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Charakterizace separačních systémů pro dělení enantiomerů

Characterization of separation systems for determination of enantiomers

Dizertační práce

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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.

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V Praze ....

podpis

Poděkování

Dlouze jsem uvažoval, jak správně a upřímně vyjádřit své poděkování všem lidem, kteří mě celé ty roky podporovali.

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Klíčová slova

Chirální separace, enantioselektivita, chirální stacionární fáze, vysokoúčinná kapalinová chromatografie, superkritická fluidní chromatografie, polysacharidy.

Keywords

Chiral separation, enantioselectivity, chiral stationary phase, high performance liquid chromatography, supercritical fluid chromatography, polysaccharides.

Abstrakt (CZ)

Dizertační práce je zaměřena na výzkum a charakterizaci retenčních a enantiodiskriminačních mechanismů chirálních chromatografických systémů. Detailní charakterizace systémů s moderními stacionárními fázemi poskytne komplexní pohled na interakce uplatňující se při separaci. Hlubší pochopení retenčního/separačního chování významným způsobem usnadní vývoj a optimalizaci metod pro separaci a stanovení celé řady sloučenin.

Enantiodiskriminační potenciál polysacharidových stacionárních fází byl studován v podmínkách reversního a normálního módu HPLC. Stacionární fáze lišící se povahou polymerního řetězce (amylosa *versus* celulosa), navázáním chirálního polymeru na silikagelový nosič (pokrytá *versus* imobilizovaná stacionární fáze) či typem derivatizační skupiny byly porovnávány z hlediska retence a selektivity. V obou separačních módech vykazovaly amylosové chirální stacionární fáze vyšší enantioselektivitu především pro kyselé a bifunkční analyty. Chirální stacionární fáze na bázi derivatizované celulosy naopak ukázaly vyšší enantioseparační potenciál pro bazické analyty. Srovnání chromatografických dat získaných v reversním módu ukázalo, že pokrytá i imobilizovaná amylosová stacionární fáze vykazovala podobný enantioselektivitu pro kyselé analyty, zatímco pro enantioseparaci bazických látek byla vhodnější pokrytá chirální stacionární fáze. Jednotlivé chirální stacionární fáze jsou vzájemně komplementární a jejich kombinace umožňuje enantioseparaci strukturně odlišných chirálních analytů.

Další část práce se zabývá separací širokého spektra bazických chirálních sloučenin v systémech superkritické fluidní chromatografie. Byly sledovány různé chemické a fyzikální faktory ovlivňující separační systém. Chirální stacionární fáze na bázi imobilizované derivatizované amylosy se projevila jako vhodný nástroj pro enantioseparaci chirálních bází.

Praktické využití získaných poznatků bylo ukázáno na vývoji a validaci dvou chromatografických metod: (i) Chirální separace a stanovení antidepresiva citalopramu a jeho prekursoru citadiolu, (ii) SFC metoda pro systematickou toxikologickou analýzu kanabinoidů a jejich metabolitů.

Abstract (EN)

The dissertation thesis is focused on the research and characterization of retention and enantiorecognition mechanisms of chiral stationary phases based on derivatized polysaccharides. The separation systems with a variety of modern stationary phases (both achiral and chiral) were characterized in detail to provide a comprehensive view of the interactions participating in the separation process. The study of the retention/separation behavior significantly facilitates the development and the optimization of new enantioselective methods for a wide variety of compounds.

The work deals with the comparison of enantioselective performance of polysaccharide-based chiral stationary phases. The objectives are to show the differences of separation behavior among these chiral stationary phases, as they differ by the nature of the polysaccharide backbone (amylose *versus* cellulose), by binding of chiral polymer to silica support (coated *versus* immobilized stationary phase) and by the phenyl moiety in the reversed and normal phase HPLC. In both separation modes amylose-based chiral stationary phases exhibited higher enantioselectivity, especially for acidic and bifunctional analytes. Chiral stationary phases based on derivatized cellulose showed higher enantiodiscrimination potential for basic analytes. Comparing the results obtained on the polysaccharide-based chiral stationary phases under reversed and normal phase separation conditions, the coated and immobilized chiral stationary phases had comparable enantiorecognition ability for acidic analytes, while coated chiral stationary phase seemed to be a better choice for the separation of basic enantiomers. The studied polysaccharide-based chiral stationary phases often exhibited complementary separation properties and their combination enabled enantioseparation of structurally diverse compounds.

The dissertation thesis also deals with the separation of a wide range of chiral basic compounds in supercritical fluid chromatography. Various physical and chemical parameters affecting the separation system were studied. The immobilized amylose-based chiral stationary phase proved to be a useful tool for the enantioseparation of a broad spectrum of chiral bases.

In order to demonstrate practical impact of the research carried out in the thesis, two chromatographic methods were developed, optimized and validated: (i) HPLC method for chiral separation and quantification of antidepressant citalopram and its precursor citadiol (ii) Enantioselective separation of biologically active basic compounds in ultra-performance supercritical fluid chromatography.

Obsah

Seznam použitých zkratek a symbolů

α	enantioselektivita
AcOH	kyselina octová
AMK	aminokyselina
CD	cyklodextrin
CE	kapilární elektroforéza
CEC	kapilární elektrochromatografie
CF	cyklofruktan
CIT	citalopram
CS	chirální selektor
CTD	citadiol
CSF	chirální stacionární fáze
DEA	diethylamin
GC	plynová chromatografie
HILIC	hydrofilní interakční kapalinová chromatografie
HPLC	vysokoúčinná kapalinová chromatografie
IPA	isopropylamin
k_1	retenční faktor prvního eluujícího enantiomeru
LFER	model lineárních vztahů volných energií
MA	makrocyklická antibiotika
MEKC	micelární elektrokinetická chromatografie
MF	mobilní fáze

NP	normální mód
PO	polárně-organický mód
P_c	kritický tlak
$R_{1/2}$	rozlišení
RP	reversní mód
SC	syntetické kanabinoidy
SF	stacionární fáze
SFC	superkritická fluidní chromatografie
T_c	kritická teplota
TEA	triethylamin
TFA	kyselina trifluoroctová
THC	Δ^9 tetrahydrokanabinol
TLC	tenkovrstvá chromatografie
Trp	tryptofan
UHPLC	ultra vysokoúčinná kapalinová chromatografie

1 Úvod

Chiralita je unikátní fenomén zasahující do mnoha oblastí lidského života. Odlišná biologická aktivita enantiomerů, tedy dvou forem jedné látky, které nejsou identické se svými zrcadlovými obrazy, se může projevovat např. různou chutí, vůní nebo terapeutickým účinkem [1]. Z tohoto důvodu se stává enantioseparace a následná purifikace biologicky aktivních látek jedním z klíčových úkolů v oblastech farmaceutické, toxikologické či potravinářské analýzy [2]. Mezi nejpoužívanější metody sloužící k separaci enantiomerů patří vysokoúčinná kapalinová chromatografie (HPLC), superkritická fluidní chromatografie (SFC) [3,4] a kapilární elektroforéza (CE) [5].

HPLC a SFC jsou moderní chromatografické metody, které se využívají k analýze širokého spektra chirálních látek v analytickém i preparativním měřítku. Mezi jejich hlavní přednosti patří možnost ovlivňovat separační proces volbou stacionární (SF) i mobilní fáze (MF). Vyznačují se vysokou účinností, dobrou opakovatelností, spolehlivostí a robustností.

Vývoj, optimalizace a následná validace chirálních separačních metod je časově i finančně náročný proces. Pro dělení enantiomerů v HPLC se stejně jako v SFC využívají přednostně chirální selektory (CS) zakotvené/vázané na vhodném nosiči. Navíc většina SF používaných v HPLC je kompatibilní se superkritickou/subkritickou MF. Mezi nejpoužívanější patří chirální SF (CSF) na bázi derivatizovaných polysacharidů. Velmi často se též používají CSF na bázi cyklických oligosacharidů (cyklodextriny, CD; cyklofruktany, CF) a makrocyklických antibiotik (MA) [1,6].

Předkládaná dizertační práce se zabývá studiem důležitých faktorů ovlivňujících složitý enantioselektivní separační mechanismus (povaha CSF, způsob navázání CS na silikagelový nosič, složení MF, teplota a tlak). Experimentální a semiempirické studie separačního chování poslouží k lepšímu pochopení distribučního procesu, a následně povedou ke snadnějšímu vývoji a optimalizaci metod pro separace širokého spektra analytů.

2 Cíle práce

Hlavní cíl práce je zaměřen na výzkum a charakterizaci retenčních a enantioselektivních mechanismů CSF na bázi derivatizovaných polysacharidů v systémech HPLC a SFC.

Dílčí cíle

- Určení enantioselektivního potenciálu imobilizovaných CSF na bázi derivatizovaných polysacharidů v reversním módu (RP) HPLC.

- Porovnání enantioselektivního potenciálu dvou imobilizovaných polysacharidových CSF lišících se povahou polymerního řetězce (amylosa *versus* celulosa) v normálním (NP) a RP.

- Podrobná charakterizace a porovnání CSF na bázi derivatizované amylosy lišící se navázáním CS na silikagelový nosič.

- Studium enantiodiskriminačních možností polysacharidové CSF v systému SFC.

- Vývoj a optimalizace chromatografických podmínek pro separaci konkrétních chirálních látek a směsi achirálních analytů.

3 Chiralita

3.1 Chiralita a optická isomerie

Přírodní látky se často vyskytují ve dvou prostorových formách, jejichž vzájemný vztah je totožný se vztahem pravé a levé ruky. Tyto molekuly se označují jako chirální (z řeckého slova *cheiros* – ruka, dlaň). Chirální molekula nemá střed, osu ani rovinu symetrie [7]. Typ chirality látek se vyjadřuje přítomností různých prvků chirality. Mezi ně patří chirální centrum (centrální chiralita), chirální osa (axiální chiralita) a chirální rovina (planární chiralita).

Centrální chiralita se vyskytuje u látek, jejichž atom tetraedrálně váže čtyři různé substituenty. Tento atom se označuje jako stereogenní centrum nebo centrum chirality. Střed chirality se může vyskytovat také vázaný v oktaedrální struktuře molekul či v tetragonální bipyramidě [8].

Axiální chiralita derivátů *o,o*-disubstituovaných bifenylů, binaftylů či allenů je dána stericky bráněnou rotací atomů nebo skupin atomů kolem jednoduché vazby mezi atomy uhlíku, spojující dvě aromatické struktury, tzv. atropoisomerismus [9,10].

Planární chiralita (např. u spiranů) je vyvolána dvojicí různých substituentů ležících ve společné rovině (rovině chirality) a minimálně jednoho substituentu (tzv. pivota) ležícího mimo tuto rovinu a spojeného s touto rovinou chemickou vazbou [11].

Inherentní chiralitu vykazují látky, které jsou chirální bez přítomnosti prvků chirality. Patří mezi ně sloučeniny s helikální strukturou, tzv. heliceny. Koncové kruhy helikálního skeletu se ze sterických důvodů odpuzují a dochází k vychýlení nad a pod rovinu systému [12].

Chiralita je základní vlastností živé hmoty. Lidský organismus se skládá z nepřeberného množství opticky aktivních látek, jako jsou aminokyseliny, enzymy či bílkoviny. Molekuly, které nejsou ztotožnitelné se svými zrcadlovými obrazy, se nazývají enantiomery (z řeckého slova *enantio* – opačný). Objev enantiomerů se datuje do roku 1848, kdy si Luis Pasteur všiml rozdílných krystalových struktur soli kyseliny vinné. Enantiomery (optické isomery) stáčí rovinu polarizovaného světla buď doleva, značí se afixem (-) nebo doprava (+). Látky, které obsahují shodné množství pravotočivé a levotočivé formy označujeme jako racemát. Racemát má nulovou hodnotu optické aktivity. Optické isomery

mají v achirálním prostředí shodné fyzikálně-chemické vlastnosti, ale při interakci v chirálním prostředí mohou vyvolat rozdílnou biologickou odpověď. Kromě čichových či chuťových vjemů se mohou lišit závažněji, např. farmakologickým účinkem léčiv.

3.2 Chirální léčiva

V dnešní době obsahuje velké množství léčiv chirální aktivní složku. Popis interakcí léčiv a cílových receptorů vede k vývoji farmak, jejichž prostorová struktura dokonale odpovídá aktivnímu místu. Znalost prostorového uspořádání molekul je rozhodující pro pochopení enantioselektivních interakcí v organismu. Jednotlivé enantiomery mohou mít rozdílné účinky. Jeden z enantiomerů může poskytovat žádoucí farmakologické, farmakokinetické či farmakodynamické vlastnosti, zatímco druhý z isomerů může být neúčinný, méně účinný nebo dokonce toxický [13].

V poslední době sílí snaha převádět racemická farmaka na léčiva s jednou enantiomerní formou, tzv. chirální záměna (*chiral switch)*, která by měla zlepšit terapeutický profil daných léčiv [14]. Pokrok spojený s přípravou čistých enantiomerů způsobil zvýšení počtu registrovaných léčiv, které obsahují pouze aktivní enantiomer. V důsledku toho se na trhu s farmaky vyskytují jak racemické, tak i pouze účinné enantiomerní formy jednotlivých léčiv. Jako příklad lze uvést léčivo, jež se využívá jako inhibitor protonové pumpy, omeprazol, který je klasicky užíván jako racemát. Jeho *S*-enantiomer je podstatně účinnější než jeho *R*-isomer (u člověka asi 10% účinek *S*-omeprazolu) [15]. Mezi hlavní výhody používání enantiomerně čistých preparátu patří především snížení dávky oproti racemické směsi či rychlejší nástup účinku.

3.3 Chirální separace

Existují dva základní přístupy pro enantioselektivní separaci chirálních látek v chromatografii. První způsob, tzv. nepřímá separace, představuje derivatizaci analytu opticky čistým derivatizačním činidlem za tvorby diastereoisomerních derivátů, které mohou být separovány pomocí konvenčních achirálních separačních metod. Přímá metoda dělení enantiomerů spočívá v zavedení CS do enantioseparačního systému. CS lze přidat jako aditivum do MF nebo častěji lze využít CSF zakotvené/vázané na vhodném nosiči.

Pro separaci jednotlivých enantiomerů se nejčastěji využívají chromatografické separační metody, jako jsou HPLC (*high performance liquid chromatography*, popřípadě

ultra high performance liquid chromatography, UHPLC), plynová chromatografie (*gas chromatography*, GC), chromatografie na tenké vrstvě (*thin-layer chromatography*, TLC) a SFC (*supercritical fluid chromatography*). Dále se uplatňují elektromigrační techniky jako CE (*capillary electrophoresis*), micelární elektrokinetická chromatografie (*micellar electrokinetic chromatography*, MEKC) a elektrochromatografické techniky, tj. kapilární elektrochromatografie (*capillary electrochromatography*, CEC).

Předkládaná dizertační práce se zabývá separací biologicky aktivních látek převážně pomocí chromatografických metod HPLC a SFC. Proto budou detailněji popsány způsoby enantiomerního dělení právě v těchto systémech. Podkapitoly 3.3.1 respektive 3.3.2 shrnují základní charakteristiky těchto chromatografických technik a jejich aplikační možnosti.

