schopny interagovat s barnatými ionty.

Analogický separační systém s IP-CF6 jako CS s a bez přídavku Ba^{2+} iontů byl zkoumán i v CE. V systému CE bylo dosaženo separace na základní linii pouze pro

jeden analyt (označený BNP, struktura je uvedena v Publikaci V). I v tomto systému přídavek Ba^{2+} iontů do základního elektrolytu vedl ke zvýšení rozlišení atropizomerů. Výsledky jasně ukazují, že navázání cyklofruktanového selektoru na silikagelový nosič má fundamentální význam pro enantioselektivitu. Příčinou je pravděpodobně vyšší rigidita vázaného selektoru v CSP oproti volnému selektoru v kapiláře.

Publikace V

Isopropyl Derivative of Cyclofructan 6 as Chiral Selector in Liquid Chromatography and Capillary Electrophoresis

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Journal of Chromatography A **2014**, 1338, 197–200

Journal of Chromatography A, 1338 (2014) 197–200

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Short communication

Isopropyl derivative of cyclofructan 6 as chiral selector in liquid chromatography and capillary electrophoresis☆

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ARTICLE INFO

Article history:
Received 9 December 2013
Received in revised form 18 February 2014
Accepted 19 February 2014
Available online 28 February 2014

Keywords:
Chiral separation
Cyclofructan
Isopropyl-cyclocfructan-6
Capillary electrophoresis
High performance liquid chromatography
Atropisomer

ABSTRACT

Cyclofructans and preferentially their derivatives can serve as chiral selectors for the separation of different enantiomers/atropisomers. Moreover, the strong ionophoric nature of the 18-crown-6 ether core of cyclofructan 6 for barium cations may be exploited to enhance or modify enantioselectivity. In this work isopropyl-cyclofructan-6 was used as a chiral selector for the separation of binaphthyl atropisomers in HPLC and CE. The data from both separation systems were compared with each other. While in HPLC the chiral selector was bonded to silica gel to afford a chiral stationary phase, in capillary electrophoresis it was freely mobile in the background electrolytes (BGE). This significant difference is reflected in the separation potential of the two separation systems. All five analytes could be baseline separated in HPLC (reversed phase mode) while only one derivative was baseline resolved in CE. This result was attributed to the more rigid nature of the immobilized chiral selector. Addition of Ba^{2+} to the mobile phase or BGE improved chiral separations in both systems. The results may help to elucidate the interaction mechanism in these systems with cyclofructan derivatives and to gain some general knowledge of their separation potential.

1. Introduction

When appropriate chiral selectors are used in high performance liquid chromatography (HPLC) and capillary electrophoresis (CE), these separation techniques provide powerful methods for enantiomeric separations. Over the years, many chiral selectors have been designed for HPLC and CE. Saccharide-based chiral selectors (e.g. polysaccharides in HPLC, cyclodextrins in CE) are among the best, exhibiting broad enantioselectivity for structurally diverse analytes. The newest macrocyclic oligosaccharide based chiral selectors are cyclofructan (CF) derivatives [1]. Native cyclofructan 6 (CF6) is comprised of an 18-crown-6 ether core, spiro-anellated with six D-fructofuranose units. In its native form, CF6, as an HPLC chiral selector, exhibited poor enantioselectivity. However, upon derivatizing the primary hydroxyl groups (with isopropylcarbamoyl-, *R*-naphthylethylcarbamoyl- or in the case of CF7, dimethylphenylcarbamoyl-) of CF, the derivatives exhibited pronounced and broad enantioselectivity for primary amines in the polar organic mode and a variety of other chiral entities in the normal phase (NP) HPLC mode and SFC [1–10].

Though derivatized cyclofructan-based chiral stationary phases (CF CSPs) have been shown to be suitable for chiral separation of atropisomers in normal phase or polar organic separation modes [2–9], reports of reversed phase (RP) mode separations are limited. Similarly, the first use of isopropylated CF6 (IP-CF6) as a chiral selector in CE appeared only recently where Perera et al. investigated the enantiomeric separations of 17 tetrahydrobenzimidazoles [8]. Separation conditions were optimized so as to separate as many compounds as possible from the set. The aim of their work was

☆ Presented at the 20th International Symposium on Electro- and Liquid Phase-Separation Techniques (ITP 2013), 6–9 October 2013, Puerto de la Cruz, Tenerife, Canary Islands, Spain.

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http://dx.doi.org/10.1016/j.chroma.2014.02.061
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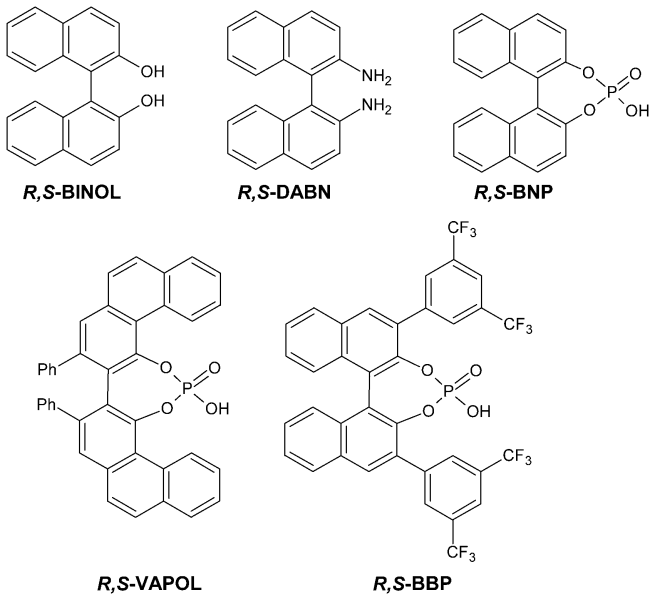

Fig. 1. Structures of the studied compounds.

just successful separation of different racemates in enantioselective separation systems of HPLC and CE with cyclofructan and cyclodextrin derivatives as chiral selectors. However, a deeper study on the interaction mechanism has not been performed.

In HPLC the chiral selector is immobilized onto silica gel, while in CE the chiral selector is freely mobile in the BGE. The chiral selector in HPLC has fewer degrees of freedom and thus is more structurally rigid. Despite the fact that the rigidity of a chiral selector is very important in chiral recognition, CFs and their derivatives can still be used as chiral selectors also in CE. In addition, when CS is free in the BGE, ternary complexes between CS and analytes may occur.

The interaction of CF6 with various metal ions has been described in several papers [11–14], with Ba^{2+} having the strongest binding constant [12]. As with synthetic crown ethers the binding constant increases with increasing organic solvent content (e.g. acetonitrile or methanol) [15]. However, unlike synthetic crown ethers, CFs do not bind metals in the plane of the crown ether core, but rather between it and the hydroxyl groups in 3 position [12]. The presence of Ba^{2+} in the separation systems could conceivably afford a different mechanism of interaction, and thus prevent or contribute to enantioresolution. Recently it has been shown that barium complexed cyclofructan CSPs exhibit unique enantioselectivity toward chiral phosphoric and sulfonic acids in the polar organic mode [16]. The use of barium salts in the reversed phase mode is reported for the first time in this publication.

In this work we selected five atropisomers of similar but different structural features, 1,1′-binaphthalene-2,2′-diyl hydrogen phosphate (BNP), 2,2′-diphenyl-3,3′-biphenanthryl-4,4′-diyl hydrogen phosphate (VAPOL), 3,3′-bis[3,5-bis(trifluoromethyl)phenyl]-1,1′-binaphthyl-2,2′-diyl hydrogen phosphate (BBP), 1,1′-bi-2-naphthol (BINOL), 1,1′-binaphthyl-2,2′-diamine (DABN) (see Fig. 1 for the structures) as model chiral compounds for evaluation of the chiral separation ability of IP-CF6 (Fig. 2) in HPLC and CE. Of special interest was the addition of Ba^{2+} to the separation environment and evaluation of its effect on chiral separation of the selected analytes. The separation results for the HPLC and CE methods were compared to gain some insight into the interaction mechanism.

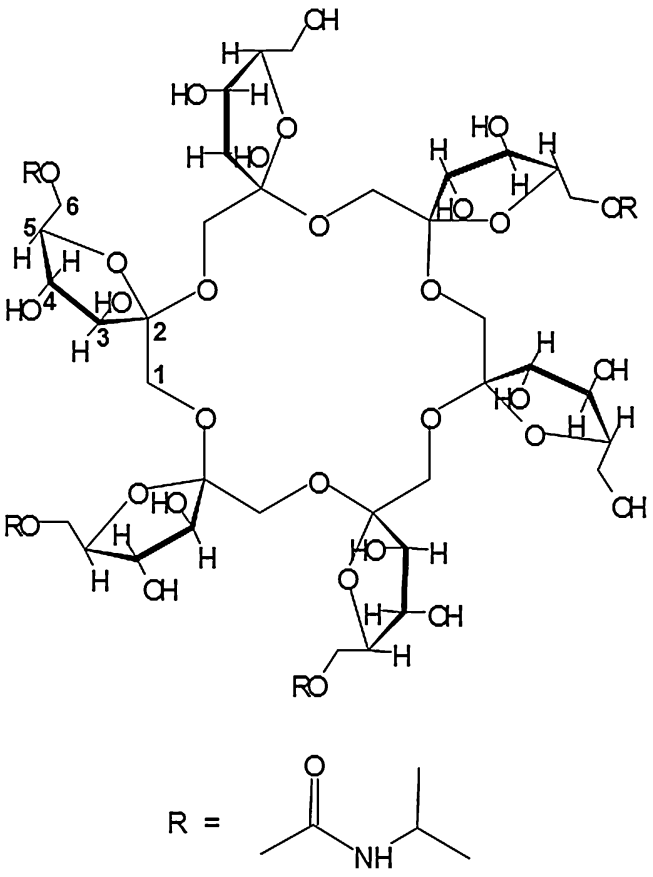

Fig. 2. Structure of IP-CF6 with a degree of substitution of four.

2. Materials and methods

2.1. Chemicals

Solvents of HPLC grade, acetonitrile (ACN) and methanol (MeOH), were purchased from Sigma–Aldrich (Steinheim, Germany). Trifluoroacetic acid (TFA) of 99.8% purity was product of Merck (Darmstadt, Germany). Boric acid, phosphoric acid, sodium hydroxide, ammonium acetate, *R,S*-BNP, *R,S*-BBP, *R,S*-VAPOL, *R,S*-BINOL, *R,S*-DABN and barium acetate, all p.a. purity, were purchased from Sigma (St. Louis, MO, USA). IP-CF6 was obtained from AZYP (Arlington, TX, USA).