3.3.1 HPLC

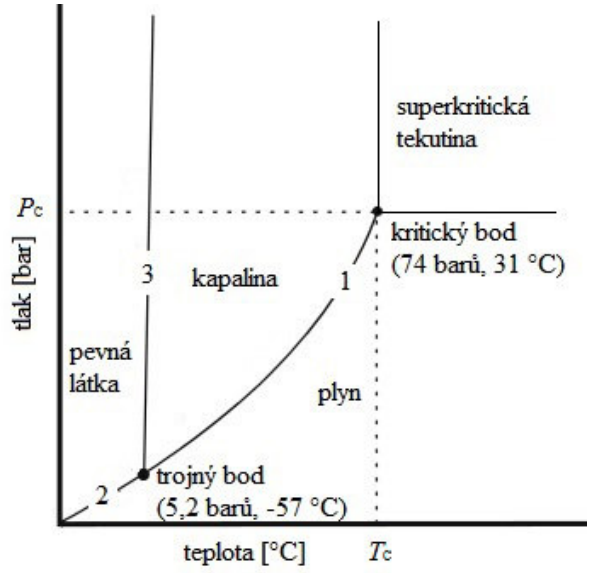
HPLC je stále nejpoužívanější separační technikou pro dělení enantiomerů. Metoda je založená na distribuci složek vzorku mezi nepohyblivou SF a pohyblivou MF na základě fyzikálně-chemických interakcí. Mezi její hlavní přednosti patří možnost ovlivňovat separace volbou jak SF, tak i MF, která na rozdíl od GC není pouhým inertním nosičem analytů. Dále vyniká vysokou účinností, dobrou opakovatelností a robustností. Chirální separace analytů se v HPLC systému nejčastěji provádí přímým dělením pomocí CSF navázaných na pevném nosiči.

Pro separaci enantiomerů se v systému HPLC nejčastěji používají tři základní separační módy. NP mód využívá polárnější SF a směs nepolárních či méně polárních organických rozpouštědel (např. *n*-hexan a propan-2-ol) a kyselých či bazických aditiv jako složek MF. V RP systému se naopak uplatňuje méně nepolární SF oproti polárnější MF, která se skládá z polární směsi vody či vodných pufrů s organickými rozpouštědly (nejčastěji acetonitril, methanol). Posledním hojně využívaným separačním módem je polárně-organický mód (PO), ve kterém je polárně-organická MF tvořena acetonitrilem či methanolem (popřípadě jejich směsí) s malým přídavkem kyselého a bazického aditiva (nejčastěji kyselina octová; AcOH, triethylamin; TEA) [16]. Kromě výše zmíněných módů se ještě rozlišuje hydrofilní, interakční chromatografie (*hydrophilic interaction liquid chromatography*, HILIC). MF zde obsahují obvykle vysoký podíl acetonitrilu (50-99%) ve vodě či pufru. SF je hydrofilní, přičemž dochází k rozdělování analytu mezi na vodu bohatší SF a na vodu chudší MF [17].

3.3.2 SFC

SFC zaznamenala v posledních letech nárůst popularity na poli separačních metod, především v oblasti farmaceutického průmyslu či klinické a forensní toxikologie [18,19,20,21]. Mezi hlavní přednosti této metody patří poměrně krátký čas analýzy, vysoká separační účinnost a v neposlední řadě šetrnost používaných složek MF k životnímu prostředí. Tato moderní separační technika (znovu „objevená", poté, co se podařilo překonat řadu instrumentálních a technických problémů neřešitelných v minulosti) využívá superkritickou tekutinu jako hlavní složku MF. Vlastnosti superkritické MF se výrazně liší od kapalných nebo plynných MF používaných v HPLC respektive v GC. Superkritické tekutiny jsou definovány jako sloučeniny ve stavu nad jejich kritickým tlakem (P_c) a kritickou teplotou (T_c). Tyto tekutiny mají viskozitu a difusní koeficienty podobné plynům, jejich hustota a solvatační schopnosti jsou naopak více podobné vlastnostem kapalin [22]. Superkritický CO_2 je zdaleka nejpoužívanější složka superkritické MF [23]. Standardními a technologicky nenáročnými postupy lze dosáhnout jeho kritických veličin (74 bar, 31 °C), navíc je netoxický, nehořlavý, inertní, relativně levný a dostupný v dobré čistotě. Fázový diagram pro CO_2 je znázorněn na Obrázku 1. Polarita superkritického CO_2 je srovnatelná s *n*-hexanem, který je majoritní složkou MF v chromatografii s NP. Zvýšení eluční síly subkritické/superkritické MF lze dosáhnout přídavkem organického modifikátoru, nejčastěji methanolu, ethanolu, propan-2-olu či acetonitrilu [24,25]. Kromě superkritického CO_2 a polárních rozpouštědel se do MF přidávají kyselá (např. kyselina mravenčí, AcOH, trifluoroctová; TFA) či bazická (např. TEA, diethylamin; DEA, isopropylamin; IPA, ethanolamin) aditiva [26,27]. Aditiva ovlivňují nejen polaritu použité MF, ale také disociaci či protonizaci separovaných analytů a funkčních skupin SF, a tím výrazně zlepšují separační účinnost a symetrii píků [28]. Další klíčové parametry superkritického systému tvoří teplota a zpětný tlak, které mají zásadní vliv na hustotu a viskozitu použité MF, čímž ovlivňují její solvatační schopnosti a ve svém důsledku separaci analytů [29,30]. Pro dělení enantiomerů v SFC se stejně jako v HPLC využívají přednostně CS zakotvené/vázané na vhodném nosiči [31,32]. Navíc většina SF používaných v HPLC je kompatibilní se superkritickou/subkritickou MF. SFC systém může nahrazovat jednak NP HPLC, částečně i RP HPLC. Avšak ve srovnání s dobře zavedenou a detailně popsanou metodou RP chromatografie, použití metody SFC je stále ještě omezeno v důsledku nedostatečného popsání separačního mechanismu. SFC poskytuje komplementární selektivitu k RP HPLC,

což umožňuje ortogonální přístup ve vývoji metod pro analýzu komplexních směsí strukturně příbuzných analytů, ale s jiným rozsahem polarity [33].



Obrázek 1. Fázový diagram CO_2. Upraveno z cit. [34]. 1 - křivka vypařování; 2 - křivka sublimační; 3 - křivka tání.

4 Charakterizace separačních systémů

Detailní charakterizace chromatografických systémů s moderními SF poskytuje komplexní pohled na interakce uplatňující se při separaci. Lepší pochopení retenčního/separačního chování významným způsobem usnadní vývoj a optimalizaci metod pro separaci a stanovení celé řady sloučenin. Pro charakterizaci separačních systémů byla navržena řada chromatografických testovacích modelů. Používají se relativně jednoduché modely podle Galushky [35], Tanaky [36], Engelhardta [37] či Walterse [38], které pro získání základních charakteristik separačního systému využívají třeba jen dvojici testovacích analytů.

4.1 Model lineárních vztahů volných energií

Komplexnější přístup představuje model lineárních vztahů volných energií (*linear free energy relationship*, LFER) [39]. Metoda LFER je založena na vztahu mezi přenosem analytu z MF do SF a změnou Gibbsovy energie (ΔG) systému.

$$\ln k = -\frac{\Delta G}{RT} + \ln \theta \qquad (1)$$

kde k je retenční faktor příslušného analytu, ΔG změna Gibbsovy energie systému, R univerzální plynová konstanta, T termodynamická teplota, θ fázový poměr. Tento model umožňuje charakterizovat separační systémy z hlediska jejich interakčních možností a popsat kvalitativně i kvantitativně příspěvky jednotlivých interakcí k celkové retenci. K popisu těchto interakcí se používají fyzikálně-chemické charakteristiky analytů (deskriptory). V separačním systému je retence analytů dána součtem všech interakcí, kterých se v systému účastní. Rovnice LFER vyjadřuje korelaci experimentálně zjištěných logaritmů retenčních faktorů s deskriptory analytů [40].

$$\log k = c + e\mathrm{E} + s\mathrm{S} + a\mathrm{A} + b\mathrm{B} + v\mathrm{V} \qquad (2)$$

kde k reprezentuje retenční faktor příslušného analytu, členy E, S, A, B, V jsou deskriptory příslušného analytu, parametry e, s, a, b, v vyjadřují regresní koeficienty a příspěvek c náleží hodnotě úseku na ose y. V rovnici (2) se nachází pět na sobě nezávislých deskriptorů, které charakterizují schopnost analytu podílet se na jednotlivých typech interakcí. Deskriptor E se získává z indexu lomu analytu a symbolizuje rozsah molární refrakce. Vyjadřuje schopnost analytu interagovat pomocí n- a π-elektronových párů. S je parametr dipolarity/polarizability a popisuje interakce dipól-dipól a dipól-indukovaný dipól. A je efektivní nebo celková acidita vodíkové vazby, zatímco B je efektivní nebo celková bazicita vodíkové vazby. Deskriptory A a B reprezentují schopnost analytu přijmout (deskriptor A) respektive poskytnout (deskriptor B) atom vodíku pro tvorbu vodíkové vazby. V je McGowanův charakteristický objem popisující dispersní a kohezní interakce (schopnost tvorby kavity). Regresní koeficienty v rovnici (2) jsou určeny multivariační regresní analýzou a zohledňují různé typy interakcí ve studovaném systému. Člen e vystihuje rozdílnou schopnost mezi MF a SF interagovat s n- a π-elektronovými páry; a vyjadřuje rozdíl ve schopnosti působit jako akceptor vodíku; b vystihuje rozdíl v donorové aciditě vodíku; koeficient v reflektuje rozdíl v dispersních interakcích mezi MF a SF (v RP módu popisováno jako hydrofobicita), člen c popisuje všechny zbylé vlivy na retenční chování, jež nejsou popsány regresními koeficienty [41]. Pozitivní hodnota koeficientu ukazuje na silnější molekulární interakci se SF vedoucí ke zvýšení retence analytů. Pokud je regresní člen záporný, interakce analyt/MF je silnější a způsobuje snižování retence. Výběr reprezentativní sady analytů má zásadní význam pro

správné hodnocení separačního systému. Analyty by měly být strukturně různorodé a distribuce deskriptorů by měla pokrývat širokou škálu interakcí. Kromě stanovení jednotlivých interakčních příspěvků na retenci v daném separačním systému může být model LFER využit ke vzájemnému porovnání SF, při zachování fixního složení MF, teploty a průtoku.

Žádný ze jmenovaných přístupů však jasně nepopisuje stereoselektivní interakce, a tudíž nevede k jednoznačné charakterizaci SF vzhledem k použitelnosti pro separaci enantiomerů. Proto byla základní LFER rovnice rozšířena přidáním dvou parametrů (globularity a flexibility), které zahrnují sterické efekty (chování analytu a separačního systému). Deskriptor G charakterizuje kompaktnost molekuly, deskriptor F vyjadřuje flexibilitu analytu. [42,43]. Je nutné však podotknout, že i tento rozšířený model LFER nevede k detailnímu pochopení enantioseparačního mechanismu.

$$\log k = c + e\mathrm{E} + s\mathrm{S} + a\mathrm{A} + b\mathrm{B} + v\mathrm{V} + g\mathrm{G} + f\mathrm{F} \qquad (3)$$

5 Chirální stacionární fáze

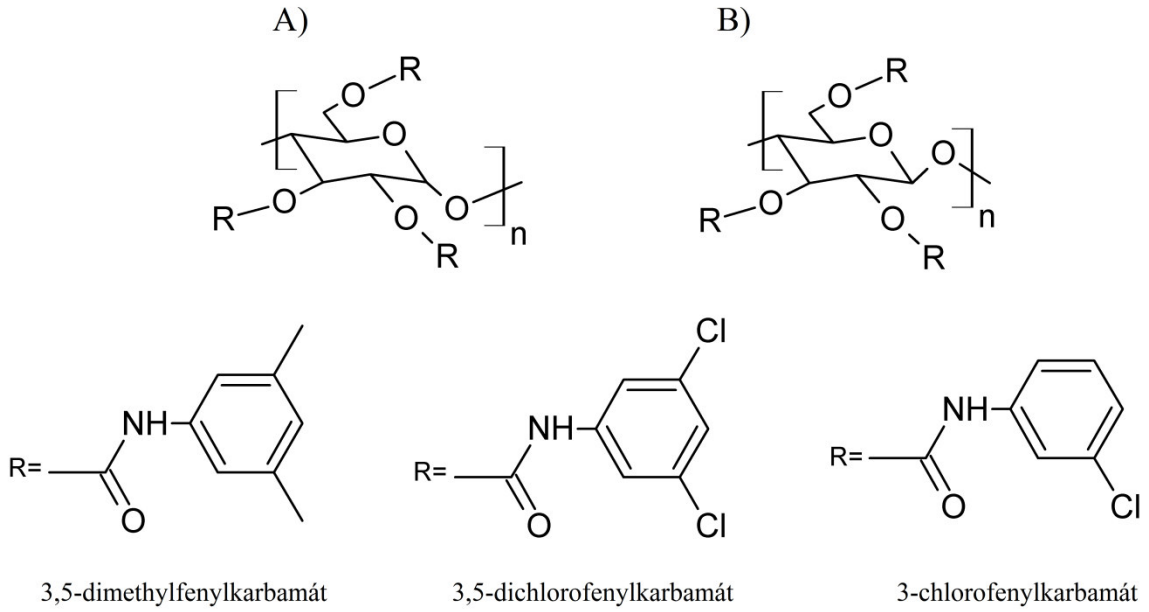
Vývoj CSF schopných chirální diskriminace širokého spektra biologicky aktivních látek se stal jedním z klíčových oblastí enantioseparačních chromatografických technik. CSF mohou být tvořeny CS přírodního původu nebo synteticky připravenými látkami. Mezi nejpoužívanější patří CSF na bázi derivatizovaných polysacharidů [44]. Dále jsou často používané CSF na bázi cyklických oligosacharidů či MA. Jelikož v této práci byly použity především CSF na bázi derivatizovaných polysacharidů, bude jim v následujícím textu věnována větší pozornost.

5.1 Polysacharidové chirální stacionární fáze

První prakticky použitá polysacharidová CSF na bázi mikrokrystalické celulosy byla připravena Hessem a Hagelem v roce 1973 [45]. Základní kostru polysacharidových CSF tvoří derivatizovaný polysacharid, amylosa či celulosa. Amylosa je tvořena glukosovými jednotkami spojenými α-1,4 vazbami, zatímco v molekule celulosy jsou glukosové jednotky spojené β-1,4 vazbami. Odlišné konstituce amylosy a celulosy významným způsobem ovlivňují sterické uspořádání makromolekul polysacharidů a tedy i CSF, které se na jejich

základě připravují. Enantioselektivní rozlišovací schopnost nativní amylosy a celulosy je nízká, proto byly připraveny a uvedeny na trh jejich deriváty (např. trifenylkarbamáty či tribenzoáty) [46,47]. Derivatizace podstatně zlepšuje jejich rozpoznávací schopnosti, které závisejí jak na struktuře polymerního řetězce, tak i na druhu derivatizačních skupin [48,49]. Enantioselektivní potenciál derivatizovaných polysacharidů na bázi aromatických esterů a fenylkarbamátů se výrazně zvýší, pokud jsou zavedeny elektron-donorové (např. methylové) či elektron-akceptorové (např. halogenové) substituenty v *meta* pozici na fenylové části molekuly [50,51]. Obrázek 2 ukazuje základní struktury nejpoužívanějších, komerčně dostupných polysacharidových CSF.

Polysacharidové CSF mohou interagovat prostřednictvím vodíkových vazeb, π-π nebo dipól-dipól interakcí, sterických interakcí či dispersních sil [52,53]. Jelikož dřívější generace polysacharidových fází byla připravena pokrytím silikagelového nosiče CS na základě fyzikální interakce, mohla být daná SF použita buď v RP, nebo v NP módu HPLC [54]. Tyto tzv. pokrývané (zakotvené, *coated*) polysacharidové CSF mohou být využity pouze s omezenou škálou rozpouštědel, obecně s alkoholy, acetonitrilem, *n*-alkany a s jejich směsmi. Rozpouštědla střední polarity jako methyl *terc*-butyl ether, tetrahydrofuran, 1,4-dioxan či chlorovaná rozpouštědla mohou částečně či úplně rozpustit chirální polymer [55]. Modernější způsob využívá chemické navázání chirálního polymeru na silikagelový nosič. Chirální kolony s imobilizovanými fázemi jsou multimodální, což zaručuje použití dané polysacharidové fáze v jakémkoliv separačním módu, a také snadný přechod mezi jednotlivými módy [56,57]. Imobilizované CSF kombinují výhody polysacharidových CS, tj. široký aplikační rozsah či preparativní potenciál, s benefity imobilizačního procesu, mezi něž patří robustnost a prakticky neomezená kompatibilita s rozpouštědly [58,59].