2.2. Equipment and experimental conditions

2.2.1. HPLC

Chromatographic column, 250 mm × 4.6 mm ID; based on isopropyl-cyclofructan-6 (commercial name: LARIHC-CF6-P) bonded on silica gel support, particle size 5 μm, was obtained from AZYP (Arlington, TX, USA).

The HPLC used was an Agilent 1200 HPLC (Agilent Technologies, Palo Alto, CA, USA) consisting of a diode array detector, a temperature controlled column chamber, auto sampler, quaternary pump and fraction collector. Data acquisition and analysis were controlled by ChemStation software (Rev. B.03.02[341], Agilent Technologies 2001–2008) in Microsoft Windows XP Professional OS. Unless stated otherwise, all HPLC separations were carried out at 25 °C with an injection volume of 5 μL and a flow rate of 1.0 mL/min (isocratic). The following UV wavelengths were monitored: 230, 254, 265 and 280 nm.

MeOH was used in the volume range of 20–60% with 20 mM ammonium acetate buffer (pH 4.5) for MS compatibility. Barium

Table 1
Chromatographic parameters; k_1, retention factor of the first eluted atropisomer; α, selectivity; R_s, resolution; for RP separation of atropisomers on IP-CF6 CSP with and without barium acetate present in the mobile phase. Mobile phase: MeOH/buffer, buffer: 20 mM ammonium acetate, pH 4.5.

Compound	Mobile phase	k_1	α	R_s	1st eluting atropisomer
No barium acetate added					
BINOL	20/80 MeOH/buffer	6.43	1.12	1.5	*R*
DABN	30/70 MeOH/buffer	5.15	1.19	2.4	*S*
BNP	30/70 MeOH/buffer	2.64	1.20	2.0	*R*
BBP	40/60 MeOH/buffer	5.04	1.44	3.3	*R*
VAPOL	40/60 MeOH/buffer	7.54	1.56	3.3	*R*
5 mM barium acetate added to buffer					
BINOL	20/80 MeOH/buffer	5.59	1.11	1.4	*R*
DABN	30/70 MeOH/buffer	4.52	1.18	2.3	*S*
BNP	30/70 MeOH/buffer	6.39	1.17	2.5	*R*
BBP	40/60 MeOH/buffer	13.09	1.37	3.9	*R*
VAPOL	40/60 MeOH/buffer	No elution within 60 min			

acetate was added to the mobile phase at a concentration of 5 mM. Columns were conditioned for at least 30 min prior to sample injection. Then the dynamic coating yielded stabile separation conditions. (The stationary phase could be easily recovered by washing the column with aqueous 0.1 M ammonium acetate solution for 30 min. After this procedure the complexed Ba^{2+} was entirely displaced.)

The void volume was estimated by the first disturbance in the baseline which was at t_0 = 3.0 min.

The concentrations of sample solutions were 1 mg/mL in MeOH. All measurements were repeated three times.

2.2.2. CE

All CE experiments were carried out using a HP3D CE, Agilent Technologies (Waldbronn, Germany) system equipped with a DAD. Bare-fused-silica capillary (50 μm ID × 365 μm OD, 33 cm the total length, 24.5 cm to the detector) was maintained at 25 °C during analysis. UV detection was accomplished at 214 nm. The capillary was initially conditioned by rinsing with 1 M sodium hydroxide for 15 min, water for 15 min and with running electrolyte. Between each sample run the capillary was flushed with 1 M sodium hydroxide for 2 min, water for 2 min and running electrolyte for 2 min. Separations were performed at negative polarity −15 kV if acidic buffer pH 2.5 was used, and normal polarity voltage +15 kV in case of alkaline buffer pH 10.0. Samples were injected hydrodynamically at 50 mbar for 5 s.

Phosphate and borate buffers were tested in the concentration range of 10–100 mM. IP-CF6 chiral selector was added as the last BGE component in the 10–80 mM range. Barium acetate was added up to 8 mM concentration in BGE in experiments specified later. Stock solutions (5 mg/mL) of the standards were prepared and diluted with methanol. All measurements were performed three times.

3. Results and discussion

3.1. HPLC

Though only few papers mention the use of cyclofructan CSPs in the RP HPLC mode [1,8], here it proved to be excellent for the enantiomeric separation of the selected atropisomers of binaphthyl derivatives (Table 1). A mobile phase composition of 20 mM ammonium acetate buffer, pH 4.5, and, preferably, methanol afforded the best resolution values. All analytes exhibited typical RP retention behavior, i.e. increasing organic modifier in the mobile phase resulted in decreased retention, which was mostly accompanied by reduced resolution values. Baseline (or near-baseline) separation of all five atropisomers was obtained under optimized conditions (Table 1 and Fig. 3A).

It was already pointed out in our previous paper dealing with chiral separation on CF CSPs in NP HPLC [9] that the substituents in close vicinity of the axis of atropisomers play an important role in the chiral discrimination process. However, it must be taken into account that interactions participating in the separation process differ in NP and RP separation modes of HPLC.

The effect of adding Ba^{2+} to the mobile phase on retention, enantioselectivity and resolution was investigated subsequently (Table 1). Illustrative chromatograms in Fig. 3 compare the separation of the selected atropisomers without (Fig. 3A) and with (Fig. 3B) Ba^{2+} present in the mobile phase. In the case of BINOL and DABN, the presence of Ba^{2+} decreases retention slightly and has negligible effect on resolution. However, for the phosphates (BNP, BBP, VAPOL), the presence of Ba^{2+} in the mobile phase increases retention. (VAPOL did not eluted within 60 min in the comparable

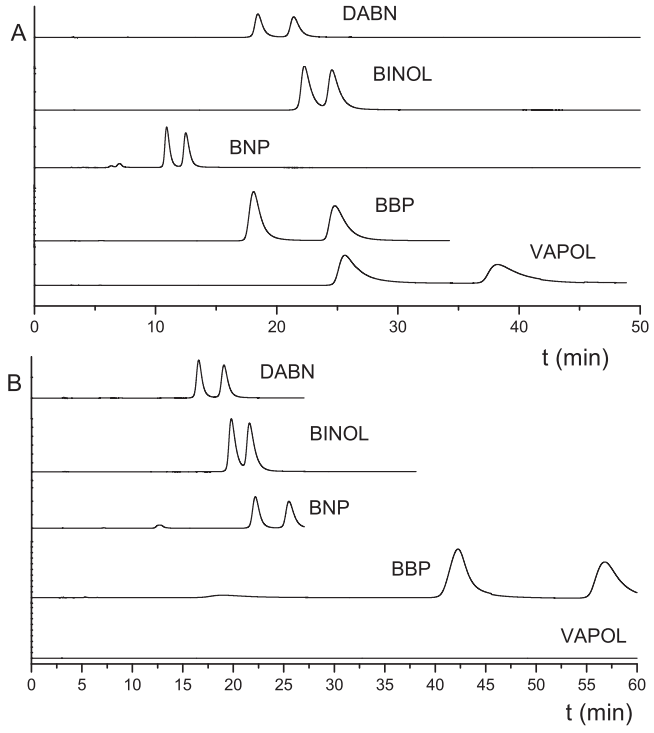

Fig. 3. Comparison of separations of the atropisomers using mobile phases without addition of Ba^{2+} (A) and with Ba^{2+} (B). Mobile phase compositions for the individual analytes separated are as follows: DABN using MeOH/buffer 30/70 (v/v), BINOL using MeOH/buffer 20/80 (v/v), BNP using MeOH/buffer 30/70 (v/v), BBP and VAPOL using MeOH/buffer 40/60 (v/v). Buffer: 20 mM ammonium acetate, pH 4.5. *Note*: VAPOL did not elute in the mobile phase with Ba^{2+} until 60 min.

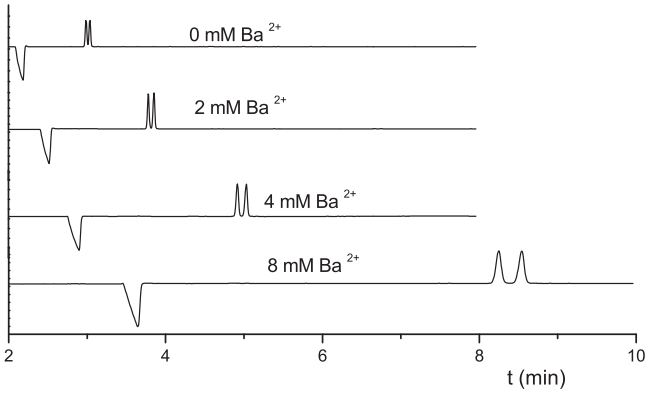

Fig. 4. Effect of barium acetate addition to BGE on enantioseparation of *R,S*-BNP in 100 mM sodium borate, pH 10.0, with addition of 20 mM IP-CF6. U = +15 kV, injection 50 mbar/5 s, λ = 214 nm.

mobile phase.). For BNP and BBP the enantioselectivity decreased slightly but overall resolution was better due to increased retention. The observed retention for the above analytes may be explained as follows: when the neutral CF macrocycle is complexed by Ba^{2+}, it is transformed into a cationic macrocycle. Accordingly the anionic phosphates undergo ionic interactions with the Ba^{2+} cation and are retained longer [16]. BINOL and DABN are relatively unchanged, and so have less opportunity to interact with the positively charged macrocycle.

The order of enantiomeric elution remained unchanged regardless of the presence of barium (see Table 1).

3.2. CE

While all atropisomers could be almost baseline resolved in RP HPLC, in CE the only baseline separation achieved was for *R,S*-BNP. The elution order was opposite to that obtained in RP HPLC and also remained unchanged regardless of the presence of barium. A partial enantiomeric separation was also obtained for *R,S*-VAPOL (R_s = 0.5). Significantly worse enantiomeric separations were observed for CE than for RP HPLC. This confirmed the presumption that structural rigidity, present in the HPLC CSP but absent in CE, was necessary for chiral recognition. Thus we focused only on optimizing the *R,S*-BNP separation.

The most promising BGEs were 50 mM sodium phosphate, pH 2.5, as acidic BGE, and 100 mM sodium borate, pH 10.0, as alkaline electrolyte. The best results yielded the alkaline BGE so, it was studied in more detail. The increasing amount of IP-CF6 in BGEs led to increased migration times and improved resolution of the separated atropisomers. Concentration of 20 mM IP-CF6 was sufficient to reach baseline resolution of *R,S*-BNP. Moreover, the concentrations of the IP-CF6 and BGE were shown to be related to each other. At higher buffer concentration (100 mM) lower amount of the IP-CF6 was sufficient to achieve baseline separation. At a given concentration of the IP-CF6 an increase of the borate concentration resulted in increased migration time and resolution.

The influence of Ba^{2+} on resolution of *R,S*-BNP under the optimized (alkaline) separation conditions also was considered. Strong interaction of Ba^{2+} with CF6 in CE system has been described already before [12]. In our study the addition of Ba^{2+} to the BGE led to substantial improvement of chiral resolution. The results of separation of *R,S*-BNP achieved by CE measurements correspond with those obtained in RP HPLC. The electropherograms showing the influence of addition of Ba^{2+} to BGE with 20 mM of IP-CF6 are depicted in Fig. 4. As a side effect, decreases in the electroosmotic flow with increasing amounts of Ba^{2+} in BGE were observed. We can speculate that incorporation of Ba^{2+} into the IP-CF6 core makes the chiral selector's structure more rigid, in addition to being cationic, and so more amenable for chiral recognition with anionic analytes. The results obtained in this study support this hypothesis.

4. Conclusion

The isopropyl derivative of cyclofructan–6 was shown to be a promising neutral chiral selector for the chiral separations of the selected atropisomers of binaphthyl derivatives. While baseline separation was obtained for all analytes in HPLC, the only baseline separation achieved in CE was for *R,S*-BNP. Interestingly, when Ba^{2+} was added to the mobile phase the retention for the phosphate atropisomers increased and resolution for atropisomers of BBP and BNP improved. This effect was observed in both HPLC, with IP-CF6 CSP, and in CE, where the same chiral selector was added to BGE. Further experiments must be carried out in order to get better understanding of the separation mechanism.

Acknowledgement

The authors acknowledge financial support of the KONTAKTAM 2010 project LH11018.

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5.5 Publikace VI - Chirální separace chlorthalidonu metodou SFC

Vývoj metody pro separaci enantiomerů je prvotním krokem pro klinické testy, které se zaměřují na odlišné biologické vlastnosti jednotlivých enantiomerních forem v živých systémech. HPLC poskytuje velmi účinné chirální separace, nicméně je v současné době často nahrazována SFC. **Publikace VI** je zaměřena na enantioselektivní separaci chlorthalidonu, látky používané pro léčbu hypertenze a edémů. V rámci vývoje metody byly použity dvě cyklofruktanové CSP, DMP-CF7 CSP a IP-CF6 CSP, dále dvě CSP na bázi polysacharidů, Chiralpak AD s amylosou derivatizovanou tris(3,5-dimethylfenyl karbamátem) jako CS a Chiralcel OD-H, kde je CS tvořen celulosou s tris(3,5-dimethylfenyl karbamátem).

Na základě podrobné literární rešerše a prvotního testování různých CSP byla pro další optimalizaci separace vybrána kolona Chiralpak AD.

Optimalizovaný separační systém byl tvořen kolonou Chiralpak AD a mobilní fází CO_2/MeOH 50/50 (*v/v*), průtok 4 ml/min, teplota 40 °C, tlaková restrikce 120 bar. Za těchto podmínek bylo dosaženo enantioseparace na základní linii s následujícími parametry: retenční faktor prvního eluujícího enantiomeru k_1 = 0,90; faktor enantioselektivity α = 1,71; rozlišení Rs = 2,61. Dále byly stanoveny základní validační parametry - limit detekce, limit kvantifikace, robustnost, lineární rozsah, opakovatelnost a reprodukovatelnost. Navržená metoda poskytuje jednoznačně nejrychlejší separaci enantiomerů chlorthalidonu (do 2,5 min) v porovnání s publikovanými HPLC a SFC metodami.

Publikace VI

Rapid Supercritical Fluid Chromatography Method for Separation of Chlorthalidone Enantiomers

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Analytical Letters **2013**, 46, 2860–2869

Analytical Letters, 46: 2860–2869, 2013

ISSN: 0003-2719 print/1532-236X online
DOI: 10.1080/00032719.2013.814058

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Chromatography

RAPID SUPERCRITICAL FLUID CHROMATOGRAPHY METHOD FOR SEPARATION OF CHLORTHALIDONE ENANTIOMERS

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Supercritical fluid chromatography employing chiral stationary phases is a popular separation technique to perform enantioselective separations. The main advantages of supercritical fluid chromatography are low analysis time, low consumption of organic modifiers, and therefore lower costs and higher environmental friendliness. A novel method for the separation of chlorthalidone enantiomers, widely used diuretic drug, is reported that clearly demonstrates the advantages of supercritical fluid chromatography. The effects of the amount and type of organic modifiers, temperature, and back pressure on enantioselectivity and resolution of the enantiomers were evaluated. The baseline separation was achieved in less than 2.5 min in the optimized system composed of Chiralpak AD column, mobile phase CO_2/ MeOH 50/50 (v/v), temperature 40° C, a flow rate of 4.0 mL/min, and 120 bar back pressure. Moreover, enantiomers of chlorthalidone were determined in two commercially available pharmaceuticals. The proposed method may be easily transferred to a semi-preparative scale.

Keywords: Chiral separation; Chlorthalidone; Saccharide based chiral stationary phase; SFC

INTRODUCTION

Single enantiomeric forms can show different biological activities that can cause unexpected and often very serious consequences in organisms. Therefore, enantioselective separations and reliable procedure for determination of single enantiomers are

Received 29 April 2013; accepted 4 June 2013.
The authors have declared no conflict of interest.
The Grant Agency of the Charles University in Prague, project No. 356411; the Ministry of Education, Youth, and Sports of the Czech Republic, project KONTAKT LH11018; and the long-term project MSM0021620857 are gratefully acknowledged for financial support. Prof. M. Schmid from the Department of Pharmaceutical Chemistry, University of Graz, is thanked for his generous gift of chlorthalidone. Furthermore, Prof. Ch. Roussel and Dr. N. Vanthuyne from the Chirosciences team of Aix-Marseille University are thanked for their kind help during the SFC experiments. The international cooperation between Charles University in Prague and Aix-Marseille University was established in the frame of the LPP - ERASMUS PROGRAMME.
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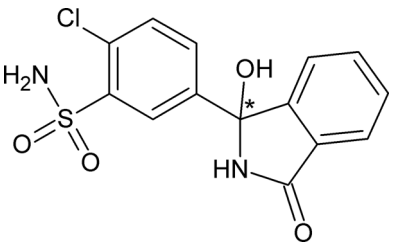

Figure 1. Chemical structure of chlorthalidone.

very significant in the pharmaceutical industry. High performance liquid chromatography (HPLC) using chiral stationary phases (CSPs) can be considered the primary technique that enables separation of enantiomers in analytical, semi-preparative, and preparative scales (Cavazzini et al. 2011; Chankvetadze 2012). However, another technique, supercritical fluid chromatography (SFC), is becoming more and more popular in this field (R. Wang, Ong, Tang, and Ng 2012a; De Klerck, Mangelings, and Heyden 2012). SFC utilizes carbon dioxide in a supercritical state as the main component of the mobile phase. The advantages of supercritical fluids over liquids are low viscosity, better diffusion properties, and a relatively low price (Lesellier 2009). Supercritical mobile phases modified with commonly used organic modifiers offer faster analysis and therefore, a lower consumption of organic solvents. An additional benefit of the use of supercritical mobile phases is the ease of collection of concentrated fractions in a semi-preparative mode (Miller 2012). On the other hand, the construction of a SFC system has had technical difficulties (mainly back pressure regulation and overall more sophisticated hardware demands) in the past. These drawbacks have been already resolved (Taylor 2009).

As a demonstration of the benefit of SFC for chiral separations, we introduce an analytical method for separation of chlorthalidone (CT) enantiomers. Chlorthalidone, (RS)-2-chloro-5-(1-hydroxy-3-oxo-2,3-dihydro-1H-isoindol-1-yl)benzene-1-sulfonamide (see Figure 1), is a diuretic drug widely used for a treatment of hypertension and edema (Sweetman 2009). To the best of our knowledge, an analytical SFC method for enantioseparation of chlorthalidone containing optimization and validation processes has not been published yet. The proposed SFC method is compared with respect to basic chromatographic parameters with HPLC and SFC analysis data obtained from the literature. A basic validation of the SFC method was also performed.

EXPERIMENTAL

Materials

Organic solvents of HPLC grade propan-2-ol (isopropanol, IPA), ethanol (EtOH), and methanol (MeOH), were purchased from Sigma-Aldrich (Steinheim, Germany).

The carbon dioxide used for SFC from Air Liquide (Paris, France) was Alphagaz CO_2 SFC, L50TP, purity : 99.998% with maximum impurities : $H_2O < 5$ ppm, $O_2 < 2$ ppm, $CO < 5$ ppm, $H_2 < 0.5$ ppm, $C_nH_m < 2$ ppm, $NO + NO_x < 2$ ppm and

total sulfur < 1 ppm. Chlorthalidone of analytical grade purity was provided by Prof. Martin Schmid from the Department of Pharmaceutical Chemistry, University of Graz (Austria). The analyzed tables Amicloton and Tenoretic were products of Zentiva (Hlohovec, Slovakia) and Astra Zeneca (Macclesfield, Cheshire, Great Britain), respectively.

Instrumentation

Overall four chiral columns were tested. Two columns were based on derivatized cyclofructan, namely dimethylphenyl carbamate cyclofructan 7 and isopropyl carbamate cyclofructan 6 bonded to silica gel support, Larihc CF7-DMP and Larihc CF6-P, respectively, both from AZYP (Arlington, TX, USA). Two polysaccharide based CSPs, Chiralpak AD, amylose tris(3,5-dimethylphenyl carbamate), and Chiralcel OD-H, cellulose tris(3,5-dimethylphenyl carbamate) bonded on silica gel, both products of Chiral Technologies Europe (Illkirch, France) were examined. The dimensions of the first three columns were 250 mm × 4.6 mm i.d.; particle size 5 μm, while the dimensions of the last column were of 150 mm × 4.6 mm i.d.; particle size 5 μm.

The SFC measurements were achieved on a system SFC-PicLab Analytic from Pic Solution (Avignon, France). The amount of the co-solvent in the mobile phase was adjusted by a piston pump, the co-solvent was directly added in the CO_2 feeding, and the mixture of co-solvent and CO_2 was pumped by another piston at a total flow rate of 4 mL/min. The head of this pump was cooled to –7°C by a cryostat. The SFC equipment also contained an autosampler, oven, UV DAD detector, and a back-pressure regulator to control the outlet pressure. The outlet tube was heated to 55°C to avoid ice formation during the carbon dioxide depressurization.

The temperature was maintained at 40°C, and the back pressure was set at 120 bar with the exception of temperature and back pressure optimization processes. The injection volume was 20 μL. The UV detection was performed at 254 nm. The void volume was determined using the solvent peak. All retention measurements were carried out in triplicate and from these data retention factors were calculated.

Data were recorded with SFC PicLab Analytic Online 3.1.2 and processed with Analytic Offline 3.2.0. The one-way ANOVA analysis was carried out using NCSS software (Kaysville, USA) (Hintze 2007).