Obrázek 2: Struktura CS na bázi amylosy (A) a celulosy (B) a nejvýznamnější typy derivatizačních skupin.

5.2 Cyklodextrinové chirální stacionární fáze

CD jsou cyklické oligosacharidy tvořené obvykle 6-8 glukosovými jednotkami spojenými α-1,4 vazbami. Geometrické uspořádání D-glukopyranosových jednotek tvoří dutý kužel, tzv. kavitu. Vnitřní prostor kavity vykazuje hydrofobní charakter, povrch je hydrofilní [60]. Tato struktura umožňuje vytvářet inkluzní komplexy se širokou škálou chirálních sloučenin. Inkluze chirálního analytu do hydrofobní kavity se primárně uplatňuje v RP HPLC [61]. Nativní CD mají omezený enantioselektivní potenciál, zatímco jejich derivatizované formy vykazují výrazně vyšší enantioselektivní charakter pro separaci nejen biologicky aktivních látek [62].

5.3 Cyklofruktanové chirální stacionární fáze

CF jsou cyklické oligosacharidy skládající se ze 6 či více D-fruktofuranosových jednotek spojených β-2,1 vazbou, které vytvářejí základní crown-etherový skelet [63]. Chirální rozlišovací schopnost nativních CF je opět značně limitována. Derivatizací hydroxylových skupin CF dojde k rozevření struktury, čímž se zpřístupní crown-etherový kruh a tím se významně zlepší enantioselektivní vlastnosti [64]. Typ derivatizační skupiny zásadním způsobem ovlivňuje selektivitu cyklofruktanových SF vůči konkrétním skupinám analytů [65,66]. Přítomnost aromatických a karbonylových funkčních skupin umožňuje dipólové, π-π či sterické interakce, které hrají významnou roli především pro chirální dělení v nepolárních rozpouštědlech [67,68].

5.4 Makrocyklická antibiotika

MA lze podle struktury rozdělit na čtyři základní skupiny zahrnující ansamyciny (rifamycin B), glykopeptidy (ristocetin A, vankomycin, teikoplanin), polypeptidy (thiostrepton) a aminoglykosidy (streptomycin) [69]. Glykopeptidy patří mezi nejpoužívanější CSF na bázi MA. Glykopeptidová antibiotika jsou přírodní molekuly s unikátní strukturou, které jsou syntetizovány různými druhy bakterií. Všechny glykopeptidy se skládají z aglykonové části spojené do makrocyklických kruhů, které vytvářejí charakteristický košovitý tvar, a liší se počtem a typem sacharidových skupin, které mohou volně rotovat. Vankomycin ve své struktuře obsahuje tři makrocykly, zatímco ristocetin A a teikoplanin obsahují čtyři makrocykly [70].

6 Výsledky a diskuse

Publikace I: Enantioselektivní separace kódovaných a nekódovaných aminokyselin pomocí HPLC

Publikace II: Přímé CE a HPLC metody pro enantioseparaci tryptofanu a jeho v přírodě se nevyskytujících derivátů

Velké množství kódovaných a nekódovaných aminokyselin (AMK) se nachází v tělech rostlin či mikroorganismů a vznikají jako koncové produkty metabolických drah. Tyto AMK hrají významnou roli v potravinářském a farmaceutickém průmyslu či jako stavební bloky pro syntézu biologicky aktivních peptidů a antibiotik [71]. V rámci **Publikace I** byly vyvinuty HPLC metody vhodné pro enantioseparaci a možnou purifikaci studovaných AMK. Cílem **Publikace II** bylo vyvinout vhodné enantioselektivní metody pro analýzu tryptofanu (Trp) a jeho strukturně příbuzných derivátů. Trp a jeho v přírodě se nevyskytující deriváty jsou důležité stavební materiály při vývoji nových typů léčiv či chirálních katalyzátorů. Ve většině případů jsou syntetizovány jako racemáty, a proto jejich enantioseparace a následná purifikace je zásadní krok pro jejich praktické využití. Vývoj a optimalizace CE metod měly sloužit pro posouzení enantiomerní čistoty, zatímco HPLC metody umožnily enantioseparaci a purifikaci studovaných látek v semipreparativním měřítku.

V rámci **Publikace I** byly separovány následující AMK: D,L-fenylalanin, D,L-4-F-fenylalanin, *N*-benzyloxykarbonyl-D,L-4-F-fenylalanin, *N*-benzyloxykarbonyl-D,L-4-F-fenylalanin ethylester, *N*-benzyloxykarbonyl-D,L-3-F-fenylalanin, *N*-benzyloxykarbonyl-D,L-2-F-fenylalanin, D,L-4-Cl-fenylalanin, D,L-tyrosin a α-methyl-D,L-tyrosin. Pro vývoj HPLC metod byly použity CSF na bázi teikoplaninu lišící se pokrytím silikagelového nosiče (CHIROBIOTIC T a CHIROBIOTIC T2) v podmínkách RP a PO módu. Imobilizované polysacharidové CSF s odlišnou konstitucí polymerního řetězce či typem derivatizačních skupin (CHIRALPAK IA, CHIRALPAK IB, CHIRALPAK IC a CHIRALPAK ID) byly použity v méně obvyklém (pro dané CSF) RP módu.

Z porovnání chromatografických dat mezi CHIROBIOTIC T a CHIROBIOTIC T2 v MF obsahující methanol/octano-amonný pufr (20 mM, pH = 4,00), vykazovala chirální

kolona s nižším pokrytím silikagelového nosiče vyšší hodnoty retence a rozlišení pro všechny studované AMK s výjimkou α-methyl-D,L-tyrosinu. Nejvhodnější MF použitá v PO módu se skládala z methanolu a přídavku AcOH a TEA. Teikoplaninové CSF jsou navzájem komplementární. Využitím obou chirálních kolon jsme byli schopni separovat všechny testované enantiomery s výjimkou ethylesteru *N*-benzyloxykarbonyl-D,L-4-F-fenylalaninu, což může být způsobeno nahrazením atomu vodíku v karboxylové funkční skupině ethylovou skupinou. Z experimentálních dat vyplynulo, že kolona CHIROBIOTIC T vykazovala vyšší enantioselektivní potenciál pro blokované AMK, zatímco CSF s vyšším pokrytím nosiče (CHIROBIOTIC T2) vykazovala vyšší enantioselektivitu pro neblokované AMK.

CSF na bázi imobilizovaných polysacharidů byly použity pro separaci blokovaných AMK a ethylesteru *N*-benzyloxykarbonyl-D,L-4-F-fenylalaninu. Polysacharidové CSF jsou neutrální entity a neposkytují iontové interakce, proto bylo nezbytné udržovat molekuly analytů v nenabité formě. Studované MF se skládaly z acetonitrilu nebo methanolu a vodného roztoku kyseliny mravenčí (pH = 2,20) či vody (pro separaci ethylesteru *N*-benzyloxykarbonyl-D,L-4-F-fenylalaninu). S ohledem na retenci a rozlišení bylo dosaženo nejlepších výsledků na koloně CHIRALPAK IC (CS na bázi *tris*(3,5-dichlorofenylkarbamátu) celulosy).

Eluční pořadí jednotlivých enantiomerů bylo zjištěno na všech testovaných CSF. Teikoplaninové CSF vykazovaly nižší retence pro L-enantiomery, zatímco eluční pořadí na polysacharidových kolonách se lišilo v závislosti na konstituci polysacharidového skeletu (amylosa *versus* celulosa) či druhu derivatizačních skupin.

Optimalizované HPLC metody jsou vhodné pro separaci enantiomerů kódovaných a nekódovaných AMK či stanovení enantiomerní čistoty jednotlivých látek.

V rámci **Publikace II** byly využity CD a jejich deriváty jako CS pro separace v CE. Sulfatovaný γ-CD (S-γ-CD) vykazoval nejvyšší hodnoty rozlišení a enantioselektivity pro enantioseparaci esterů Trp (vyjma oktylesteru Trp) a amfoterních derivátů Trp. Chirální separace oktylesteru Trp bylo dosaženo v duálním selektorovém systému (β-CD + S-γ-CD). Pro enantioseparaci kyselého *N*-(*terc*-butoxy)karbonyl-Trp byl vybrán CS na bázi heptakis(6-amino-6-deoxy)-β-CD. Enantiomery všech analytů byly rozděleny až na základní linii a migrační čas nepřekročil 8 min.

Při vývoji HPLC metod byly testovány chirální kolony na bázi derivatizovaných polysacharidů (CHIRALPAK AD-RH, CHIRALPAK IB), oligosacharidů CD (CYCLOBOND I 2000 HP-RSP), CF (LARIHC CF7-DMP, LARIHC CF6-RN) a MA (CHIROBIOTIC T, CHIROBIOTIC T2). Nejlepších výsledků bylo dosaženo pro enantioseparaci Trp a jeho amfoterních derivátů v separačním systémů tvořeném CSF na bázi teikoplaninu a MF obsahující čistý methanol. Optimalizované podmínky mohou sloužit ke stanovení enantiomerní čistoty jednotlivých látek či pro semipreparativní aplikace.

Publikace I

Enantioselective separation of unusual amino acids by high performance liquid chromatography

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Enantioselective separation of unusual amino acids by high performance liquid chromatography

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ABSTRACT

Unusual amino acids, *i.e.* amino acids not encoded by DNA, play fundamental roles in many scientific fields. Since the single enantiomeric form can cause different and often serious response of organisms, chiral separations of unusual amino acids are irreplaceable tools in their study and their employment. Two types of chiral stationary phases, two teicoplanin-based and four polysaccharide-based columns, were used. Separation conditions of reversed phase mode, polar organic mode and hydrophilic interaction chromatography were evaluated and compared. All columns used exhibited significantly different enantioselectivities. Teicoplanin-based chiral stationary phases, especially Chirobiotic T column, were able to separate almost all enantiomers tested, with the exception of *Z*-D,L-4-F-phenylalanine ethyl ester. No partial enantioseparation of this analyte was obtained on teicoplanin-based chiral stationary phases, while baseline enantioresolution was achieved on polysaccharide-based columns. Change of elution order of L- and D-enantiomers was proved regarding the chiral stationary phase used.

1. Introduction

Peptides with their functional variability belong undoubtedly to the most important chemical entities in nature. One of the widely used ways for study of peptides, their conformations and functions, is an employment of unusual amino acids (AAs), which are not encoded by DNA. Integration of such AAs into the peptide chain enables intentional changes, which provide a valuable insight into the mechanisms of peptide activity [*e.g.* 1–4]. This approach has already brought a lot of findings and enabled development of important drugs [*e.g.* 5–7]. Particularly, a variety of impressive applications of fluoro-phenylalanines can be found in the literature. For instance, their homogeneous antibody-drug complexes showed enhanced effectiveness against target cell types [8]. Fluoro-phenylalanines were also introduced as a class of promising carriers for *in vitro* cell cultures/drug delivery [9].

However, the use of unusual AAs brings also some difficulties. Since the unusual AAs are often synthesized as racemates, prepared substances may contain many impurities. Therefore, purification followed by enantioseparation of unusual AAs stays at the very beginning of peptide/potential drug design. HPLC as a widely used and reliable separation technique is a good choice for this challenge [10–16].

The aim of our work was to develop HPLC methods suitable for enantioseparation and possible purification of a set of unusual amino acids. The set contained fluorine derivatives of phenylalanine, D,L-4-Cl-phenylalanine and α-methyl D,L tyrosine. Other compounds in the set were *N*-protected fluorine derivatives of phenylalanine including *N*-blocked D,L-4-F-phenylalanine ethyl ester. As a protection group benzyloxycarbonyl (*Z*) substituent was used. Phenylalanine and tyrosine were used as reference standards. The complete set of AAs is depicted in Fig. 1.

Initially, chiral stationary phases (CSPs) based on the macrocyclic antibiotic teicoplanin, namely Chirobiotic T and T2 columns, were chosen since they are considered to have extraordinary enantioseparation potential for amino acids [17–19]. Reversed phase (RP) and polar organic (PO) modes were used. However, after the initial investigation four additional CSPs based on derivatized polysaccharides [20–23], namely Chiralpak IA, Chiralpak IB, Chiralpak IC and Chiralpak ID columns, were employed since no enantiosep-

Abbreviations: ACN, acetonitrile; AA, amino acid; CSP, chiral stationary phase; HOAc, acetic acid; HILIC, hydrophilic interaction liquid chromatography; MeOH, methanol; PO, polar organic; RP, reversed phase; TEA, triethylamine.

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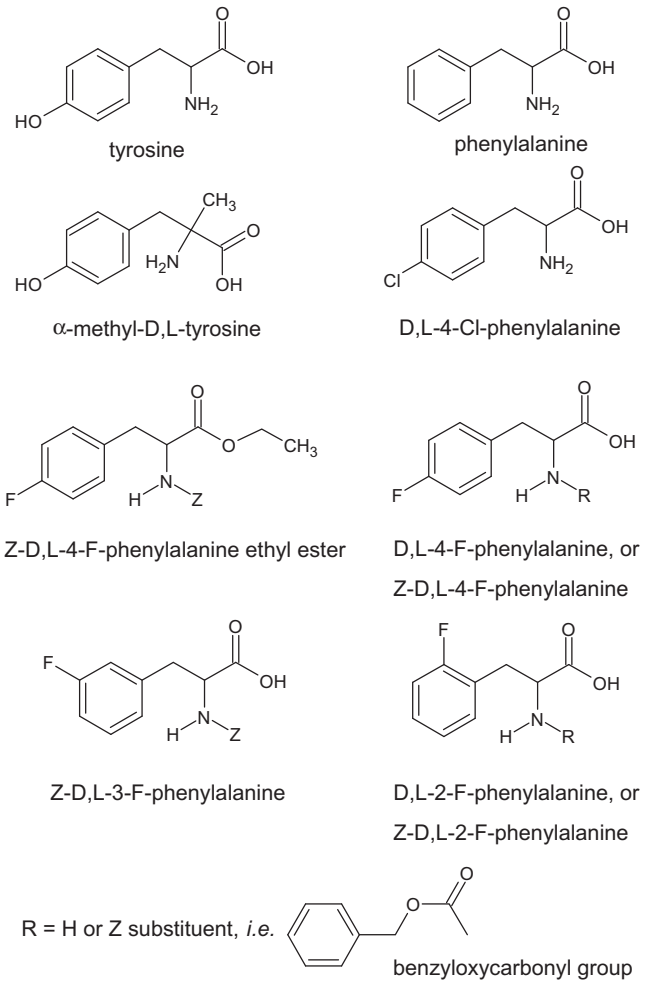

Fig. 1. Chemical structures of studied amino acids and *Z*-D,L-4-F-phenylalanine ethyl ester.

aration of *Z*-D,L-4-F-phenylalanine ethyl ester could be obtained on teicoplanin-based CSPs.

2. Experimental

2.1. Instrumentation

All chromatographic measurements were performed on Waters Alliance® system (Waters Corporation, Milford, USA) consisting of 2690 Separation Module, UV–VIS 2-channel detector 2487, 717 plus autosampler, and Alliance® Series column heater. Empower® software was used for data acquisition and analyses. Two teicoplanin-based CSPs were used, *i.e.* Astec Chirobiotic® T and Astec Chirobiotic® T2 columns (both column sizes 250 × 4.6 mm i.d., particle sizes 5 μm) from SUPELCO® (Bellefonte, USA). Other four polysaccharide CSPs used were based on (i) amylose tris(3,5-dimethylphenylcarbamate) – Chiralpak® IA column, (ii) cellulose tris(3,5-dimethylphenylcarbamate) – Chiralpak® IB column, (iii) cellulose tris(3,5-dichlorophenylcarbamate) – Chiralpak® IC column and (iv) amylose tris(3-chlorophenylcarbamate) – Chiralpak® ID column. The later CSPs were immobilized on 5 μm silica gel, columns sizes were 250 × 4.6 mm i.d., and they were obtained from Chiral Technologies Europe (Illkirch, France).

The columns were thermostated at 25 °C. The detection was performed at 254 nm. The flow rate was 1 mL/min and the injection volume was 10 μL in all experiments.

2.2. Chemicals and reagents

Methanol (MeOH, Chromasolv® for HPLC), acetonitrile (ACN, gradient grade), ammonium acetate (purity ⩾ 99%), triethylamine (TEA, purity ⩾ 99%), acetic acid (HOAc, purity > 99.8%) and formic acid (reagent grade, ⩾95%) were supplied by Sigma–Aldrich (St. Louis, USA). The deionized water used was purified by a Milli-Q water purification system from Millipore (Bedford, USA). D,L-tyrosine, D,L-phenylalanine, L-tyrosine, L-phenylalanine and potassium iodide were purchased from Sigma–Aldrich (St. Louis, USA). D,L-4-F-phenylalanine, *N*-benzyloxycarbonyl-D,L-4-F-phenylalanine, *N*-benzyloxycarbonyl-D,L-4-F-phenylalanine ethyl ester, D,L-4-Cl-phenylalanine, *N*-benzyloxycarbonyl-D,L-3-F-phenylalanine, D-2-F-phenylalanine, L-2-F-phenylalanine, *N*-benzyloxycarbonyl-D,L-2-F-phenylalanine, α-methyl-D,L-tyrosine, *N*-benzyloxycarbonyl-L-4-F-phenylalanine, *N*-benzyloxycarbonyl-D-3-F-phenylalanine, *N*-benzyloxycarbonyl-L-2-F-phenylalanine, L-4-Cl-phenylalanine and L-4-F-phenylalanine were prepared at the Institute of Molecular Biology, Bulgarian Academy of Sciences (Sophia, Bulgaria). Abbreviation *Z* stands for benzyloxycarbonyl group bonded to amino group in the following text.

2.3. Procedures

All measurements were performed in triplicates. Void volume was determined by injection of potassium iodide. Stock solutions of samples were prepared in concentration of 1 mg/mL using MeOH or mixture of MeOH and water as solvents.

Ammonium acetate buffer was prepared by dissolving appropriate amount of ammonium acetate in deionized water and adjusted with HOAc to reach the required pH value.

For teicoplanin-based CSPs, mobile phases in RP mode consisted of MeOH and deionized water or ammonium acetate buffer, while mobile phases in PO mode were composed of MeOH/HOAc/TEA in various volume ratios. For polysaccharide-based CSPs, MeOH or ACN and water or aqueous solution of formic acid, pH 2.20, in various volume ratios were used.

3. Results and discussion

3.1. Teicoplanin-based chiral stationary phases

3.1.1. Enantioseparation in RP/HILIC mode

Initially, pH (3.00; 4.00; 5.00) and concentration (15 mM; 20 mM; 25 mM) of ammonium acetate buffer as an aqueous mobile phase component were tested for the separation of AAs enantiomers on teicoplanin-based CSPs. Organic part of the mobile phases was formed of MeOH. At first, two chiral columns (Chirobiotic T and T2), were used differing in higher teicoplanin coverage, longer spacer and presence of additional carbamate moiety of the later CSP [24,25]. Based on the obtained results 20 mM ammonium acetate buffer pH 4.00 was chosen for further investigation. Table 1 summarizes obtained results of enantioseparation, baseline or partial, in the optimized mobile phases. Injections of single enantiomers proved that L-enantiomers eluted first in all cases. By comparing the data in Table 1, it is obvious that Chirobiotic T and Chirobiotic T2 columns show different enantioseparation potential for the tested analytes under RP conditions. Chirobiotic T with lower teicoplanin coverage exhibits higher retention and enantioresolution for all AAs enantiomers, except for α-methyl-D,L-tyrosine at identical mobile phase composition. For illustration see Fig. 2. The substituent type on the benzene ring of phenylalanine has interesting influence on retention and resolution values on Chirobiotic T column (see Table 1). The highest resolution value was obtained for non substituted D,L-phenylalanine. D,L-phenylalanine substituted by chlorine had lower resolution value despite the

Table 1
Chromatographic data of the studied amino acids separated on Chirobiotic T and T2 columns in buffer methanolic and aqueous methanolic mobile phases. Retention factor of the first eluted enantiomer (k_1), resolution (R) and optimized mobile phase composition.

Analyte	Column	k_1	R	Mobile phase composition
D,L-4-F-phenylalanine	T	1.17	3.91	MeOH/buffer 70/30 (v/v)
	T	1.17	4.44	MeOH/water 70/30 (v/v)
Z-D,L-4-F-phenylalanine	T	0.13	2.37	MeOH/buffer 80/20 (v/v)
Z-D,L-3-F-phenylalanine	T	0.18	2.31	MeOH/buffer 70/30 (v/v)
D,L-2-F-phenylalanine	T	1.16	3.99	MeOH/buffer 70/30 (v/v)
	T	1.18	4.80	MeOH/water 70/30 (v/v)
	T2	0.88	0.61	MeOH/buffer 70/30 (v/v)
Z-D,L-2-F-phenylalanine	T	0.19	1.88	MeOH/buffer 70/30 (v/v)
D,L-4-Cl-phenylalanine	T	1.32	3.46	MeOH/buffer 70/30 (v/v)
	T	1.36	4.14	MeOH/water 70/30 (v/v)
D,L-phenylalanine	T	1.24	4.24	MeOH/buffer 70/30 (v/v)
	T	1.22	4.19	MeOH/water 60/40 (v/v)
D,L-tyrosine	T	1.03	4.15	MeOH/buffer 70/30 (v/v)
	T	1.07	4.99	MeOH/water 70/30 (v/v)
	T2	0.96	3.01	MeOH/buffer 70/30 (v/v)
α-methyl-D,L-tyrosine	T2	0.68	1.04	MeOH/buffer 60/40 (v/v)

Buffer stands for 20 mM ammonium acetate buffer, pH 4.00.

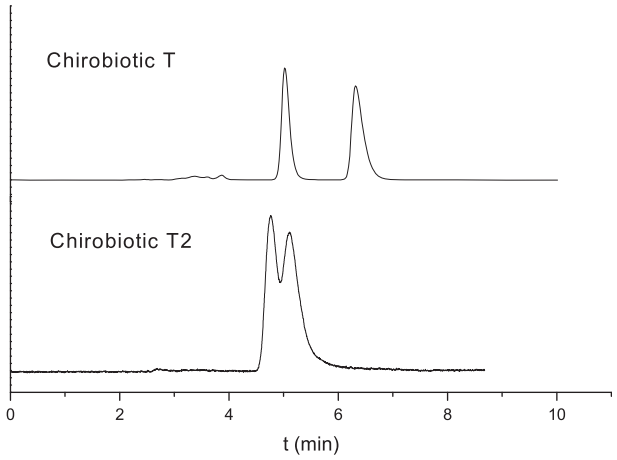

Fig. 2. Chromatograms of enantioseparation of D,L-2-F-phenylalanine on two teicoplanin-based CSPs. Mobile phase composition: ACN/20 mM ammonium acetate buffer, pH 4.00, 70/30 (v/v), flow rate: 1 mL/min, column temperature: 25 °C, UV detection: 254 nm.

fact that this analyte has the longest retention from the set of compounds. Both analytes with fluorine substituent in *ortho* or in *para* positions exhibit similarly lower retention and decrease in enantioresolution. The introduction of *Z*-group significantly decreases retention of the AAs. However, baseline resolution was preserved for some analytes. The effect of the amount of organic modifier on retention of AAs enantiomers was also tested. Generally, U-shaped dependencies of retention on the amount of organic modifier were obtained on both CSPs for non-blocked analytes, while for *N*-blocked AAs the retention just decreased with increasing MeOH content. This means that the less polar *N*-blocked AAs show a typical RP behavior. U-shaped dependencies indicate a change of the interaction/retention mechanism for the polar analytes. In the mobile phases with lower MeOH content teicoplanin-based CSPs work as RP columns, while in the mobile phases with high MeOH content as HILIC (hydrophilic interaction liquid chromatography) columns [24]. Thus, in the mobile phases with lower MeOH content non-polar interactions prevail, while at the higher MeOH content polar interactions become more important.

Use of simple mobile phases composed just of MeOH and deionized water also led to successful enantioseparation of non-blocked AAs on Chirobiotic T column – see Table 1. The majority of AAs enantiomers reached even higher resolution values while retention remained similar, however the peak shapes became worse than in the mobile phases with buffer. This behavior can be explained by the dissociation of carboxylic groups of AAs at different pH values. Dissociated carboxylic groups at the pH of water can interact as hydrogen-bond acceptors with amide groups of teicoplanin. This type of interaction has stereoselective character, as was already proved [26]. The use of ammonium acetate buffer, pH 4.00, suppresses the dissociation, which results in the decreased resolution values. The non-selective interactions remained unchanged. AAs with *Z*-group were not retained under these mobile phase compositions and eluted with the void volume.

3.1.2. Enantioseparation in PO mode

Suitable mobile phases used in PO mode were simply composed of MeOH with small additions of TEA and HOAc. The use of mobile phases composed of ACN/MeOH/HOAc/TEA had no positive effect on enantioseparation of the tested analytes. The optimized mobile phases for successful enantioseparations on Chirobiotic T and T2 columns with corresponding chromatographic parameters of individual AAs are summarized in Table 2. Chirobiotic T and T2 columns are complementary in PO mode. Chirobiotic T showed enantioselective potential only for *N*-blocked AAs, while Chirobiotic T2 for non-blocked AAs. Almost all *N*-blocked AAs were baseline

Table 2
Chromatographic data of the studied amino acids separated on Chirobiotic T and T2 columns in PO mode. Retention factor of the first eluted enantiomer (k_1), resolution (R) and optimized mobile phase composition.

Analyte	Column	k_1	R	MeOH/HOAc/TEA (v/v/v)
D,L-4-F-phenylalanine	T2	1.72	1.94	100/0.5/0.1
Z-D,L-4-F-phenylalanine	T	0.43	2.46	100/0.1/0.5
Z-D,L-3-F-phenylalanine	T	0.43	2.21	100/0.1/0.3
D,L-2-F-phenylalanine	T2	1.47	1.91	100/0.5/0.1
Z-D,L-2-F-phenylalanine	T	0.42	1.52	100/0.1/0.5
D,L-4-Cl-phenylalanine	T2	2.10	1.61	100/0.5/0.1
D,L-phenylalanine	T2	1.78	1.86	100/0.5/0.1
D,L-tyrosine	T2	1.88	1.66	100/0.3/0.1
α-methyl-D,L-tyrosine	T2	1.34	1.29	100/0.3/0.3

enantioresolved in PO mode on Chirobiotic T column, except for *Z*-D,L-4-F-phenylalanine ethyl ester. This seems to be a consequence of the presence of ethyl group instead of hydrogen on carboxylic group which results in the reduction of hydrogen bond interaction. Steric effects can also play a role. Non-blocked AAs showed no indication of enantioseparation, although they were more retained than the blocked ones under the same mobile phase composition. The best resolution of enantiomers of *N*-blocked AAs was obtained in mobile phases in which the amount of TEA was higher than that of HOAc. These results are comparable with those obtained in mobile phases with higher amounts of TEA and HOAc (volume ratio 1:1). Different results were obtained on Chirobiotic T2 column. All non-blocked AAs were baseline separated in short analysis times except for α-methyl-D,L-tyrosine that was just partially resolved ($R = 1.29$). Methyl group of this analyte probably rules out the proper steric fit with the chiral selector and thus decreases the enantioresolution. The best separations (comparing the *R* values) were obtained in mobile phases with higher amount of HOAc than TEA.

3.2. Polysaccharide-based chiral stationary phases

As no enantioseparation of *Z*-D,L-4-F-phenylalanine ethyl ester was obtained using teicoplanin-based CSPs, four polysaccharide-based CSPs, known for their good enantioselective separation abilities, were chosen for separation of *N*-blocked AAs and *N*-blocked ester. PO mode covering various mobile phase compositions was not suitable for enantioseparation of *N*-blocked AAs. The retention was very low with no sign of enantioseparation. RP mode mobile phases composed of ACN or MeOH as organic modifiers and aqueous solution of formic acid, pH 2.20, brought interesting results. Low buffer pH was used since enantiomers must be uncharged for successful enantioseparation on these CSPs [21]. Table 3 summarizes optimized mobile phase compositions for *N*-blocked AAs and the ester on Chiralpak columns. At least partial enantioseparation was obtained for all enantiomers on all four columns. The best column with respect to retention and resolution was Chiralpak IC. All analytes were baseline separated in significantly shorter analysis time as compared to the other Chiralpak columns. Both organic modifiers (ACN and MeOH) were proved to be suitable mobile phase components for this cellulose based column. Chiralpak IA column (amylose based CSP) provided better results with mobile phases composed of ACN as organic modifier rather than of MeOH. Nevertheless, only partial enantioseparations could be achieved. On the other hand, Chiralpak IB and ID columns gave better results with MeOH as organic modifier, except for enantioseparation of *Z*-D,L-3-F-phenylalanine on Chiralpak IB column where ACN was better mobile phase constituent. The enantioseparation of uncharged *Z*-D,L-4-F-phenylalanine ethyl ester is possible under very simple mobile phase compositions, ACN or MeOH and water, on all four Chiralpak columns. In separation systems with acidified aqueous part (pH 2.20) of mobile phases, hydrolysis of ester during separation occurred. The most suitable column for separation of the ester enantiomers was again Chiralpak IC. The baseline enantioseparation was obtained in the shortest analysis time – see Table 3. Fig. 3A and B compare enantioselectivity of individual Chiralpak columns under the same mobile phase compositions. Comparing amylose-based columns, Chiralpak IA and ID, we can summarize that the CSP without chlorine atom in the structure shows slightly higher enantioselectivity for all *N*-blocked AAs with the exception of *Z*-D,L-2-F-phenylalanine in mobile phase containing MeOH. In the case of cellulose-based columns, Chiralpak IB and IC, the situation is opposite. CSP with two chlorine atoms has significantly higher enantioselectivity for all *N*-blocked AAs in both mobile phase compositions. For illustration see Fig. 4.

The basic polysaccharide structure, amylose *vs.* cellulose, crucially influences the elution order of enantiomers. On Chiralpak IA, amylose tris(3,5-dimethylphenylcarbamate), L-enantiomers eluted first, while on Chiralpak IB, cellulose tris(3,5-dimethylphenylcarbamate), D-enantiomers eluted first. Introduction of chlorine atom(s) to the chiral selectors reverses the elution order in both cases. D-enantiomers eluted first using amylose based Chiralpak ID column and L-enantiomers eluted first using cellulose based Chiralpak IC column.