Standard Stock Solutions

The chlorthalidone racemic standard stock solution was prepared at concentration 1 mg/mL using methanol as a sample solvent. The other solutions were prepared from the stock solution by dilution with methanol to appropriate concentrations. The stock solution was stored in the refrigerator.

Validation of the Method

Basic validation was carried out according to the ICH guidelines (ICH 2012) with respect to following parameters. The linearity was studied over the concentration range of 0.05–2.5 mg/mL for both chlorthalidone enantiomers. All measurements

were carried out in triplicate and all the values of peak areas were subject to linear regression (peak area vs. the corresponding concentration).

The limit of detection (LOD) was calculated from the peak heights and was expressed as the concentration at a signal-to-noise ratio of 3:1. The baseline noise was recorded over a period approximately ten times the widths of the peaks. Similarly, the limit of quantification (LOQ) was taken as the concentration of analyte where signal-to-noise ratio was 10:1.

The precision of the method was verified by repeatability and reproducibility of measurements. The repeatabilities of the retention factor, concentration, and enantioselectivity values were determined as relative standard deviations (RSD) for 10 consecutive injections of the racemate solutions at the concentration levels of 0.10 mg/mL, 0.50 mg/mL, and 2.50 mg/mL for both enantiomers. The reproducibilities of retention factors, concentrations, and enantioselectivities were measured within two days at the same concentration levels as in case of repeatabilities, by two analysts on two different SFC equipments and were determined again as RSD values.

The one-way analysis of variance (ANOVA) statistical method was used for robustness testing. Selected variable parameters were methanol content ($50 \pm 1\%$) and temperature ($40 \pm 1°C$). The effects of method parameters on peak areas, peak heights and enantioselectivity were evaluated. The robustness was determined for 10 consecutive injections of both enantiomers, each at concentration level of 0.50 mg/mL.

To evaluate the accuracy of the method two different tablets (Amicloton, Tenoretic) both containing 25 mg of chlorthalidone were analyzed. Tablets were powdered and diluted in methanol to concentration of 1.00 mg/mL. The concentration of the chlorthalidone enantiomers was determined using the optimized SFC method.

RESULTS AND DISCUSSION

Method Optimization

In the frame of the method development and optimization overall four chiral columns were tested: Larihc CF7-DMP and Larihc CF6-P, based on derivatized cyclofructans and Chiralcel OD-H and Chiralpak AD columns, based on cellulose and amylose derivatives, respectively. Three organic modifiers isopropyl alcohol, ethanol, and methanol were evaluated in a volume range 5–50%. The influences of the back pressure in the range of 120–180 bar and the temperature in the range of 35–45°C on the chromatographic process were also studied to find the optimal separation conditions.

Both polysaccharide based CSPs performed good separations, whereas no enantioseparation was achieved using the cyclofructan based CSPs. The Chiralpak AD column was the best for separation of chlorthalidone enantiomers due to better resolution values and lower retention times. The comparison of alcohol modifiers revealed that the most suitable mobile phases contained MeOH (see Figure 2). The analysis time of mobile phases that contained less than 20% MeOH was higher than 15 min. The analysis time was significantly shortened by increasing of methanol content. The baseline separation ($R_s = 2.61$) was achieved in the mobile phase composed of CO_2/MeOH 50/50 (v/v) in less than 2.5 min at a flow rate of 4 mL/min, a temperature 40°C, and a back pressure of 120 bar. By increasing the back pressure,

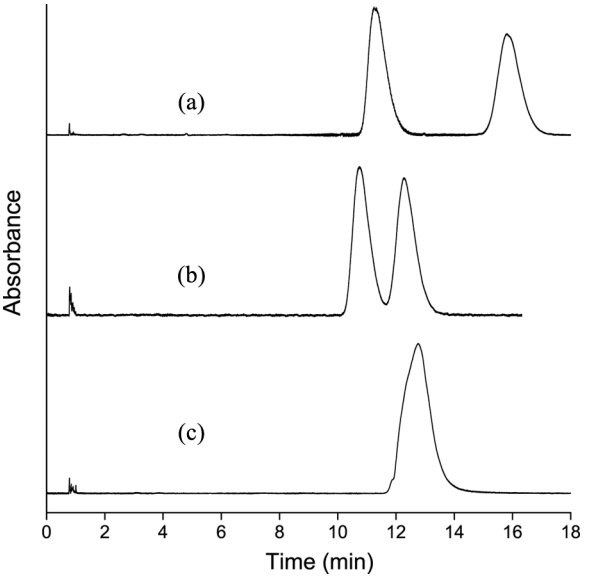

Figure 2. The influence of the organic modifier type on Chiralpak AD column. Mobile phase compositions CO_2/organic modifier 80/20 (*v*/*v*), (A) methanol, (B) ethanol, (C) isopropyl alcohol, temperature 40°C, flow 4 mL/min, and back pressure 150 bar.

the retention time slightly decreased, which was accompanied by a reduction of the resolution value. The retention time and resolution values decreased with increasing temperature. The optimal temperature was determined at 40°C. Table 1 shows the effect of back pressure and temperature on enantioselectivity and resolution values. Both were measured in mobile phase composed of CO_2/MeOH 50/50 (*v*/*v*). For pressure measurements, the temperature was constantly 40°C, whereas for temperature measurements the back pressure was 120 bar.

The chromatogram obtained under the optimized separation conditions is shown in Figure 3. The resolution and enantioselectivity values of chlorthalidone enantiomers were $R_s = 2.61$ and $\alpha = 1.71$, respectively, and the retention factor of the first eluted enantiomer $k_1 = 0.90$. The previous HPLC and SFC enantioselective separations of chlorthalidone using CSPs, which are summarized in Table 2, clearly indicate that the proposed SFC method provides the highest enantioselectivity, one of the highest

Table 1. Effect of back pressure and temperature on enantioselectivity and resolution values

	Back pressure (bar)			Temperature (°C)		
	120	150	180	35	40	45
α	1.71	1.63	1.42	1.77	1.71	1.59
Rs	2.61	2.26	1.91	2.72	2.61	2.49

Note: Chiralpak AD column, mobile phase CO_2/MeOH 50/50 (*v*/*v*), flow 4 mL/min, 40°C for the pressure measurements, 120 bar for the temperature measurements. α, enantioselectivity; *Rs*, resolution.

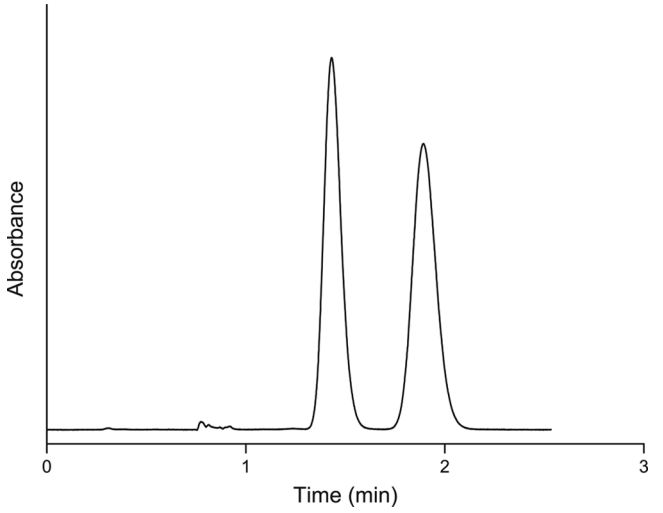

Figure 3. Chiral separation of chlorthalidone enantiomers under the optimized separation conditions. Chiralpak AD column, mobile phase composition CO_2/methanol 50/50 (*v*/*v*), temperature 40°C, flow rate 4.0 mL/min, and back pressure 120 bar.

resolution values, and an analysis time of less than 2.5 minutes. The enantioselective SFC separation of CT performed by Liu et al. (2002) provided higher enantioselectivity and resolution values $\alpha = 1.82$ and $R_s = 3.60$; however, the analysis time (retention time of the first eluted enantiomer was 4.72 min) is much higher than in our SFC method.

Linear Range

A graph of the peak areas plotted against the concentrations of chlorthalidone enantiomers was linear in the studied concentration range (0.05–2.50 mg/mL). Overall 10 points were used for linear regression. The resulting linear regression equations were as follows:

$$\text{Enantiomer 1 } Y = 1667.7X - 13.765;\ R^2 = 0.9999$$

$$\text{Enantiomer 2 } Y = 1692.6X - 20.904;\ R^2 = 0.9997$$

where X is the concentration of the enantiomer (mg/mL), Y is the peak area (mV s), and R^2 is the coefficient of determination.

Precision

Table 3 summarizes verification of the repeatability and the reproducibility of the method at three concentration levels. The results suggest that the method is suitable for both qualitative and quantitative analysis of the chlorthalidone enantiomers.

Table 2. HPLC and SFC separations of CT enantiomers obtained from the literature research since 2000 until now