4. Conclusions

Enantiomers of all unusual AAs studied were baseline resolved except for α-methyl-D,L-tyrosine which was only partially separated on Chirobiotic T2 column. It was proved that teicoplanin-based CSPs work for these compounds as HILIC/RP mixed mode CSPs. Complementarity of teicoplanin-based CSPs with different

Table 3
Chromatographic data of the studied amino acids separated on Chiralpak columns in optimized mobile phases. Retention factor of the first eluted enantiomer (k_1), resolution (R) and optimized mobile phase composition.

Analyte	Column	k_1	R	Mobile phase composition
Z-D,L-4-F-phenylalanine	CH IA	3.11	1.27	ACN/aq. part 40/60 (*v*/*v*)
	CH IB	7.52	0.68	MeOH/aq. part 55/45 (*v*/*v*)
	CH IC	1.38	1.83	ACN/aq. part 42/58 (*v*/*v*)
		1.33	1.92	MeOH/aq. part 70/30 (*v*/*v*)
	CH ID	4.10	1.63	MeOH/aq. part 60/40 (*v*/*v*)
Z-D,L-3-F-phenylalanine	CH IA	2.94	1.10	ACN/aq. part 40/60 (*v*/*v*)
	CH IB	10.52	0.67	ACN/aq. part 30/70 (*v*/*v*)
	CH IC	1.32	1.67	ACN/aq. part 42/58 (*v*/*v*)
		1.26	1.74	MeOH/aq. part 70/30 (*v*/*v*)
	CH ID	4.27	0.82	MeOH/aq. part 60/40 (*v*/*v*)
Z-D,L-2-F-phenylalanine	CH IA	3.09	0.76	ACN/aq. part 40/60 (*v*/*v*)
	CH IB	6.41	0.91	MeOH/aq. part 55/45 (*v*/*v*)
	CH IC	1.63	1.73	ACN/aq. part 40/60 (*v*/*v*)
		1.33	1.68	MeOH/aq. part 70/30 (*v*/*v*)
	CH ID	4.79	1.40	MeOH/aq. part 60/40 (*v*/*v*)
Z-D,L-4-F-phenylalanine ethylester	CH IA	13.81	3.24	ACN/water 40/60 (*v*/*v*)
	CH IB	10.19	1.69	ACN/water 40/60 (*v*/*v*)
	CH IC	6.22	4.19	ACN/water 45/55 (*v*/*v*)
	CH ID	23.59	1.41	MeOH/water 60/40 (*v*/*v*)

Aq. part means aqueous solution of HCOOH, pH 2.20.

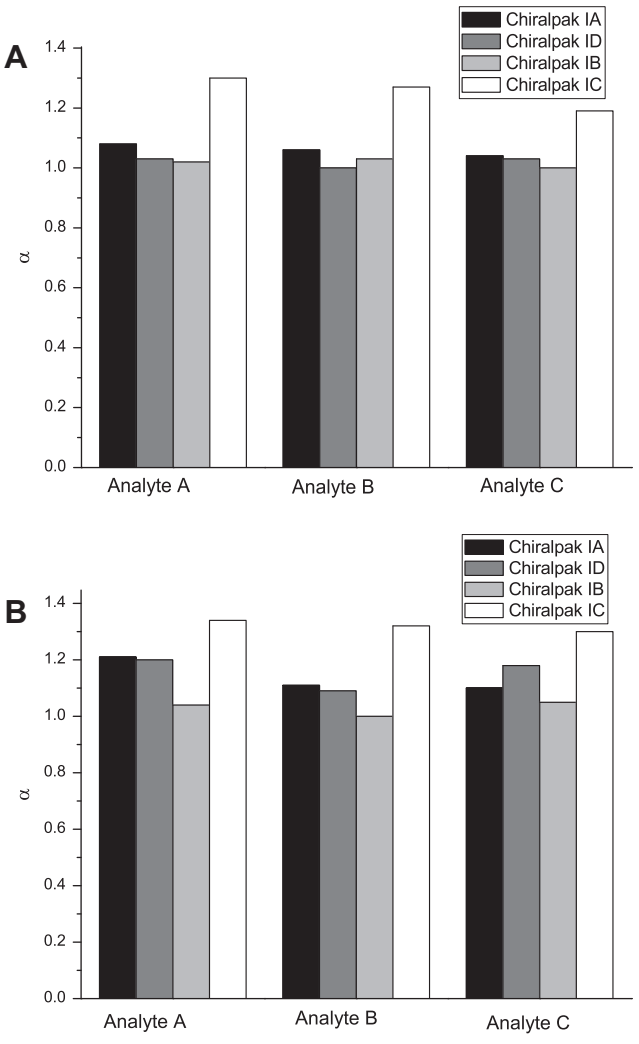

Fig. 3. The dependence of enantioselectivity (α) for *N*-blocked AAs on polysaccharide based CSPs. (A) mobile phase composition: ACN/aqueous solution of formic acid, pH 2.20, 30/70 (*v*/*v*); (B) mobile phase composition: MeOH/aqueous solution of formic acid, pH 2.20, 60/40 (*v*/*v*). Analyte A – *Z*-D,L-4F-phenylalanine; Analyte B – *Z*-D,L-3F-phenylalanine; Analyte C – *Z*-D,L-2F-phenylalanine.

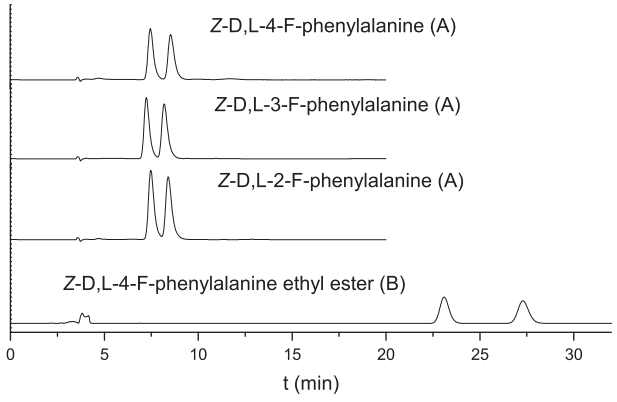

Fig. 4. Chromatograms of enantioseparation of *N*-blocked analytes on Chiralpak IC column. (A) Mobile phase composition: MeOH/aqueous solution of formic acid, pH 2.20, 70/30 (*v*/*v*); (B) mobile phase composition: ACN/water 45/55 (*v*/*v*). For more details see Experimental.

chiral selector coverage was observed. The best enantioseparation systems for non-blocked AAs are Chirobiotic T column in RP mode and/or Chirobiotic T2 column in PO mode. The most suitable conditions for enantioseparation of *N*-blocked AAs are either Chirobiotic T column in PO mode or Chiralpak IC column in RP mode. While L-enantiomers eluted first on teicoplanin-based CSPs, the elution order of enantiomers varied on polysaccharide-based CSPs depending on the subtle structural differences of the chiral selector. Chirobiotic T2 column was proved to be the only column suitable for enantioseparation of α-methyl-D,L-tyrosine. On the other hand, only polysaccharide-based columns are usable for separation of *Z*-D,L-4-F-phenylalanine ethyl ester enantiomers. The optimized HPLC methods are convenient for enantiomeric purity control and consequent sample purification.

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References

[1] A. Hasuoka, Y. Nishikimi, Y. Nakayama, K. Kamiyama, M. Nakao, K. Miyagawa, O. Nishimura, M. Fujino, Total synthesis of novel antibiotics pyloricidin A, B and C and their application in the study of pyloricidin, J. Antibiot. 55 (2002) 322–336.
[2] L. Wang, P.G. Schultz, Expanding the genetic code, Angew. Chem. Int. Ed. Engl. 44 (2005) 34–66.
[3] M. Tanaka, Design and synthesis of chiral α, α/disubstituted amino acids and conformational study of their oligopeptides, Chem. Pharm. Bull. 55 (2007) 349–358.
[4] W. Leuchtenberger, K. Huthmacher, K. Drauz, Biotechnological production of amino acids and derivatives: current status and prospects, Appl. Microbiol. Biot. 69 (2005) 1–8.
[5] D. Venugopal, D. Klapper, A.H. Srouji, J.B. Bhonsle, R. Borschel, A. Mueller, A.L. Russell, B.C. Williams, R.P. Hicks, Novel antimicrobial peptides that exhibit activity against select agents and other drug resistant bacteria, Bioorg. Med. Chem. 18 (2010) 5137–5147.
[6] M.D. Seo, H.S. Won, J.H. Kim, T. Mishig-Ochir, B.J. Lee, Antimicrobial peptides for therapeutic applications: a review, Molecules 17 (2012) 12276–12286.
[7] R.P. Hicks, J.J. Abercrombie, R.K. Wong, K.P. Leung, Antimicrobial peptides containing unnatural amino acid exhibit potent bactericidal activity against ESKAPE pathogens, Bioorg. Med. Chem. 21 (2013) 205–214.
[8] W.H. Zhang, G. Otting, C.J. Jackson, Protein engineering with unnatural amino acids, Curr. Opin. Struct. Biol. 23 (2013) 581–587.
[9] Y. Wang, Z. Zhang, L. Xu, X. Li, H. Chen, Hydrogels of halogenated Fmoc-short peptides for potential application in tissue engineering, Colloids Surf. B 104 (2013) 163–168.
[10] X. Wang, Y. Liu, Ch.B. Ching, Kinetic and equilibrium study of enantioseparation of propranolol in preparative scale chromatography, Sep. Purif. Technol. 50 (2006) 204–211.
[11] H. Khan, T. Khan, J.K. Park, Separation of phenylalanine racemates using d-phenylalanine imprinted microbeads as HPLC stationary phase, Sep. Purif. Technol. 62 (2008) 363–369.
[12] S. Shi, Y. Ma, Y. Zhang, L. Liu, Q. Liu, M. Peng, X. Xiong, Systematic separation and purification of 18 antioxidants from *Pueraria lobata* flower using HSCCC target-guided by DPPH–HPLC experiment, Sep. Purif. Technol. 89 (2012) 225–233.
[13] I. Ilisz, A. Aranyi, A. Péter, Review: chiral derivatizations applied for the separation of unusual amino acids enantiomers by liquid chromatography and related techniques, J. Chromatogr. A 1296 (2013) 119–139.
[14] I. Ilisz, A. Aranyi, Z. Pataj, A. Péter, Recent advances in the direct and indirect liquid chromatographic enantioseparation of amino acids and related compounds: a review, J. Pharm. Biomed. Anal. 69 (2012) 28–41.
[15] K. Kalíková, M. Riesová, E. Tesařová, Recent chiral selectors for separation in HPLC and CE, Cent. Eur. J. Chem. 10 (2012) 450–471.
[16] A. Cavazzini, L. Pasti, A. Massi, N. Marchetti, F. Dondi, Recent applications in chiral high performance liquid chromatography: a review, Anal. Chim. Acta 706 (2011) 205–222.
[17] A. Berthod, Y. Liu, Ch. Bagwill, D.W. Armstrong, Facile liquid chromatographic enantioresolution of native amino acids and peptides using a teicoplanin chiral stationary phase, J. Chromatogr. A 731 (1996) 123–137.
[18] A. Péter, G. Török, D.W. Armstrong, High-performance liquid chromatographic separation of enantiomers of unusual amino acids on a teicoplanin chiral stationary phase, J. Chromatogr. A 793 (1998) 283–296.

[19] I. Ilisz, R. Berkecz, A. Péter, Review: retention mechanism of high-performance liquid chromatographic enantioseparation on macrocyclic glycopeptide-based chiral stationary phases, J. Chromatogr. A 1216 (2009) 1845–1860.
[20] T. Zhang, D. Nguyen, P. Franco, T. Murakami, A. Ohnishi, H. Kurosawa, Cellulose 3,5-dimethylphenylcarbamate immobilized on silica. A new chiral stationary phase for the analysis of enantiomers, Anal. Chim. Acta 557 (2006) 221–228.
[21] T. Zhang, D. Nguyen, P. Franco, Reversed-phase screening strategies for liquid chromatography on polysaccharide-derived chiral stationary phases, J. Chromatogr. A 1217 (2010) 1048–1055.
[22] T. Zhang, P. Franco, D. Nguyen, R. Hamasaki, S. Miyamoto, A. Ohnishi, T. Murakami, Complementary enantiorecognition patterns and specific method optimization aspects on immobilized polysaccharide-derived chiral stationary phases, J. Chromatogr. A 1269 (2012) 178–188.
[23] B. Chankvetadze, Recent developments on polysaccharide-based chiral stationary phases for liquid-phase separation of enantiomers, J. Chromatogr. A 1269 (2012) 26–51.
[24] M. Honetschlägerová-Vadinská, S. Srkalová, Z. Bosáková, P. Coufal, E. Tesařová, Comparison of enantioselective HPLC separation of structurally diverse compounds on chiral stationary phases with different teicoplanin coverage and distinct linkage chemistry, J. Sep. Sci. 32 (2009) 1704–1711.
[25] Chirobiotic Handbook , Advanced Separation Technology, 5th ed., Whippany, New York, 2004.
[26] A. Cavazzini, G. Nadalini, F. Dondi, F. Gasparrini, A. Ciogli, C. Villani, Study of mechanisms of chiral discrimination of amino acids and their derivatives on a teicoplanin-based chiral stationary phase, J. Chromatogr. A 1031 (2004) 143–158.

Publikace II

Direct CE and HPLC methods for enantioseparation of tryptophan and its unnatural derivatives

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Direct CE and HPLC methods for enantioseparation of tryptophan and its unnatural derivatives

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ABSTRACT

Tryptophan and its eight derivatives considered in this work are biologically important compounds. Since their enantiomers can exhibit different behavior, efficient enantioselective separation methods are needed for both analytical and preparative purposes. In capillary electrophoresis cyclodextrins and their derivatives were proved to be suitable chiral selectors. Two pH values of background electrolytes were tested in order to affect ionization of the analytes and consequently their enantioseparation. Enantiomers of all analytes in this study were baseline separated within 8 min using capillary electrophoresis. However, different separation systems/conditions were required. In HPLC various separation modes and columns (based on derivatized polysaccharides, cyclofructan, cyclodextrin and teicoplanin) were used. The best results of enantioseparation of tryptophan and its amphoteric derivatives were achieved with teicoplanin based chiral stationary phases and methanol as a mobile phase. Proposed conditions were suitable even for purification purposes. This study can serve as a tool for simplifying the method development for enantioseparation of tryptophan and its derivatives.

1. Introduction

In a large number of biochemical processes the essential amino acid (AA) tryptophan (Trp) plays a crucial role [1]. The indolic side chain of tryptophan residue has unique structural and chemical properties. Trp and its unnatural (unusual) derivatives are important building blocks for the total synthesis of various products and development of new drugs, biological probes, and chiral small molecule catalysts [2–4]. Introduction of the unusual AAs in peptide chains has attracted a considerable interest to overcome the pharmacological limitations of bioactive peptides [5,6]. Various specific applications of Trp derivatives can be found in the literature. For example, 5-hydroxy Trp can serve as an effective antioxidant and radioprotector [7]; L-enantiomer of tryptophanol is used as a chiral synthon to assemble indolo[2,3-*a*]quinolizidine alkaloids [8]; L-Trp methyl ester was used as starting compound for synthesis of compounds with antimicrobial activity [9], various esters of tryptophan can be used for synthesis of antimicrobial peptides [10]; *N*-BOC-Trp (*N*-(tert-butoxy)carbonyl-Trp) is used in peptide synthesis [11,12].

Unnatural AAs, as many other pharmaceutical substances, can be chiral. They are often synthesized as racemates if prepared by non-asymmetric synthesis. For this reason, enantioseparation and purification are essential before further application. CE and HPLC techniques represent good choices for solving these tasks [13–22].

The aim of this work was to screen possibilities for the development of fast and effective separation environments of CE and HPLC for Trp and its structurally related but different derivatives. The analytes of our interest were D,L-Trp, D,L-Trp methyl ester (MET), D,L-Trp butyl ester (BUT), D,L-Trp benzyl ester (BEN), D,L-Trp octyl ester (OCT), 5-F-D,L-Trp (5FL), 5-OH-D,L-Trp (5OH), *N*-BOC-D,L-Trp (BTrp) and D,L-tryptophanol (OL). The structures are shown in Supplementary material, Fig. S1. Development and optimization of CE methods were focused especially on design of fast analyses for the enantiomeric purity screening. Developed HPLC methods are proposed for enantioseparation and further purification of Trp and its derivatives for semipreparative purposes. Widely used

Abbreviations: AcOH, acetic acid; ACN, acetonitrile; BEN, D,L-Trp benzyl ester; BOC, *N*-(tert-butoxy)carbonyl; BTrp, *N*-BOC-D,L-Trp; BUT, D,L-Trp butyl ester; CF, cyclofructan; CS, chiral selector; CSP, chiral stationary phase; DS, degree of substitution; EA, ethanolamine; HEX, n-hexane; IPA, propane-2-ol; MeOH, methanol; MET, D,L-Trp methyl ester; OCT, D,L-Trp octyl ester; OL, D,L-tryptophanol; 5FL, 5-F-D,L-Trp; 5-OH, 5-OH-D,L-Trp; TEA, triethylamine.

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cyclodextrin (CD) and its derivatives [23–25] were used as chiral selectors (CSs) in CE experiments. For HPLC analyses, application of different types of chiral stationary phases (CSPs) and separation modes was essential for successful enantioseparation due to significant structural differences of tested analytes.

2. Experimental

2.1. Chemicals and reagents

Methanol (MeOH, Chromasolv®, gradient grade, for HPLC, ⩾99.9%), acetonitrile (ACN, Chromasolv®, gradient grade, for HPLC, ⩾99.9%), *n*-hexane (HEX, Chromasolv® for HPLC, ⩾97%), propane-2-ol (IPA, Chromasolv® for HPLC, ⩾99.8%), ammonium acetate (purity ⩾ 99%), acetic acid (AcOH, purity > 99.8%), ammonium hydroxide solution (ACS reagent, 28–30%), ethanolamine (EA, ACS reagent ⩾ 99.0%), triethylamine (TEA, minimum 99%), trifluoroacetic acid (TFA, 99%), dimethylsulfoxide (DMSO, ACS reagent, 99.9%), lithium hydroxide monohydrate, (2-hydroxypropyl)-β-cyclodextrin (HP-β-CD) of 0.8 M substitution and average M_r = 1460, heptakis(2,6-di-O-methyl)-β-cyclodextrin (DM-β-CD), β-cyclodextrin (β-CD), 6-O-α-maltosyl-β-cyclodextrin hydrate (Malt-β-CD) and sulfated β-cyclodextrin (S-β-CD) sodium salt (12–15 mol per mol β-CD) were supplied by Sigma Aldrich (St. Louis, USA). Orthophosphoric acid 85% was purchased from Lachema (Neratovice, Czech Republic) and 0.1 M sodium hydroxide solution was product of Agilent Technologies (Waldbronn, Germany). Sulfated α-cyclodextrin (S-α-CD) sodium salt (DS (degree of substitution) ∼12), sulfated γ-cyclodextrin (S-γ-CD) sodium salt (DS∼14) and heptakis(6-amino-6-deoxy)-β-cyclodextrin (A-β-CD) heptahydrochloride were purchased from CycloLab (Budapest, Hungary). Water for solution preparation was deionized by the Watrex Rowapur and Ultrapur system (Prague, Czech Republic). Tryptophan and its derivatives were obtained as follows: D-tryptophan methyl ester hydrochloride, L-tryptophan methyl ester hydrochloride, D-tryptophan benzyl ester, L-tryptophan benzyl ester, D-tryptophanol, 97%, L-tryptophanol, 97%, D,L-tryptophan, 99%, 5-fluoro-D,L-tryptophan were purchased from Sigma–Aldrich (St. Louis, USA). D,L-tryptophan butyl ester hydrochloride was obtained from Pfaltz&Bauer (Waterbury, USA) and D,L-tryptophan octyl ester from Santa Cruz Biotechnology (Heidelberg, Germany). 5-hydroxy-D,L-tryptophan was purchased from Molekula (München, Germany) and *N*-α-BOC-L-tryptophan, *N*-α-BOC-D-tryptophan from Fluka (Buchs, Switzerland).

2.2. Instrumentation

CE experiments were carried out using Agilent 3D^{CE} capillary electrophoresis instrument operated by ChemStation software from Agilent Technologies (Waldbronn, Germany). Detection was performed with a built-in diode array detector at a wavelength of 217 nm. Fused silica capillary with i.d. of 50 μm and o.d. of 375 μm (Polymicro Technologies, Phoenix, AZ) was used. The total and effective lengths of the capillary were 49.5 cm and 41.0 cm, respectively.

All chromatographic measurements were performed on Waters Alliance System (Waters, Milford, USA) composed of Waters 2695 Separation Module, Waters 2996 Photodiode Array Detector, an autosampler 717 Plus, and Waters Alliance Series column heater, controlled by Empower software, which was used for data acquisition and analyses. In this study, following columns were used: two teicoplanin-based columns Astec CHIROBIOTIC™ T (T) and Astec CHIROBIOTIC™ T2 (T2); a cyclodextrin-based CSP containing *R,S*-hydroxypropylether derivatized β-CD - Astec CYCLOBOND™ I 2000 HP-RSP column (HP CD) (SUPELCO®, Bellefonte, USA); two cyclofructan (CF) -based columns Larihc CF7-DMP and Larihc CF6-RN (AZYP, Arlington, TX, USA) containing 3,5-dimethylphenylcarbamate functionalized cyclofructan 7 (CF7) and *R*-naphthylethyl carbamate modified CF6, respectively; two polysaccharide-based columns with guard columns, CHIRALPAK AD-RH (AD RH) and CHIRALPAK IB (IB) obtained from Chiral Technologies Europe (Illkirch, France), containing tris(3,5-dimethylphenylcarbamate) of amylose and tris(3,5-dimethylphenylcarbamate) of cellulose, respectively. All tested columns and guard columns were sized 250 × 4.6 mm i.d. and 10 × 4.6 mm i.d, respectively, both particle size 5 μm.

2.3. Software

Computer program PeakMaster 5.3 [26,27] was used to design and optimize the composition of buffers for CE and HPLC measurements and for calculation of their ionic strength and the theoretical pH values. For CE data evaluation programs Origin 8.1 (OriginLab Corporation, Northampton, MA) and Microsoft Office Excel 2010 were used. MarvinSketch online calculator (ChemAxon Kft., Budapest, Hungary) was utilized for acid dissociation constants estimations. Resolution *R* was calculated by the means of the operating software of CE and HPLC instruments.

2.4. Procedures

All CE and HPLC experiments were carried out in triplicates. The temperature of capillary or column was maintained at 25 °C. All buffers employed in CE and HPLC experiments were filtered with Minisart syringe filters (Sartorius Stedim Biotech, Goettingen, Germany), pore size 0.45 μm. Stock solutions of samples for both CE and HPLC experiments were prepared in concentration of 1 mg/mL using MeOH for MET, BUT, OCT, BEN, BTrp, OL or MeOH/water 80/20 (*v*/*v*) for Trp, 5FL, 5OH as solvents.

2.4.1. CE measurements

A new capillary was conditioned with deionized water for 20 min, with 0.1 M NaOH for 10 min and again with water for 5 min. Prior to each run, the capillary was flushed with separation buffer for 3 min. Samples were injected hydrodynamically at 10 mbar × 9 s. Separation voltage was −15 kV (anode at the detector side) or +25 kV (cathode at the detector side) depending on the charge of the particular analyte. When indicated, additional pressure of 30 or 40 mbar was applied to shorten the analysis time. Two acidic background electrolytes (BGEs) with a 10 mM ionic strength were employed for CE measurements: the acetate buffer composed of 10 mM LiOH and 20 mM acetic acid with theoretical pH 4.72 and the phosphate buffer composed of 20 mM phosphoric acid with theoretical pH 2.07. Experimental pH values of acetate and phosphate buffers were 4.74 and 2.09, respectively. Appropriate amount of particular CS was dissolved directly in BGEs and filtered. The pH of the BGE was verified again after addition of CSs as addition of even neutral CS to BGE can substantially change the buffer pH [28,29]. No significant pH changes were observed. Stock solutions of analytes were diluted by BGE to inject sample containing 0.05 mg/mL of individual enantiomers. When electrophoretic mobilities were calculated, 0.1% DMSO was present as electroosmotic flow marker in the sample injected. Migration times of analytes were determined as a time of the peak apex. Only for the electrophoretic mobility calculations, migration times were obtained by fitting the peak by Haarhoff-Van der Linde function [30,31].

2.4.2. HPLC measurements

Chromatographic measurements were carried out at a flow rate 1.0 mL/min and UV detection at wavelengths of 220 and 280 nm.

Sonication for 30 min was used for degassing the aqueous and HEX parts of the mobile phases. Ammonium acetate buffers (10 mM ammonium acetate/52 mM acetic acid, pH 4.00 and 10 mM ammonium acetate/3.2 mM ammonium hydroxide, pH 8.80) were prepared by dissolving the appropriate amount of ammonium acetate in water and mixing it with calculated amount of acetic acid or ammonium hydroxide. The correctness of buffer preparation was verified by measurements of experimental pH value. The 1% TEA-acetate (TEAA) buffer was prepared by titration of 1% (by volume) aqueous solution of TEA with acetic acid to pH 4.00. Void volume was determined using the solvent peak.

3. Results and discussion

The acid-base properties of analytes are decisive for the selection of suitable separation environment, mainly in CE techniques. Different acid-base behavior obviously requires different conditions for successful enantioseparation of Trp and its derivatives. There are basic (MET, BUT, OCT, BEN, OL), acidic (BTrp) or amphoteric (Trp, 5FL, 5OH) analytes in the set of Trp derivatives. Based on the calculated values of acidic dissociation constants of Trp and its derivatives a suitable pH of separation systems, mainly in CE, was designed; see Table S1 in Supplementary material.

3.1. CE separation

3.1.1. Basic Trp derivatives

Acetate buffer composed of 10 mM LiOH/20 mM acetic acid was selected as a suitable BGE for the enantioseparation of OL and Trp esters. Acetate buffer at pH 4.74 maintains all basic derivatives fully positively charged while its pH is not too low to cause hydrolysis of the esters. Regarding that Trp derivatives contain indolic part suitable for fitting the CD cavity, cyclodextrins (CDs) were the CSs of choice. Although published strategies for enantioselective separation of basic compounds recommend starting with sulfated derivatives of CDs [32–34], in this work, the enantioselective capabilities of four neutral CDs were screened first. The advantage of neutral CS lies mainly in the fact that it does not increase the ionic strength of the BGE. Constant ionic strength (even at various concentration levels of CS) allows to measure at constant voltage without a danger of excessive Joule heating. Moreover, the eventual determination of complexation constants of the CS with analytes is easier when constant ionic strength conditions are obeyed [35–39].

The interaction capabilities of four neutral CD derivatives, namely native β-CD, HP-β-CD, DM-β-CD and Malt-β-CD towards five basic Trp derivatives – MET, BUT, OCT, BEN and OL were tested. All tested analytes showed some interaction with all four neutral CD derivatives, which proved that CDs are a suitable group of CSs for our purpose. Baseline enantioseparation of BUT and BEN in the presence of 5 mM HP-β-CD and a partial enantioseparation of MET and BUT with 5 mM DM-β-CD were observed. Although we tried to further optimize the separation conditions, none of the tested neutral CDs was enantioselective to all of the five basic Trp derivatives.

As a next step, sulfated CDs were used as CSs. All three sizes of sulfated CDs (S-α-CD, S-β-CD, S-γ-CD) at three concentration levels (2.5 mM, 5.0 mM and 7.5 mM) were tested for enantioseparation of basic analytes. Negative separation voltage of −15 kV was used because the interaction of positively charged Trp derivatives with multiply negatively charged sulfated CDs leads to a negative effective mobility of the basic Trp derivatives. Additional pressure of +30 mbar was applied during the measurements for reducing the analysis time. S-α-CD separated only enantiomers of BUT within 8 min with resolution values $R_{2.5}$ = 1.45, $R_{5.0}$ = 1.45 and $R_{7.5}$ = 1.25 at 2.5, 5.0 and 7.5 mM concentrations of S-α-CD, respectively. Under given separation condition, S-β-CD caused partial enantioseparation of MET ($R_{2.5}$ = 1.27, $R_{5.0}$ = 1.13 and $R_{7.5}$ = 1.02), BUT ($R_{2.5}$ = 1.09, $R_{5.0}$ = 1.04 and $R_{7.5}$ = 0.99) and BEN ($R_{2.5}$ = 1.29, $R_{5.0}$ = 1.12 and $R_{7.5}$ = 1.10) derivatives. In the separation system containing S-β-CD, OCT repeatedly showed deformed, extra broaden peak. All separations took less than 9 min. In the presence of S-γ-CD MET, BUT, BEN and OL derivatives were baseline separated at all three concentration levels of the CS. OCT provided only partial separation in the presence of S-γ-CD. Migration times and resolution values for all basic derivatives in separation system with S-γ-CD are summarized in Table 1.

Resolution values of enantiomers of MET, BUT, BEN and OL derivatives were high even at the lowest consumption of S-γ-CD and there was no need for further optimization. Illustrative electropherograms of enantioseparation of MET, BUT, BEN and OL derivatives by means of 5.0 mM S-γ-CD are shown in Fig. 1A.

Enantioseparation of OCT was further optimized. Prolongation of separation time (by no additional pressure application) did not lead to baseline separation because of bad peak shape of OCT enantiomers. In the course of first screening of interaction possibilities of neutral CDs, strong interaction and good peak shape were observed with native β-CD. Therefore, mixed CSs system composed of S-γ-CD and β-CD was tested. The best concentration ratio of S-γ-CD to β-CD was 4.0–1.0 mM. Keeping the same separation conditions, the enantioseparation was significantly improved (R = 1.76) as shown in Fig. 1B.

3.1.2. Trp and amphoteric derivatives

The enantioseparation ability of sulfated CDs towards Trp derivatives was proved already for the basic derivatives and thus,

Table 1
Resolution and migration times of basic and ampholytic Trp derivatives in acetate buffer (10 mM LiOH/20 mM acetic acid, pH 4.74) or phosphate buffering solution (20 mM phosphoric acid, pH 2.09) containing 2.5, 5.0 or 7.5 mM S-γ-CD.

Analyte	2.5 mM S-γ-CD			5.0 mM S-γ-CD			7.5 mM S-γ-CD		
	R	t_1/min	t_2/min	R	t_1/min	t_2/min	R	t_1/min	t_2/min
pH 4.74									
MET	8.08	6.18	8.36	6.96	5.51	6.98	6.54	5.32	6.60
BUT	4.09	5.09	5.93	3.62	4.73	5.34	3.33	4.62	5.16
OCT	n/a	4.18	4.27	0.61	4.01	4.14	0.60	3.99	4.10
BEN	8.52	6.29	8.58	7.30	5.65	7.26	6.79	5.48	6.88
OL	9.28	4.38	6.20	8.46	4.23	5.63	8.05	4.21	5.47
pH 2.09									
Trp	4.61	4.92	5.84	4.30	4.65	5.43	4.10	4.57	5.30
5FL	4.35	4.84	5.66	4.02	4.59	5.29	3.91	4.49	5.15
5OH	2.36	5.91	6.49	2.31	5.62	6.14	2.26	5.38	5.86

n/a – partial separation was observable but R was not possible to calculate.