	Chiral stationary phase	Mobile phase composition	k_1	α	Rs	Reference
HPLC	azide-modified β-CD	ACN/H_2O (0.5% HAc) 8/92 (v/v)	3.03	1.33	1.71	Y. Zhang et al. 2008
HPLC	Chiralcel OD-H, cellulose tris(3,5-dimethylphenyl carbamate)	hept/EtOH/TFA 90/10/0.1 ($v/v/v$)	–	–	0.93	Younes, Mangelings, and Heyden 2011
HPLC	Sepapak-5, cellulose tris(3,5-dichlorophenyl carbamate)	ACN/EtOH 95/5 (v/v)	–	–	1.33	Ates et al. 2013
HPLC	Chiralpak IC, cellulose tris(3,5-dichlorophenyl carbamate)	methyl t-butyl ether/hex 80/20 (v/v)	1.65	1.64	4.19	T. Zhang et al. 2008
HPLC	6^A-(3-vinylimidazolium)-6-deoxyperphenylcarbamoyl-β-CD chloride	hex/IPA 70/30 (v/v)	–	1.50	1.04	R. Wang, Ong, Tang, and Ng 2012b
HPLC	6^A-(*N,N*-allylmethylammonium)-6-deoxyperphenylcarbamoyl-β-CD chloride	hex/IPA 70/30 (v/v)	–	1.27	0.67	R. Wang, Ong, Tang, and Ng 2012b
HPLC	2,4-dinitrophenyl ether substituted β-CD, carbamate linkage	MeOH/TEAA(pH 4.1) 30/70 (v/v)	1.10	1.44	1.45	Zhong et al. 2006
HPLC	3,4-dinitrophenyl ether substituted β-CD, ether linkage	MeOH/TEAA (pH 4.1)15/85 (v/v)	4.89	1.45	2.46	Zhong et al. 2006
HPLC	2,6-dinitro-4-(trifluoromethyl)phenyl ether substituted β-CD, carbamate linkage	ACN/TEAA (pH 4.1) 15/85 (v/v)	3.29	1.20	1.35	Zhong et al. 2006
HPLC	2,6-dinitro-4-(trifluoromethyl)phenyl ether substituted β-CD, ether linkage	ACN/TEAA (pH 4.1)15/85 (v/v)	1.66	1.10	1.50	Zhong et al. 2006
HPLC	2,4-dinitro-6-(trifluoromethyl)phenyl ether substituted β-CD, ether linkage	ACN/TEAA (pH 4.1) 5/95 (v/v)	4.68	1.12	0.80	Zhong et al. 2006
HPLC	2,4-dinitrophenyl ether substituted β-CD, carbamate linkage	hept/IPA/TFA 50/50/0.1($v/v/v$)	2.76	1.13	0.84	Zhong et al. 2006
HPLC	CD-click sil, azide-modified β-CD bonded to poly(2-methyl-3-butyl-2-ol methacrylate)	MeOH/TEAA (pH 4.9) 10/90 (v/v)	9.60	1.44	1.58	H. Wang et al. 2011
HPLC	CD-click RAM, CD-click sil derivatized by poly(glycidyl methacrylate)	MeOH/TEAA (pH 4.9) 10/90 (v/v)	8.13	1.29	1.34	H. Wang et al. 2011
HPLC	Chirobiotic T, teicoplanin	MeOH/H_2O 20/80 (v/v)	2.73	1.14	1.98	Visegrády et al. 2002
HPLC	Sepapak-2, cellulose tris(3-chloro-4-methylphenyl carbamate)	ACN/DEA/TFA 100/0.1/0.1 ($v/v/v$)	0.47	1.24	1.30	Dossou et al. 2012
SFC	Chirobiotic R, ristocetin	CO_2/MeOH 70/30 (v/v)	–	1.08	1.00	Liu et al. 2002
SFC	Chirobiotic TAG, teicoplanin aglycone	CO_2/MeOH 55/45 (v/v)	–	1.82	3.60	Liu et al. 2002
SFC	Chirobiotic T, teicoplanin	CO_2/MeOH/TEA/TFA 60/40/0.1/0.1 ($v/v/v/v$)	–	1.37	2.90	Liu et al. 2002
SFC	6^A-(3-vinylimidazolium)-6-deoxyperphenyl carbamate-β-CD chloride	CO_2/MeOH 90/10 (v/v)	–	1.29	2.44	R. Wang, Ong and Ng 2012

ACN, acetonitrile; CD, cyclodextrin; DEA, diethylamine; HAc, acetic acid; hex, n-hexane; hept, n-heptane; TEA, triethylamine; TEAA, triethylammonium acetate buffer; TFA, trifluoroacetic acid; - the chromatographic parameter was not available.

Table 3. Repeatability and reproducibility, expressed as relative standard deviation (RSD) values of retention factors, selectivities and concentrations at three different concentration levels

	Repeatability								
	R.S.D. %	(*k*)		R.S.D. %	(c)		R.S.D. %	(α)	
c (mg/mL)	0.10	0.50	2.50	0.10	0.50	2.50	0.10	0.50	2.50
Enantiomer 1	0.23	0.47	0.15	0.47	0.49	0.19	0.41	0.42	0.39
Enantiomer 2	0.53	0.48	0.49	0.74	0.74	0.24			
	Reproducibility								
	R.S.D. %	(*k*)		R.S.D. %	(c)		R.S.D. %	(α)	
c (mg/mL)	0.10	0.50	2.50	0.10	0.50	2.50	0.10	0.50	2.50
Enantiomer 1	0.35	0.42	0.40	0.43	0.42	0.28	0.38	0.36	0.39
Enantiomer 2	0.58	0.53	0.50	0.78	0.75	0.62			

Note: *k*, retention factor; c, concentration; α, enantioselectivity.

Limit of Detection and Limit of Quantification

The limits of detection (LOD) determined at wavelength 254 nm were 7.9 μg/mL for the first chlorthalidone enantiomer and 10.9 μg/mL for the second enantiomer. The limits of quantitation (LOQ) at the same wavelength were 26.4 μg/mL for the first enantiomer and 36.2 μg/mL for the second one. These values are somewhat high, which is related to the detector type. However, the determined LOD and LOQ values are sufficient from the point of view that a 1% impurity was detected.

Method Robustness

The hypothesis that errors resulted from a normal distribution was tested first. This hypothesis can be accepted in all cases at significance level $\alpha = 0.05$. Consequently, the robustness of the method was examined using the one-way ANOVA. The impact of methanol content ($50 \pm 1\%$) and the temperature ($40 \pm 1°C$) on peak areas, peak heights, and enantioselectivity were evaluated. The calculated *p*-values are summarized in Table 4. The proposed analytical method for determination of chlorthalidone enantiomers was proven to be robust to all the variations tested in this work because the resulting *p*-values were higher than the significance level $\alpha = 0.05$.

Table 4. Statistical *p*-values obtained from one-way ANOVA

	p-values				
	$A(E_1)$	$A(E_2)$	$H(E_1)$	$H(E_2)$	α
Temperature	0.10	0.25	0.81	0.46	0.17
MeOH content	0.15	0.28	0.63	0.57	0.06

A, peak area; *H*, peak height; E_1, first eluted enantiomer; E_2, second eluted enantiomer; α, enantioselectivity.

Accuracy

For accuracy testing, two commercially available drugs were analyzed. The accuracy of the method, regarded as the closeness of the agreement between the claimed contents of the active components in the Amicloton tablet, was found to be 103.3% for the first chlorthalidone enantiomer and 103.5% for the second one. The accuracy of the method for the Tenoretic tablet was found 102.3% for the first chlorthalidone enantiomer and 102.2% for the second enantiomer.

CONCLUSIONS

SFC can be considered a promising alternative to HPLC because it enables fast analysis along with low consumption of organic modifiers, and saves analysis time and reagent costs. As a good example of this statement a simple, rapid, and robust method for separation of chlorthalidone enantiomers was introduced in this work. The baseline separation of the enantiomers was achieved in less than 2.5 min using Chiralpak AD column, a mobile phase composed of CO_2/MeOH 50/50 (v/v) at flow rate 4 mL/min, a temperature 40°C, and a back pressure 120 bar. The basic validation parameters were evaluated. The comparison of our method with those obtained from the literature clearly demonstrates the advantage of SFC. Despite the absence of particular retention times in the literature, it can be assumed from the given retention factors and enantioselectivity values that the SFC method provides one of the fastest baseline separations of chlorthalidone enantiomers. In summary, the proposed SFC method offers fast separation with high resolution of chlorthalidone enantiomers, which can be easily transferred to a semi-preparative scale.

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5.6 Publikace VII - Chirální separace citalopramu a jeho prekurzoru citadiolu metodami HPLC a SFC

Citalopram tvoří aktivní složku preparátů předepisovaných k léčbě deprese, panické úzkosti či kompulzivní obsesivní poruchy. Z jednotlivých enantiomerních forem citalopramu je pouze *S*-enantiomer aktivní, zatímco *R*-enantiomer nevykazuje odpovídající biologickou aktivitu a do jisté míry působí proti *S*-enantiomeru. V rámci **Publikace VII** je navržena metoda pro separaci enantiomerů citalopramu a jeho syntetického prekurzoru citadiolu.

Pro vývoj separační metody bylo testováno celkem šest CSP v HPLC. CSP na bázi makrocyklických antibiotik - vankomycinu (Chirobiotic V, Chirobiotic V2) a teikoplanin aglykonu (Chirobiotic TAG), které byly testovány v POM HPLC společně s CSP na bázi cyklofruktanu DMP-CF7 (Larihc DMP-CF7). Polysacharidové CSP na bázi celulosy byly testovány v NP (Chiralcel OD-H) a RP (Chiralcel OD-RH) HPLC. Dále byly testovány tři CSP v podmínkách SFC, a to CSP na bázi celulosy (Chiralcel OD-H), kolona Chiralpak AD na bázi amylosy a kolona Larihc DMP-CF7 na bázi cyklofruktanu.

Na základě prvotních HPLC experimentů byla vybrána pro optimalizaci a následnou validaci kolona Chiralcel OD-H v podmínkách NP HPLC. V rámci SFC experimentů bylo dosaženo separace na základní linii pouze pro enantiomery citadiolu.

Optimalizovaný separační systém byl tvořen kolonou Chiralcel OD-H a mobilní fází hex/IPA/TEA 96/4/0,1 (*v/v/v*), průtok 1 ml/min, teplota 25 °C. Za těchto podmínek bylo dosaženo enantioseparace obou analytů na základní linii během jedné analýzy. Chromatografické parametry pro enantiomery citalopramu byly: retenční faktor prvního eluujícího enantiomeru k_1 = 3,45; faktor enantioselektivity α = 1,23; rozlišení *Rs* = 2,16. Pro enantiomery citadiolu byly retenční faktor prvního eluujícího enantiomeru k_1 = 5,96; faktor enantioselektivity α = 1,18; rozlišení *Rs* = 1,50. Pro optimalizovanou metodu byly stanoveny základní validační parametry - limit detekce, limit kvantifikace, robustnost, lineární rozsah, opakovatelnost a reprodukovatelnost. Dále byla ověřena aplikovatelnost metody pro stanovení enantiomerní čistoty léčiv.

Publikace VII

HPLC Method for Chiral Separation and Quantification of Antidepressant Citalopram and Its Precursor Citadiol

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Chromatographia **2013**, 76, 483-489

Chromatographia
DOI 10.1007/s10337-013-2426-6

ORIGINAL

HPLC Method for Chiral Separation and Quantification of Antidepressant Citalopram and Its Precursor Citadiol

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Received: 3 October 2012 / Revised: 1 February 2013 / Accepted: 8 February 2013

Abstract HPLC method enabling chiral separation and determination of citalopram (CIT), a widely used antidepressant, and its synthetic precursor citadiol in one analysis was developed and validated. Moreover, supercritical fluid chromatography was also tested and was proved to be less effective for this separation purpose. The optimized HPLC system was composed of Chiralcel OD-H column and *n*-hexane/propane-2-ol/triethylamine 96/4/0.1 (*v*/*v*/*v*) as mobile phase, column temperature 25 °C, flow rate 1.0 mL min^{-1}, UV detection at 250 nm. The effects of amount of propane-2-ol, triethylamine addition, and temperature on enantioselectivity and resolution of the enantiomers were evaluated. The method was found to be suitable for determination of the enantiomeric purity of CIT in bulk drugs. Enantiomers of CIT were determined in two commercially available pharmaceuticals.

Keywords HPLC · SFC · Chiral separations · Citalopram · Citadiol · Chiralcel OD-H column

Introduction

Citalopram (CIT), chemically 1-[3-(dimethylamino)propyl]-1-(4-fluorophenyl)-1,3-dihydro-5-isobenzofurancarbonitrile (Fig. 1), one of the widely used antidepressants from the selective serotonin reuptake inhibitors (SSRI) class, can serve as a typical demonstration of different biological activity of individual enantiomeric forms. While *S*-enantiomer of CIT (*S*-CIT), so-called escitalopram, has the mentioned biological activity, *R*-enantiomer is not active and even counteracts *S*-enantiomer. *S*-CIT is approximately twice as potent as CIT [1–6]. Drugs based on CIT are used for treatment of depression, panic anxiety or obsessive compulsive disorder of pathological laughing and crying. It has been demonstrated that enhancing serotonin neurotransmission may form the basis of the response to certain antidepressant treatments. SSRIs like CIT bounded to the serotonin transporter prevent reuptake of serotonin into neurons, and therefore is responsible for raising of extracellular concentration of serotonin in various brain regions [4, 7–10].

Nowadays, the growing trend in the pharmaceutical industry is to produce drugs in enantiomerically pure forms. However, CIT is commercially available as racemic drug, e.g. Seropram, as well as enantiomerically pure drug, e.g. Cipralex, containing only *S*-enantiomer of CIT as the active constituent. Chemical preparation of CIT is based on dehydration of citadiol (CTD), chemically 4-[4-(dimethylamino)-1-(4-fluorophenyl)-1-hydroxy-1-butyl]-3-(hydroxymethyl)benzonitrile (Fig. 1), chiral synthetic precursor of CIT [11, 12]. Consequently, the final enantiomeric purity of CIT depends on the enantiomeric composition of CTD used.

Mostly electrophoretic separation techniques were used for enantioselective separations of CIT and CTD enantiomers [13–15]. Just few papers consider HPLC. Various methods dealing with determination of CIT in biological matrix can be found in the literature. This issue is described in detail in recent reviews [16, 17] and in a recent research article [18]. Some papers deal with chiral HPLC separation of CIT [19, 20]. However, only few works are focused on analytical determination of CIT and/or CTD in pharmaceuticals. Raman et al. [21] presented a work dealing with a

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Published online: 23 February 2013

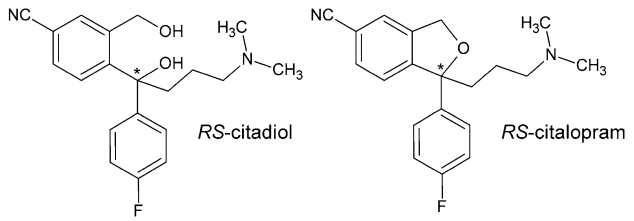

Fig. 1 Structures of the separated analytes

structural elucidation of process-related impurities in *S*-CIT by LC/ESI–MS and NMR. Solares et al. [12] determined enantiomeric excess for the acetyl derivative of CTD using Chiralcel OD column. Semreen [22] studied enantioselective potential of Chiralcel OC column for chiral separation of CIT enantiomers in pharmaceuticals. Rao et al. [23] introduced HPLC method for the determination of enantiomeric purity of CIT in bulk drugs and pharmaceuticals using Chiralcel OD-H (250 mm × 4.6 mm) column in normal phase mode HPLC (NP HPLC). The same column was tested for chiral separation of CTD in NP HPLC under similar conditions like in case of CIT. The authors did not succeed in baseline separation of the enantiomers of CTD on the Chiralcel OD-H (250 mm × 4.6 mm) column. Therefore, they tested chiral separation of CTD on Chiralpak AD-H (250 mm × 4.6 mm) column containing derivatized amylose as chiral selector, while Chiralcel OD-H contains derivatized cellulose. The developed analytical method was consequently validated [24].

In this work, we show the results of testing diverse separation systems using seven different CSPs. We introduce a new analytical HPLC method enabling simultaneous determination of CIT and CTD enantiomers in one analysis using Chiralcel OD-H (150 mm × 4.6 mm) column. The influence of mobile phase composition and temperature were evaluated to optimize the separation process. In addition, supercritical fluid chromatography (SFC) technique was tested for enantioseparation of CIT and CTD as an alternative which usually allows to obtain separations with short analysis time and high separation efficiency [25, 26]. The optimized HPLC method was validated and applied to analysis of different tablet formulations of CIT and *S*-CIT.

Experimental

Chemicals and Reagents

Organic solvents of HPLC grade, *n*-hexane (hex), propane-2-ol (isopropanol, IPA), methanol, ethanol and triethylamine (TEA), and glacial acetic acid were purchased from Sigma-Aldrich (Steinheim, Germany). The carbon dioxide (CO_2) used for SFC from Air Liquide (Paris, France) was Alphagaz CO_2 SFC, L50TP, purity: 99.998 % with maximum impurities: $H_2O < 5$ ppm, $O_2 < 2$ ppm, $CO < 5$ ppm, $H_2 < 0.5$ ppm, $C_nH_m < 2$ ppm, $NO + NO_x < 2$ ppm and total sulphur < 1 ppm.

RS-Citalopram (CIT), *S*-CIT and *RS*-Citadiol (CTD) were obtained from Prof. G. K. E. Scriba from University of Jena, Germany. The tablets of Seropram (CIT, 20 mg) and Cipralex (*S*-CIT, 10 mg) were products of Lundbeck (Valby, Denmark).

Standard and Sample Preparation

Stock solutions of *S*-CIT, CIT and CTD were dissolved in methanol to concentration of 1.00 mg mL^{-1} and diluted with methanol to appropriate concentrations. The stock solutions were kept in the refrigerator at 4 °C. Aliquots of powdered tablet samples were dissolved in methanol to concentration of 0.20, 1.00 and 5.00 mg mL^{-1} for CIT and 0.10, 0.50 and 2.50 mg mL^{-1} for *S*-CIT. The samples were sonicated for 15 min to provide complete dissolution. The prepared samples were filtered through 0.45-μm membrane filter before injection into the separation system.

Equipment

All HPLC measurements were carried out on two systems. The first system, Waters HPLC chromatograph Breeze System (Waters, MA, USA) was composed of HPLC gradient pump 1525, autosampler 717 Plus, column oven Jetstream 2 Plus, and UV–VIS 2-channel detector 2487, controlled by Breeze software.

The second system, Waters Alliance System with Waters 2695 Separation Module (Waters, MA, USA) composed of Waters 2996 Photodiode Array Detector, an autosampler 717 Plus, and Waters Alliance Series column heater, controlled by Empower software, was used for study of reproducibility of the method. Data were measured and consequently processed with Origin 8.1 (OriginLab, Northampton, UK) and one-way analysis of variance (ANOVA) was processed in program MiniTab Pro (Minitab Inc., PA, USA). The SFC measurements were performed on a system SFC-PICLAB Analytic from PIC SOLUTION (Avignon, France). The proportion of the co-solvent in the mobile phase was adjusted by a piston pump, the co-solvent was directly added in the CO_2 feeding, and the mixture of co-solvent and CO_2 was pumped by another piston pump at the total flow rate. The head of this pump was cooled to −7 °C by a cryostat. The unit was also composed of autosampler, oven, UV DAD detector and back-pressure regulator to control the outlet pressure. The outlet tube was heated at 55 °C to avoid ice formation during the CO_2 depressurization. Data were recorded with SFC PicLab Analytic Online 3.1.2 and processed with Analytic Offline 3.2.0.

Chromatographic Conditions

Altogether seven chromatographic columns were tested in HPLC and/or SFC. Larihc DMP-CF7 containing dimethylphenyl carbamate cyclofructan 7 immobilized on silica gel support was obtained from AZYP (Arlington, TX, USA). Chirobiotic TAG containing teicoplanin aglycone, Chirobiotic V and Chirobiotic V2 (V2 stands for higher vancomycin coverage composed of vancomycin bonded to silica gel support were purchased from Advanced Separation Technologies (Whippany, NJ, USA). Chiralpak AD with amylose tris(3,5-dimethylphenyl carbamate) chiral selector was a product of Chiral Technologies Europe (Illkirch, France). The dimensions of all these columns were 250 mm × 4.6 mm i.d.; particle size 5 μm. Furthermore, Chiralcel OD-RH and Chiralcel OD-H based on cellulose tris(3,5-dimethylphenyl carbamate) bonded on silica gel, column dimensions of 150 mm × 4.6 mm i.d.; particle size 5 μm, were obtained from Chiral Technologies Europe (Illkirch, France). The guard columns Chiralcel OD-H (10 mm × 4.6 mm) and Chiralcel OD-RH (10 mm × 4.6 mm) from the same company were used.

A wide variety of mobile phases and chromatographic modes: NP, reversed phase (RP) and polar-organic (PO) HPLC modes were tested with the columns. Special attention was paid to the mobile phases composed of hex/IPA/TEA in different volume ratios in the separation systems with the polysaccharide-based chiral stationary phases. In the separation system with Chiralcel OD-H column, which was the most promising, therefore studied in detail, the effect of concentration of TEA in the mobile phase with fixed ratio of hex/IPA 96/4 (*v/v*) was studied in the volume range of 0.00–0.20 %.

Temperature of the columns was kept at 25 °C in HPLC, except of the evaluation of temperature effect on separation on the Chiralcel OD-H column. Then, the temperature was changed in the range of 20–35 °C. The injection volume was 10 μL and flow rate was 1.0 mL min^{-1}. Sonication for 30 min was used for degassing hex. The detection was performed at 250 nm.

For SFC measurements, Chiralcel OD-H, Chiralpak AD and Larihc DMP-CF7 columns were tested. Mobile phases were composed of CO_2 with addition of methanol, ethanol or IPA. Small amounts of TEA (0.00–0.25 %) were added to the mobile phases to improve peak shape. The influence of the back pressure was evaluated in the range of 120–180 bars. Temperature was maintained at 40 °C. The injection volume was 20 μL and flow rate was 4.0 mL min^{-1}. Wavelength of 250 nm was used for detection.

The void volume was determined using solvent peak in both techniques.

Method Validation

Validation of the method was carried out under optimized separation conditions in HPLC according to the ICH guidelines. Stability of sample solutions, precision, linearity, limit of detection, limit of quantification, robustness and accuracy were considered.

Stability of the sample solutions was tested during the period of 2 weeks. Two equal solutions of CIT and CTD at the concentration of 1.00 mg mL^{-1} were prepared and stored at low temperature in the refrigerator.

Precision was expressed as relative standard deviation (RSD) values of retention factors and concentrations. The repeatability of the retention factors and concentrations of the enantiomers were determined for 10 consecutive injections of the racemate solutions at the concentrations of 0.10, 0.50 and 2.5 mg mL^{-1} of each enantiomer. The reproducibility of retention factors and concentrations of the enantiomers were measured in 2 days, by two analysts on two different HPLC equipments.