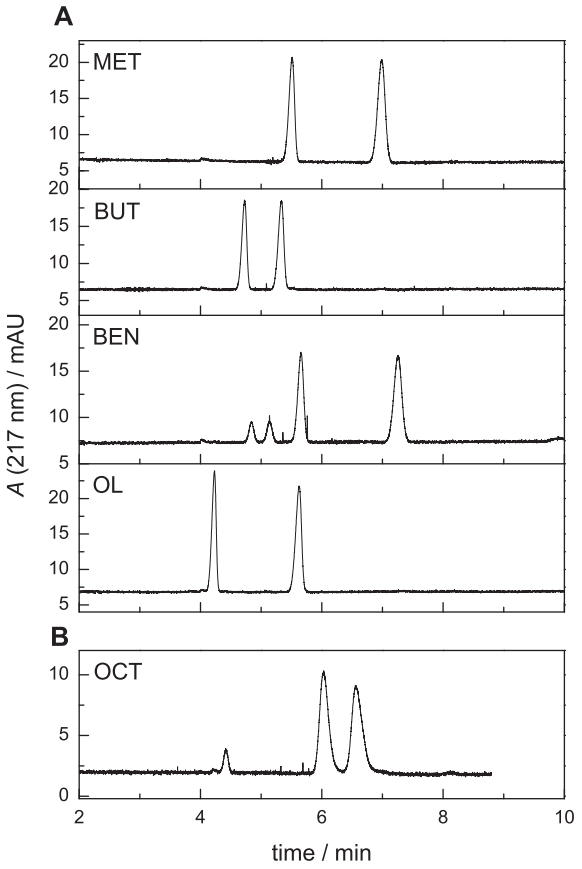

Fig. 1. Enantioseparation of basic Trp derivatives (A) MET, BUT, OCT, BEN with 5.0 mM S-γ-CD and (B) OL with mixed CSs: 1.0 mM β-CD + 4.0 mM S-γ-CD; acetate buffer (10 mM LiOH/20 mM acetic acid, pH 4.74), separation voltage −15 kV, additional pressure +30 mbar.

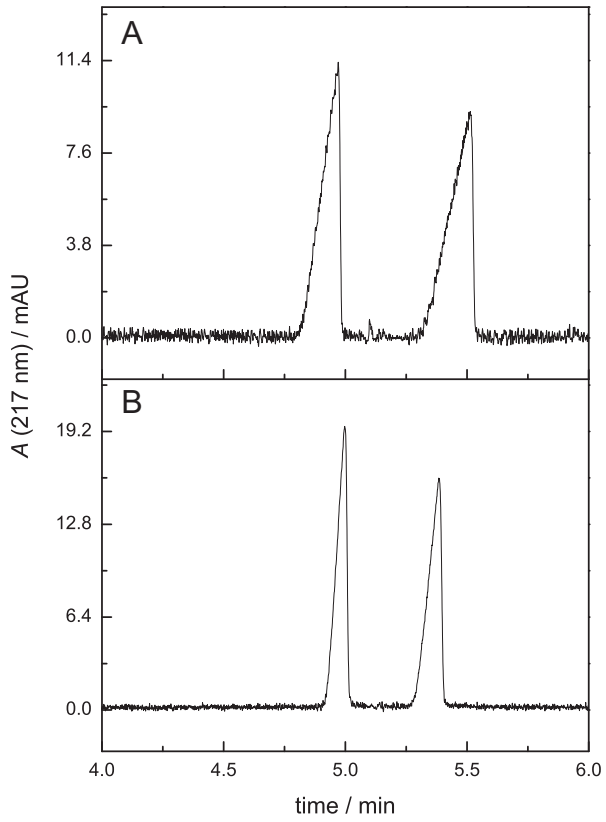

Fig. 2. Enantioseparation of BTrp in acetate buffer (10 mM LiOH/20 mM acetic acid, pH 4.74); separation voltage +25 kV, no additional pressure; (A) 1.0 mM A-β-CD; (B) 2.5 mM A-β-CD.

the same CSs were tested for Trp, 5FL and 5OH. No enantioseparation was achieved at pH 4.74 where all amphoteric analytes were neutral (for pK_A constants see Supplementary material, Table S1). Lower pH BGE, 20 mM phosphoric acid, pH 2.09, was used to keep Trp, 5FL and 5OH partially positively charged. The same concentrations of sulfated α-, β- and γ-CDs (2.5, 5.0 and 7.5 mM) under the same experimental conditions (−15 kV, +30 mbar) were tested. No enantioseparation was observed in the presence of S-α-CD. When S-β-CD was employed, partial separation of Trp enantiomers at the individual S-β-CD concentration levels ($R_{2.5}$ = 1.16, $R_{5.0}$ = 1.11, and $R_{7.5}$ = 1.08) and 5FL ($R_{2.5}$ = 0.95, $R_{5.0}$ = 0.82, and $R_{7.5}$ = 0.67) was achieved within 7 min. Only slight indication of enantioseparation was observed in the case of 5OH derivative.

S-γ-CD provided enantioseparation of all three amphoteric derivatives at all three concentration levels. Resolution values were high enough to shorten the analysis time by applying +40 mbar of additional pressure. All analysis in presence of S-γ-CD took less than 7 min. Resolution values and migration times in the presence of various concentration of S-γ-CD are listed in Table 1.

3.1.3. Acidic Trp derivative – BTrp

BTrp was the only representative of acidic derivatives of Trp. S-γ-CD was tested as potential CS in order to keep the same CS for all analytes. However, interaction of BTrp (in its neutral form) with S-γ-CD did not yield enantioselective separation. BTrp has been already successfully separated by teicoplanin [40] and the work of Vespalec et al. [41] has shown the possibility to separate BTrp enantiomers using β-CD. As the dependence of enantioselectivity on concentration of β-CD (in acetate buffer, pH 4.74) goes through a maximum [37], starting (5.0 mM) and lower (1.0 mM) concentrations of β-CD were tested. Only partial enantioseparation of BTrp within long analysis time (20 min) was achieved at 1.0 mM β-CD.

Finally, acetate buffer, pH 4.74, was used to obtain partially negatively charged BTrp and oppositely charged CS A-β-CD was employed. Positive voltage of 25 kV and no additional pressure were applied. Although 1.0 mM concentration of A-β-CD was sufficient to obtain fully separated enantiomers of BTrp derivative (R = 3.24) within 6 min, significant fronting of peaks was observed (Fig. 2A). The peak shapes of BTrp enantiomers were improved by increasing the CS concentration as shown in Fig. 2B, where electropherogram obtained with 2.5 mM A-β-CD (R = 4.20) is depicted.

3.2. HPLC separation

3.2.1. Basic Trp derivatives

Initially, reversed-phase (RP) separation mode was employed for enantioseparation of OL and Trp esters. Partial enantioseparation of all Trp esters was obtained using the amylose based Chiralpak AD-RH column with 10 mM ammonium acetate, pH 8.80 and ACN [21] or MeOH as organic modifiers (data not shown). Further optimization of mobile phase composition did not lead to baseline separation of any of the AA. Consequently, hydroxypropylether derivatized β-CD CSP (Astec Cyclobond™ I 2000 HP-RSP column) was tested. Baseline separation (R > 3) of BUT was obtained in mobile phase composed of MeOH/10 mM ammonium acetate, pH 4.00, 30/70 (*v/v*). Analysis time did not exceed 22 min and the optimized method is suitable for semipreparative purposes. Partial enantioseparation of MET and BEN was also observed with the

Table 2
The best results obtained in the NP and RP modes with respect to analysis time and resolution of enantiomers, retention factor of the first eluted enantiomer (k_1), enantioselectivity (α), resolution (R).

Analyte	NP mode					RP mode				
	CSP	k_1	α	R	MP composition (v/v/v)	CSP	k_1	α	R	MP composition (v/v)
Basic										
MET	IB	5.87	1.10	1.40	HEX/IPA/EA 85/15/0.1	HP CD	2.40	1.07	0.96	MeOH/buffer A 10/90
BUT	IB	3.05	1.10	1.51	HEX/IPA/EA 85/15/0.1	HP CD	1.90	1.18	1.85	MeOH/buffer A 40/60
OCT	IB	2.42	1.11	1.55	HEX/IPA/EA 85/15/0.3	AD RH	27.53	1.05	0.81	ACN/buffer B 40/60
BEN	IB	5.59	1.10	1.30	HEXIPA/EA 85/15/0.1	HP CD	1.50	1.06	1.00	MeOH/buffer A 20/80
OL	IB	10.71	1.37	1.56	HEX/IPA/TFA 90/10/0.1	x	x	x	x	x
Acidic										
BTrp	CF7	4.80	1.12	1.50	HEX/IPA/TFA 90/10/0.05	HP CD	14.35	1.05	1.50	MeOH/buffer C 10/90
	IB	0.88	1.23	1.79	HEX/IPA/TFA 80/20/0.1					

Buffer A: 10 mM ammonium acetate, pH 4.00.
Buffer B: 10 mM ammonium acetate, pH 8.80.
Buffer C: 1% TEAA, pH 4.00.
IB: CHIRALPAK IB column.
HP CD: CYCLOBOND™ I 2000 HP-RSP column.
AD RH: CHIRALPAK AD-RH column.
CF7: Larihc CF7-DMP column.

Table 3
The best results obtained for Trp and its ampholytic derivatives on teicoplanin-based columns in different separation modes, retention factor of the first eluted enantiomer (k_1), enantioselectivity (α), resolution (R).

Analyte					
	CSP	k_1	α	R	MP composition
Trp	T	2.14	1.94	2.99	MeOH
	T2	1.61	1.35	1.29	MeOH
5FL	T	1.73	2.24	3.88	MeOH
	T2	1.42	1.65	2.10	MeOH
5OH	T	2.52	2.47	3.80	MeOH
	T2	2.31	1.80	2.34	MeOH
	HILIC mode				
	CSP	k_1	α	R	MP composition (v/v)
Trp	T	1.63	1.93	3.45	MeOH/buffer A 95/5
	T2	1.40	1.35	1.51	MeOH/buffer A 95/5
5FL	T	1.32	2.24	4.60	MeOH/buffer A 95/5
	T2	0.85	1.61	3.00	MeOH/buffer A 80/20
5OH	T	1.81	2.41	4.63	MeOH/buffer A 95/5
	T2	1.50	1.64	3.11	MeOH/buffer A 90/10
	PO mode				
	CSP	k_1	α	R	MP composition (v/v/v)
Trp	T	2.20	1.88	1.91	MeOH/AcOH/TEA 100/0.1/0.05
	T2	1.45	1.35	1.50	MeOH/AcOH/TEA 100/0.5/0.1
5FL	T	1.87	2.12	2.46	MeOH/AcOH/TEA 100/0.1/0.05
	T2	1.31	1.63	2.32	MeOH/AcOH/TEA 100/0.5/0.1
5OH	T	2.67	2.43	2.85	MeOH/AcOH/TEA 100/0.1/0.05
	T2	2.04	1.77	2.47	MeOH/AcOH/TEA 100/0.5/0.1

Buffer A: 10 mM ammonium acetate, pH 4.00.
T: CHIROBIOTIC™ T column.
T2: CHIROBIOTIC™ T2 column.

β-CD-based CSP. Nevertheless, no enantioseparation of OL was achieved under tested separation conditions.

As a next step, normal phase (NP) separation systems were applied. First, CF-based CSP was tested as it had been reported to be suitable for the enantioseparation of analytes with primary amine functional group [42–44]. Only partial enantioseparation ($R \sim 1$) of all analytes was observed using the Larihc CF6-RN column. The best CSP for enantioseparation of basic Trp derivatives was the cellulose-based one (Chiralpak IB column) – see Table 2. Baseline separations of BUT and OCT enantiomers were achieved in reasonable analyses times (less than 15 min). Baseline separation of OL enantiomers was achieved only with the addition of TFA to mobile phase while EA was more promising mobile phase additive for enantioseparation of Trp esters.

3.2.2. Acidic Trp derivative – BTrp

BTrp enantiomers were baseline separated in NP mode with Larihc CF7 DMP or Chiralpak IB columns and also in RP mode with β-CD-based CSP– see Table 2. However, the long analysis time in the RP mode was not suitable for practical use. The best enantioseparation of BTrp enantiomers was obtained in separation system with Chiralpak IB column and mobile phase composed of HEX/IPA/TFA 80/20/0.1 (*v/v/v*).

3.2.3. Trp and amphoteric derivatives

CSPs based on teicoplanin (Chirobiotic T and T2 columns) were used for their unique capability to resolve primary and secondary amino acids in the native state [18,45]. These two columns differ in the unequal chiral selector coverage (higher on Chirobiotic T2)

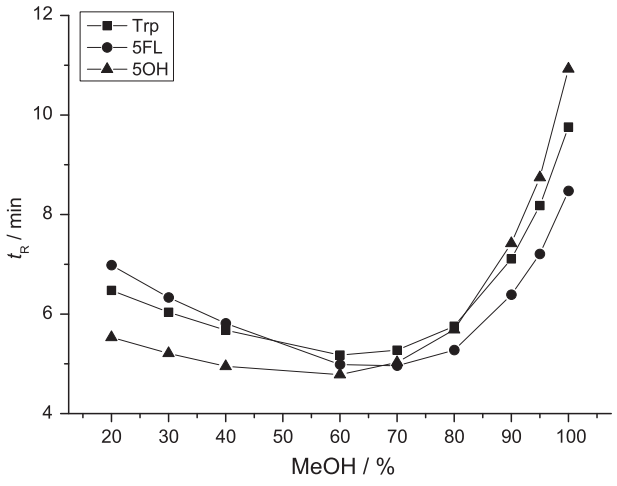

Fig. 3. The effect of MeOH content in mobile phase on retention time of the first eluted enantiomer of Trp, 5FL and 5OH.

and distinct linkage chemistry. It has been demonstrated before that higher teicoplanin coverage is not a prerequisite for better enantioseparation [46]. Silanol groups of silica gel carrier seem to participate on the retention mechanism of Chirobiotic T column. However, the extent of this contribution to retention mechanism is dependent on the mobile phase composition and analytes properties. The enantioseparation was performed on both columns in RP, HILIC and polar organic (PO) modes. Enantiomers of Trp, 5FL and 5OH were baseline separated using Chirobitotic T column with simple mobile phase composed of pure MeOH – see Table 3. Resolution values of the enantiomers ranged between 2.99 and 3.88. High resolution values and mobile phase composed of a single organic solvent indicate a possible utilization of the method for purification purposes. Enantiomers of 5FL and 5OH were also baseline separated in pure MeOH on Chirobiotic T2 column.

The effect of MeOH and buffer content in the mobile phase on retention and resolution of enantiomers was tested on both columns. Results confirmed mixed mode behavior of the teicoplanin-based CSPs [19] – see Fig. 3. The U-shaped dependencies indicate a change of the interaction/retention mechanism for all tested analytes. In the mobile phases with lower MeOH content teicoplanin-based CSPs exhibited a typical RP behavior while the mobile phases with high MeOH content resulted in a HILIC system. It is worth mentioning that higher resolution of all enantiomers was observed in HILIC mode. Baseline enantioseparation of all three analytes was achieved also in PO mode on the both CSPs. The best mobile phase composition in PO mode was not the same for both columns. Higher amounts of acidic and basic additives were required using the Chirobiotic T2 column – see Table 3. However, for both columns higher content of AcOH than of TEA in the mobile phase was necessary for successful enantioseparation of these amphoteric analytes.

4. Conclusion

CE and HPLC methods were found for separation of enantiomers of Trp and its eight (basic, amphoteric or acidic) derivatives of biological interest. The search for proper separation conditions showed that even the subtle changes in the analyte structure caused by derivatization require substantial changes of the separation system.