The linearity was tested over the concentration range 0.025–2.50 mg mL^{-1} for all enantiomers. Measurements at all concentration levels were carried out in triplicates and all the values of peak areas were subjected to linear regression.

The limit of detection (LOD), expressed as a concentration at a signal-to-noise ratio 3:1, was calculated based on the baseline noise, which was evaluated by recording the detector response over a period approximately ten times the widths of the peaks. Limit of quantification (LOQ) was taken as a concentration of analyte where signal-to-noise ratio is 10:1.

For robustness testing the selected variable parameters were column temperature (24, 25, 26 °C) and IPA content in the mobile phase (4.0 ± 0.5 %). The robustness was determined for triplicate injections of all enantiomers, each at concentration level of 0.50 mg mL^{-1}.

Results and Discussion

Method Optimization in HPLC

In the frame of the optimization procedure six different chiral columns were tested: Chirobiotic TAG, Chirobiotic V, Chirobiotic V2, Larihc DMP-CF7 in PO mode, Chiralcel OD-H in NP mode and Chiralcel OD-RH in RP mode. Chirobiotic TAG and Larihc DMP-CF7 columns were not suitable for any partial separation of the tested racemates. Baseline separation of CIT enantiomers ($R_{1/2} = 1.64$) was obtained on Chirobiotic V2 with methanol/TEA/acetic acid 100/0.05/0.05 (*v/v/v*) as mobile phase while the same separation system with Chirobiotic V

Table 1 (A) The effect of the amount of IPA in the mobile phase, hex/IPA/TEA, at constant addition of 0.1 % TEA on the separation results, (B) The effect of the column temperature on the separation results

	CIT				CTD			
	k_1	$R_{1/2}$	α	A_s	k_1	$R_{1/2}$	α	A_s
(A)[a] % IPA								
10	1.86	1.58	1.18	1.17	1.91	0.96	1.14	1.15
8	2.29	2.04	1.20	1.20	2.90	1.01	1.13	1.13
5	2.89	2.08	1.21	1.20	4.33	1.46	1.18	1.24
4	3.45	2.16	1.23	1.24	5.96	1.50	1.18	1.17
2	5.66	2.85	1.31	1.27	14.40	1.64	1.18	1.24
(B)[b] T (°C)								
20	3.92	2.34	1.28	1.29	6.56	1.49	1.19	1.21
25	3.45	2.16	1.23	1.24	5.96	1.50	1.18	1.17
30	3.12	1.85	1.20	1.20	5.54	1.50	1.17	1.19
35	2.89	1.56	1.16	1.13	5.34	1.52	1.17	1.17

[a] Chiralcel OD-H column, flow rate 1.0 mL min^{-1}, UV detection 250 nm, column temperature 25 °C, injection volume 10 µL, k_1 retention factor of the first eluted enantiomer, $R_{1/2}$ resolution, α enantioselectivity, A_s peak symmetry of the first eluted enantiomer

[b] Chiralcel OD-H column, mobile phase: hex/IPA/TEA 96/4/0.1 (*v*/*v*/*v*), other conditions and symbols as ad A)

resulted in worse enantioresolution ($R_{1/2} = 1.48$). Concerning enantioseparation of CTD just partial resolution was achieved under the same conditions on Chirobiotic V2 (the best resolution obtained was $R_{1/2} = 0.62$). The best enantioseparation of CIT and CTD in one chromatographic run was achieved on Chiralcel OD-H column (with Chiralcel OD-H guard column).

The effects of IPA amount in hex, TEA addition and temperature on enantioselectivity and resolution of the enantiomers were evaluated on the Chiralcel OD-H column. As expected, the retention factors and resolutions decreased with increasing IPA concentration in the mobile phase (see Table 1). The addition of TEA to the mobile phase reduced peak tailing, however, higher concentration of TEA (0.20 %) caused an increase of baseline noise.

The effect of column temperature was studied in the optimized mobile phase composed of hex/IPA/TEA 96/4/0.1 (*v*/*v*/*v*) in the range of 20–35 °C (see Table 1). The best resolution of both racemates was achieved at 20 °C. Decrease of temperature increased the resolution values of the enantiomers of CIT but in the case of CTD enantiomers negligible changes of resolution values and enantioselectivity were observed. Concerning retention and resolution, the optimum temperature for the analysis of both enantiomeric pairs was 25 °C.

At the end, the optimized separation conditions, found as the compromise between resolution and analysis time, were as follows: Chiralcel OD-H column, mobile phase hex/IPA/TEA 96/4/0.1 (*v*/*v*/*v*), flow rate 1.0 mL min^{-1}, detection wavelength 250 nm and column temperature 25 °C.

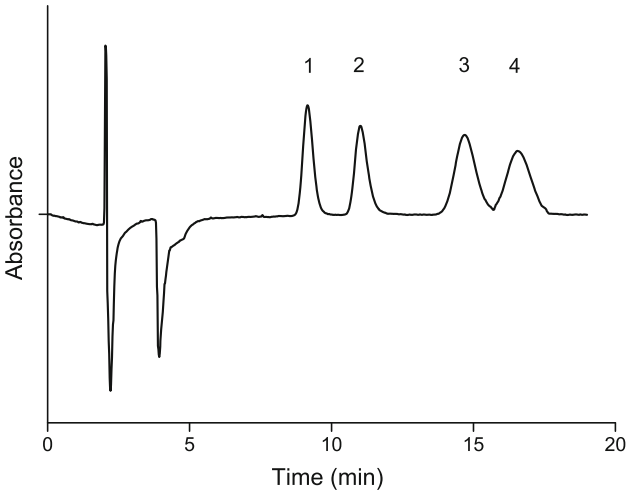

Fig. 2 HPLC separation of enantiomers of CIT and CTD under optimized conditions using Chiralcel OD-H column; mobile phase: hex/IPA/TEA 96/4/0.1 (*v*/*v*/*v*), flow rate 1.0 mL min^{-1}, UV detection at 250 nm; column temperature 25 °C, injection volume 10 µL. Resolution $R_{1/2} = 2.16$ (CIT) and 1.50 (CTD), 1 *R*-CIT, 2 *S*-CIT, 3 *S*-CTD, 4 *R*-CTD

Chromatogram of enantioseparation of CIT and CTD under optimized conditions is shown in Fig. 2. The elution order of the enantiomers of CIT was confirmed by injection of pure *S*-CIT. The elution order of the enantiomers of CTD was determined according to the literature data [24]. Namely separation of CTD enantiomers was performed under the experimental conditions described in ref. [24]. The first peak (referred as *R*-CTD) was collected and reinjected on the Chiralcel OD-H column under the optimized separation conditions, and CTD racemate was also injected for an easy comparison. Enantiomers of CTD eluted on Chiralcel OD-H column in the opposite elution order (*S*-CTD elutes first) to the Chiralpak AD-H column under the described conditions.

Method Optimization in SFC

Furthermore, we have verified the possibility of using SFC. Two CSPs based on derivatized polysaccharides, namely Chiralcel OD-H and Chiralpak AD columns, and the cyclofructan-based CSP (Larihc DMP-CF7) were studied. Overall three types of mobile phases differing in the alcohol type and amount (IPA, ethanol and methanol), with addition of TEA in some cases, were evaluated. Partial enantioseparation of CIT enantiomers was obtained on Chiralcel OD-H column, while on the other columns no enantioseparations were achieved. The most promising mobile phases contained five volume percent of IPA or

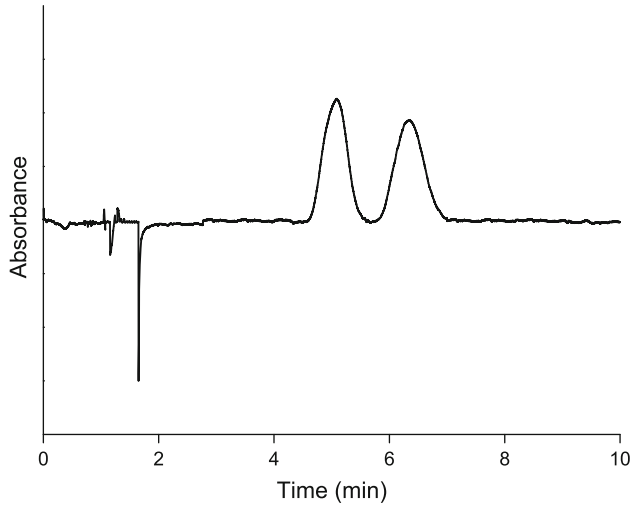

Fig. 3 SFC separation of CTD enantiomers using Chiralpak AD column; mobile phase: CO_2/methanol/TEA 90/10/0.25 (*v/v/v*), flow rate 4.0 mL min^{-1}, UV detection at 250 nm, injection volume 20 µL, column temperature 40 °C, 120 bars as back pressure

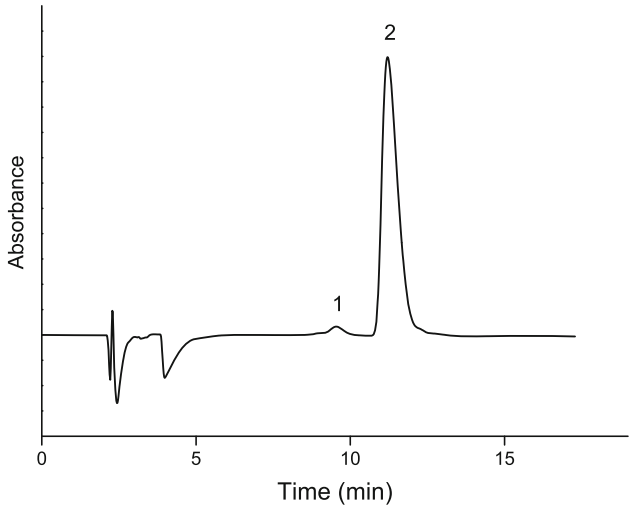

Fig. 4 HPLC chromatogram showing the separation of *S*-CIT (3.50 mg mL^{-1}) spiked with 1.0 % impurity of *R*-CIT (0.035 mg mL^{-1}) under optimized conditions—see caption to Fig. 2 for details. 1 *R*-CIT; 2 *S*-CIT

methanol in CO_2. Further reducing of the amount of the alcoholic modifier resulted in improved enantioselectivity. However, such analysis was accompanied by substantial increase of retention and peak deterioration.

CTD enantiomers were also partially separated on Chiralcel OD-H column showing the same trends like CIT enantiomers. However, baseline separation of CTD enantiomers was reached on Chiralpak AD column (see Fig. 3). Mobile phases with addition of methanol showed the best separation potential for CTD enantiomers, compared to ethanol or IPA. By increasing the back pressure the retention decreased, which was accompanied by a slight decrease of resolution values. The best enantioseparation of CTD was achieved in mobile phase composed of CO_2/methanol/TEA 90/10/0.25 (*v/v/v*), at temperature 40 °C, flow rate 4.0 mL min^{-1}, and 120 bars as back pressure. The obtained resolution and enantioselectivity values were $R_{1/2} = 1.58$ and $\alpha = 1.31$, respectively.

In summary, using Chiralpak AD column in SFC is a faster and more selective alternative for separation of CTD enantiomers as compared to the HPLC method. Unfortunately, enantiomers of CIT could not be baseline separated in SFC on any of the tested columns. Therefore, the validation was performed for the optimized HPLC method (see the previous chapter) because both analytes, CIT and its precursor CTD, could be separated in one run there.

Validation of the HPLC Method

The newly developed method was validated for determination of the enantiomers of CIT and CTD by HPLC. Validation of the method was carried out under the optimized conditions: Chiralcel OD-H column, hex/IPA/TEA 96/4/0.1 (*v/v/v*), column temperature 25 °C, flow rate 1.0 mL min^{-1}, UV detection at 250 nm. Stability of sample solutions, precision, linearity, limit of detection, limit of quantification, robustness and accuracy were investigated.

To confirm the suitability of the HPLC method enantioselective determination of distomer (*R*-CIT), its quantification in the presence of large enantiomer excess of eutomer (*S*-CIT) was carried out. The eutomer concentration was at 3.50 mg mL^{-1} whereas the distomer concentration was at 0.035 mg mL^{-1}, i.e. 1 % of the eutomer concentration (see the chromatogram in Fig. 4).

Stability of Sample Solutions

Sample solutions of both pairs of enantiomers stored at low temperature (7 °C) were proved to be stable over the period of 2 weeks.

Precision

In order to evaluate the precision of the method, repeatability and reproducibility of measurements were carried out. The values, expressed as relative standard deviation (R.S.D.) of retention factors and concentrations, obtained at three different concentration levels are summarized in Table 2. The results confirm that the method is suitable for both qualitative and quantitative analyses of the CIT and CTD enantiomers.

Linearity

The dependences obtained for the peak areas plotted against the concentrations of CIT and CTD enantiomers

Table 2 Repeatability and reproducibility, expressed as relative standard deviation (R.S.D.) values of retention factors and concentrations

	Repeatability						Reproducibility					
	R.S.D. (*k*) %			R.S.D. (*c*) %			R.S.D. (*k*) %			R.S.D. (*c*) %		
c (mg mL^{-1})	0.10	0.50	2.50	0.10	0.50	2.50	0.10	0.50	2.50	0.10	0.50	2.50
R-CIT	1.28	0.06	0.38	2.14	1.56	3.32	1.00	0.10	2.30	3.54	3.07	4.52
S-CIT	1.43	0.16	0.63	2.33	1.59	2.41	1.27	0.15	0.50	3.65	3.12	3.95
R-CTD	0.13	0.38	0.70	1.47	3.74	4.79	1.91	0.74	0.95	2.96	4.81	4.99
S-CTD	0.11	0.33	2.12	3.23	3.70	3.73	0.93	0.70	2.42	4.22	4.78	4.79

k retention factor, *c* concentration

were proved to be linear in the studied concentration range. The resulting linear regression equations were as follows:

R-CIT

$$Y = 6.721 \times 10^6 X + 7.034 \times 10^4; R^2 = 0.9997$$

S-CIT

$$Y = 6.941 \times 10^6 X + 2.963 \times 10^4; R^2 = 0.9982$$

R-CTD

$$Y = 8.421 \times 10^6 X - 32.80 \times 10^4; R^2 = 0.9985$$

S-CTD

$$Y = 8.196 \times 10^6 X - 17.82 \times 10^4; R^2 = 0.9993$$

where X is the concentration of the enantiomer (mg mL^{-1}), Y is the peak area (mV s) and R^2 is the coefficient of determination.

LOD and LOQ

The LOD and LOQ values obtained as concentrations of the analytes at a signal-to-noise ratio 3:1 and 10:1, respectively, were following: LOD values for *R*-CIT 0.68 μg mL^{-1} and for *S*-CIT 0.85 μg mL^{-1}, 1.30 μg mL^{-1} for *R*-CTD and 1.03 μg mL^{-1} for *S*-CTD. The values of LOQ were 2.26 μg mL^{-1} for *R*-CIT, 2.84 μg mL^{-1} for *S*-CIT, 4.32 μg mL^{-1} for *R*-CTD and 3.44 μg mL^{-1} for *S*-CTD, respectively.

Robustness

The parameters that had a significant impact on the results, namely column temperature and IPA amount in mobile phase, were tested for evaluation of robustness of the method. The effects of the method parameters on peak areas and enantioselectivity were evaluated. The hypothesis that errors resulted from a normal distribution was tested first. This hypothesis was accepted in all cases at significance level ($\alpha = 0.05$). Consequently, the robustness of the method was examined using the one-way ANOVA.

Table 3 Statistical *p* values obtained from one-way ANOVA

Tested factor	*p* value					
	CIT			CTD		
	A *R*-CIT	*A* *S*-CIT	α	*A* *R*-CTD	*A* *S*-CTD	α
Temperature	0.17	0.30	0.06	0.19	0.53	0.17
IPA content	0.10	0.22	0.10	0.45	0.76	0.06

Variable method parameters: column temperature (25 ± 1 °C) and IPA content in the mobile phase (4.0 % ± 0.5 %), *A* peak area, α enantioselectivity

The calculated *p* values are summarized in Table 3. Based on these results, the proposed analytical method for determination of CIT and CTD enantiomers was proved to be robust to all the variations tested in this work because the resulting *p* values are higher than the significance level $\alpha = 0.05$.

Real Samples Analysis: Accuracy

Two different tablets containing 20 mg of CIT (Seropram) and 10 mg of *S*-CIT (Cipralex) were analyzed three times diluted to concentration of 0.20, 1.00 and 5.00 mg mL^{-1} for CIT and 0.10, 0.50 and 2.50 mg mL^{-1} for *S*-CIT using the optimized HPLC method. Accuracy of the methods, regarded as the closeness of the agreement between the claimed contents of the active components in the tablets and the found values, was 98.5 % for *R*-CIT, 103.2 % for *S*-CIT at concentration level of 0.10 mg mL^{-1}, 101.7 % for *R*-CIT, 101.2 % for *S*-CIT at concentration level of 0.50 mg mL^{-1} and 99.4 % for *R*-CIT, 99.3 % for *S*-CIT at concentration level of 2.50 mg mL^{-1} in Seropram and 100.1 % for *S*-CIT at concentration level of 0.10 mg mL^{-1}, 100.1 % for *S*-CIT at concentration level of 0.50 mg mL^{-1} and 98.3 % for *S*-CIT at concentration level of 2.50 mg mL^{-1} in Cipralex. Enantiomers of CTD were not detected in the CIT drugs.

Conclusion

The new HPLC method for enantioseparation and determination of the enantiomers of CIT and CTD was found to be simple, rapid and robust. The two pairs of enantiomers were very well separated under the optimized conditions and no interference from the excipients was observed. Basic validation parameters have been evaluated. Enantioselective separation with resolution values ≥ 1.50 for both enantiomeric pairs was achieved within 20 min in single run on Chiralcel OD-H column with hex/IPA/TEA 96/4/0.1 (*v*/*v*/*v*) as mobile phase. The usage of Chiralpak AD column in SFC is a faster and more environmental friendly alternative for separation of CTD enantiomers providing similar results as HPLC. Nevertheless, the enantiomers of CIT could not be baseline separated in the SFC system. The developed HPLC method was used for analyses of two commercially available drugs based on CIT. The proposed HPLC method could be useful for routine quality control of the enatiomeric purity of drugs.

Acknowledgments The Grant Agency of the Charles University in Prague, Project No. 356411, KONTAKT AM 2010 Project LH11018, Project UNCE 204018/304018/2012 and the long-term project MSM0021620857 of the Ministry of Education, Youth and Sports of the Czech Republic are gratefully acknowledged for the financial support. The authors want to express their gratitude to Prof. G. E. Scriba for his generous gift of the enantiomers tested. Prof. Ch. Roussel and Dr. N. Vanthuyne from the Chirosciences team of Aix-Marseille University are acknowledged for their kind help during the SFC experiments. The bilateral cooperation between Charles University in Prague and Aix-Marseille University was established in the frame of LPP-ERASMUS PROGRAMME.

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6 Závěr

Předkládaná dizertační práce, tvořená komentovaným souborem sedmi publikací, se zabývá fyzikálně-chemickou charakterizací a aplikačním potenciálem CSP na bázi derivatizovaných cyklofruktanů. Výsledky práce mohou být využity k výraznému usnadnění vývoje, optimalizace a validace enantioselektivních metod využívajících cyklofruktanové CSP.

Jako výchozí přístup pro vzájemné porovnání cyklofruktanových CSP byl použit LFER model. Tento model poskytl důležité počáteční informace o typech a distribuci chromatografických interakcí tří komerčně dostupných cyklofruktanových CSP v podmínkách NP HPLC. Jako hlavní interakce přispívající v různé míře k retenci byly ve všech cyklofruktanových systémech určeny schopnost poskytovat vodík pro tvorbu vodíkové interakce a dipolarita/polarizibilita. Disperzní interakce retenci v různé míře snižují. Protože model LFER nezahrnuje sterické faktory, význam sacharidového skeletu v separačních systémech byl objasněn na základě porovnání cyklofruktanových CSP s cyklodextrinovými analogy. Cyklofruktanové CSP prokázaly v podmínkách NP HPLC mimořádnou enantioselektivitu zejména pro deriváty binaftolu a aminy.

Následně byly zkoumány vlastnosti DMP CF7 CSP v podmínkách SFC. Využitím modelu LFER byla získána data pro porovnání vlivu složení mobilní fáze (hexan *vs* superkritický oxid uhličitý) na retenční mechanizmy v HPLC a SFC. Bylo dokázáno, že dochází ke změně distribucí retenčních a tedy i enantiodiskriminačních interakcí v analogických HPLC a SFC systémech. Význam těchto změn pro chirální separace byl také demonstrován.

Dále bylo v rámci práce studováno využití cyklofruktanových CSP v podmínkách RP HPLC. Přestože cyklofruktanové CSP nejsou v tomto módu běžně používány, prokázaly značný enantioseparační potenciál. Úprava enantiodiskriminačního mechanizmu přídavkem barnatých iontů do mobilní fáze zvýšila enantioselektivitu IP-CF6 CSP pro určité typy analytů.

Kromě teoretického popisu retenčních a enantiodiskriminačních procesů jsou do práce zařazeny dva příklady zaměřené na praktický vývoj separačních metod pro stanovení enantiomerní čistoty léčiv.

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A. Seznam publikací

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