Different CDs proved their ability to serve as chiral selectors in CE. Good results of Trp esters enantioseparation were obtained with S-γ-CD. However, baseline separation of OCT was achieved only with dual selector system (β-CD + S-γ-CD). The most exacting task was the separation of BTrp enantiomers, which was successful with A-β-CD at last. Separation conditions were found for baseline resolution in an analysis time not exceeding 8 min.

In HPLC, polysaccharide-, CF-, CD- and teicoplanin-based CSPs were tested for their potential use for the enantioselective separation of Trp and its derivatives. Some of the optimized separation systems granted conditions even for semipreparative use. An easy to prepare separation system found for possible purification of Trp, 5OH and 5FL was composed of teicoplanin based CSP and pure MeOH as mobile phase.

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Appendix. A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.seppur.2015.12.012.

References

[1] A. Mollica, A. Stefanucci, F. Feliciani, G. Lucente, F. Pinnen, Synthesis of (S)-5,6-dibromo-tryptophan derivatives as building blocks for peptide chemistry, Tetrahedron Lett. 52 (2011) 2583–2585.
[2] A. Daugan, P. Grondin, C. Ruault, A.C.L. de Gouville, H. Coste, J.M. Linget, J. Kirilovsky, F. Hyafil, R. Labaudiniere, The discovery of tadalafil: A novel and highly selective PDE5 inhibitor. 2: 2,3,6,7,12,12a-hexahydropyrazino [1',2':1,6] pyrido [3,4-b]indole-1,4-dione analogues, J. Med. Chem. 46 (2003) 4533–4542.
[3] C.A. Royer, Probing protein folding and conformational transitions with fluorescence, Chem. Rev. 106 (2006) 1769–1784.
[4] M.E. Kieffer, L.M. Repka, S.E. Reisman, Enantioselective synthesis of tryptophan derivatives by a tandem Friedel-Crafts conjugate addition/asymmetric protonation reaction, J. Am. Chem. Soc. 134 (2012) 5131–5137.
[5] L. Gentilucci, A. Tolomelli, F. Squassabia, Peptides and peptidomimetics in medicine, surgery and biotechnology, Curr. Med. Chem. 13 (2006) 2449–2466.
[6] L. Gentilucci, L. Cerisoli, R. De Marco, A. Tolomelli, A simple route towards peptide analogues containing substituted (S)- or (R)-tryptophans, Tetrahedron Lett. 51 (2010) 2576–2579.
[7] R.L. Sverdlov, S.D. Brinkevich, O.I. Shadyro, Effects of tryptophan derivatives and beta-carboline alkaloids on radiation- and peroxide-induced transformations of ethanol, Radiat. Phys. Chem. 98 (2014) 77–85.
[8] O. Bassas, N. Llor, M.M.M. Santos, R. Griera, E. Molins, M. Amat, J. Bosch, Biogenetically inspired enantioselective approach to indolo [2,3-a] – and benzo[a]quinolizidine alkaloids from a synthetic equivalent of secologanin, Org. Lett. 7 (2005) 2817–2820.
[9] Y. Ma, H. Wu, J. Zhang, Y. Li, Enantioselective synthesis and antimicrobial activities of tetrahydro-B-carboline diketopiperazines, Chirality 25 (2013) 656–662.
[10] M.B. Strom, B.E. Haug, M.L. Skar, W. Stensen, T. Stiberg, J.S. Svendsen, The pharmacophore of short cationic antibacterial peptides, J. Med. Chem. 46 (2003) 1567–1570.
[11] H. Franzen, L. Grehn, U. Ragnarsson, Synthesis, properties, and use of N^{in}-BOC-tryptophan derivatives, J. Chem. Soc., Chem. Commun. (1984) 1699–1700.
[12] P. Micuch, D. Seebach, Preparation of beta(2)-homotryptophan derivatives for beta-peptide synthesis, Helv. Chim. Acta 85 (2002) 1567–1577.
[13] F. Kilar, S. Fanali, Separation of tryptophan-derivative enantiomers with iron-free human serum transferrin by capillary zone electrophoresis, Electrophoresis 16 (1995) 1510–1518.
[14] H. Hoedl, J. Koidl, M.G. Schmid, G. Guebitz, Chiral resolution of tryptophan derivatives by CE using canine serum albumin and bovine serum albumin as chiral selectors, Electrophoresis 27 (2006) 4755–4762.
[15] H. Hoedl, M.G. Schmid, G. Guebitz, Chiral separation of amino acids and glycyl dipeptides by chiral ligand-exchange capillary electrophoresis comparing Cu (II), Co(II), Ni(II) and Zn(II) complexes of three different sugar acids, J. Chromatogr. A 1204 (2008) 210–218.
[16] M. Goodarzi, M.P. Freitas, Prediction of electrophoretic enantioseparation of aromatic amino acids/esters through MIA-QSPR, Sep. Purif. Technol. 68 (2009) 363–366.
[17] Z. Zhang, M. Zhang, Y. Liu, X. Yang, L. Luo, S. Yao, Preparation of L-phenylalanine imprinted polymer based on monodisperse hybrid silica microsphere and its application on chiral separation of phenylalanine racemates as HPLC stationary phase, Sep. Purif. Technol. 87 (2012) 142–148.

[18] I. Ilisz, A. Aranyi, A. Peter, Chiral derivatizations applied for the separation of unusual amino acid enantiomers by liquid chromatography and related techniques, J. Chromatogr. A 1296 (2013) 119–139.
[19] G. Kucerova, J. Vozka, K. Kalikova, R. Geryk, D. Plecita, T. Pajpanova, E. Tesarova, Enantioselective separation of unusual amino acids by high performance liquid chromatography, Sep. Purif. Technol. 119 (2013) 123–128.
[20] I. Ilisz, Z. Gecse, Z. Pataj, F. Fulop, G. Toth, W. Lindner, A. Peter, Direct high-performance liquid chromatographic enantioseparation of secondary amino acids on *Cinchona* alkaloid-based chiral zwitterionic stationary phases. Unusual temperature behavior, J. Chromatogr. A 1363 (2014) 169–177.
[21] K. Kalikova, R. Geryk, J. Vozka, E. Tesarova, Evaluation of differences between Chiralpak IA and Chiralpak AD-RH amylose-based chiral stationary phases in reversed-phase high-performance liquid chromatography, J. Sep. Sci. 38 (2015) 711–719.
[22] T. Alizadeh, Enantioseparation of atenolol using chiral ligand-exchange chromatography on C-8 column, Sep. Purif. Technol. 118 (2013) 879–887.
[23] K. Kalikova, M. Riesova, E. Tesarova, Recent chiral selectors for separation in HPLC and CE, Cent. Eur. J. Chem. 10 (2012) 450–471.
[24] P. Rezanka, K. Navratilova, M. Rezanka, V. Kral, D. Sykora, Application of cyclodextrins in chiral capillary electrophoresis, Electrophoresis 35 (2014) 2701–2721.
[25] J. Stavrou, M.C. Mavroudi, C.P. Kapnissi-Christodoulou, Chiral selectors in CE: recent developments and applications (2012-mid 2014), Electrophoresis 36 (2015) 101–123.
[26] V. Hruska, M. Riesova, B. Gas, A nonlinear electrophoretic model for PeakMaster: I. Mathematical model, Electrophoresis 33 (2012) 923–930.
[27] echmet.natur.cuni.cz, accessed 24th April 2015.
[28] M. Benes, M. Riesova, J. Svobodova, E. Tesarova, P. Dubsky, B. Gas, Complexation of buffer constituents with neutral complexation agents: Part II. Practical impact in capillary zone electrophoresis, Anal. Chem. 85 (2013) 8526–8534.
[29] M. Riesova, J. Svobodova, Z. Tosner, M. Benes, E. Tesarova, B. Gas, Complexation of buffer constituents with neutral complexation agents: Part I. Impact on common buffer properties, Anal. Chem. 85 (2013) 8518–8525.
[30] G.L. Erny, E.T. Bergstrom, D.M. Goodall, Electromigration dispersion in capillary zone electrophoresis – Experimental validation of use of the Haarhoff-Van der Linde function, J. Chromatogr. A 959 (2002) 229–239.
[31] P.C. Haarhoff, H.J. Van der Linde, Concentration dependence of elution curves in non-ideal gas chromatography, Anal. Chem. 38 (1966) 573–582.
[32] N. Matthijs, C. Perrin, M. Maftouh, D.L. Massart, Y. Vander Heyden, Knowledge-based system for method development of chiral separations with capillary electrophoresis using highly-sulphated cyclodextrins, J. Pharm. Biomed. Anal. 27 (2002) 515–529.
[33] C.E. Evans, A.M. Stalcup, Comprehensive strategy for chiral separations using sulfated cyclodextrins in capillary electrophoresis, Chirality 15 (2003) 709–723.
[34] N. Matthijs, S. Van Hemelryck, M. Maftouh, D.L. Massart, Y. Vander Heyden, Electrophoretic separation strategy for chiral pharmaceuticals using highly-sulfated and neutral cyclodextrins based dual selector systems, Anal. Chimica Acta 525 (2004) 247–263.
[35] M. Riesova, J. Svobodova, K. Uselova, Z. Tosner, I. Zuskova, B. Gas, Determination of thermodynamic values of acidic dissociation constants and complexation constants of profens and their utilization for optimization of separation conditions by Simul 5 Complex, J. Chromatogr. A 1364 (2014) 276–288.
[36] K. Uselova-Vcelakova, I. Zuskova, B. Gas, Stability constants of amino acids, peptides, proteins, and other biomolecules determined by CE and related methods: Recapitulation of published data, Electrophoresis 28 (2007) 2145–2152.
[37] M. Benes, I. Zuskova, J. Svobodova, B. Gas, Determination of stability constants of complexes of neutral analytes with charged cyclodextrins by affinity capillary electrophoresis, Electrophoresis 33 (2012) 1032–1039.
[38] J. Ostergaard, H. Jensen, R. Hoim, Use of correction factors in mobility shift affinity capillary electrophoresis for weak analyte – ligand interactions, J. Sep. Sci. 32 (2009) 1712–1721.
[39] S. Ehala, E. Marklik, P. Toman, V. Kasicka, ACE applied to the quantitative characterization of benzo-18-crown-6-ether binding with alkali metal ions in a methanol-water solvent system, Electrophoresis 31 (2010) 702–708.
[40] E. Tesarova, Z. Bosakova, V. Pecakova, Comparison of enantioselective separation of N-tert.-butyloxycarbonyl amino acids and their non-blocked analogues on teicoplanin-based chiral stationary phase, J. Chromatogr. A 838 (1999) 121–129.
[41] R. Vespalec, S. Fanali, P. Bocek, Consequences of a maximum existing in the dependence of separation selectivity on concentration of cyclodextrin added as chiral selector in capillary zone electrophoresis, Electrophoresis 15 (1994) 1523–1525.
[42] K. Kalikova, L. Janeckova, D.W. Armstrong, E. Tesarova, Characterization of new R-naphthylethyl cyclofructan 6 chiral stationary phase and its comparison with R-naphthylethyl beta-cyclodextrin-based column, J. Chromatogr. A 1218 (2011) 1393–1398.
[43] A. Aranyi, A. Bagi, I. Ilisz, Z. Pataj, F. Fueloep, D.W. Armstrong, P. Antal, High-performance liquid chromatographic enantioseparation of amino compounds on newly developed cyclofructan-based chiral stationary phases, J. Sep. Sci. 35 (2012) 617–624.
[44] P. Sun, D.W. Armstrong, Effective enantiomeric separations of racemic primary amines by the isopropyl carbamate-cyclofructan6 chiral stationary phase, J. Chromatogr. A 1217 (2010) 4904–4918.
[45] A. Berthod, Y.B. Liu, C. Bagwill, D.W. Armstrong, Facile liquid chromatographic enantioresolution of native amino acids and peptides using a teicoplanin chiral stationary phase, J. Chromatogr. A 731 (1996) 123–137.
[46] M. Honetschlagerova-Vadinska, S. Srkalova, Z. Bosakova, P. Coufal, E. Tesarova, Comparison of enantioselective HPLC separation of structurally diverse compounds on chiral stationary phases with different teicoplanin coverage and distinct linkage chemistry, J. Sep. Sci. 32 (2009) 1704–1711.

Publikace III: Enantioselektivní potenciál chirálních stacionárních fází na bázi imobilizovaných polysacharidů v reversním módu

CSF na bázi derivatizovaných polysacharidů jsou nejpoužívanější CS pro separaci biologicky aktivních látek v HPLC. Imobilizované polysacharidové CSF se připravují navázáním chirálního polymeru na silikagelový nosič pomocí kovalentní vazby. Takto připravené CSF jsou multimodální. Nejčastěji však nacházejí uplatnění při analýzách v NP módu. Nicméně chirální separace reálných vzorků v RP módu jsou upřednostňovány především díky kompatibilitě s MS detekcí.

Publikace III je zaměřena na charakterizaci a porovnání enantioselektivního potenciálu čtyř komerčních imobilizovaných CSF lišících se jednak povahou polysacharidového skeletu (amylosa *versus* celulosa) a typem derivatizační skupiny. Testované kolony byly následující: CHIRALPAK IA, CS: *tris*(3,5-dimethylfenylkarbamát) amylosy; CHIRALPAK IB, CS: *tris*(3,5-dimethylfenylkarbamát) celulosy; CHIRALPAK IC, CS: *tris*(3,5-dichlorofenylkarbamát) celulosy; CHIRALPAK ID, CS: *tris*(3-chlorofenylkarbamát) amylosy. Sada třiceti strukturně různorodých látek zahrnující kyselé (8), bifunkční (4) a bazické analyty (18), byla použita ke studiu enantiodiskriminačních schopností jednotlivých CSF.

Kyselé analyty na bázi profenů byly separovány v MF obsahující acetonitril či methanol jako organické modifikátory a vodný roztok kyseliny mravenčí (pH = 2,10). Porovnání chromatografických dat na testovaných chirálních kolonách ukázalo, že nejvhodnější CSF pro enantioseparaci kyselých analytů je *tris*(3,5-dimethylfenylkarbamát) amylosy. Kolona CHIRALPAK IA byla schopna rozdělit z celkového počtu osmi profenů šest sloučenin až na základní linii a dvě částečně.

Thiazidová diuretika jsou skupinou bifunkčních analytů, které mohou být separovány v kyselém, neutrálním i bazickém prostředí. CSF na bázi derivatizované amylosy vykazovaly lepší enantioselektivní potenciál pro testované analyty ve srovnání s kolonami na bázi celulosy. Všechna diuretika byla separována až na základní linii na amylosové koloně CHIRALPAK ID.

Chirální báze byly separovány v MF obsahující organický modifikátor (acetonitril, methanol) a 100 mM vodný roztok KPF_6 či octano-amonný pufr (10 mM, pH = 8,80).

Přídavek chaotropní soli (KPF_6) do MF umožnil vytvořit iontový pár s pozitivně nabitým bazickým analytem. Srovnání výsledků získaných na amylosových a celulosových CSF ukazuje, že bazické biologicky aktivní látky obsahující ve své struktuře sekundární aminoskupinu (β-blokátory) byly lépe separovány na CSF na bázi celulosy (CHIRALPAK IB). Na druhou stranu, amylosová kolona (CHIRALPAK ID) vykazovala vyšší enantioselektivitu pro primární aminy (deriváty amfetaminu).

Enantioselektivní potenciál testovaných kolon se lišil podle složení MF či struktury analytů. Amylosové CSF vykazovaly vyšší enantioselektivitu především pro kyselé a bifunkční analyty. CSF na bázi derivatizované celulosy (CHIRALPAK IB) ukázala vyšší enantioselektivní potenciál pro většinu bazických analytů se sekundárními aminoskupinami.

Publikace III

Enantioselective potential of chiral stationary phases based on immobilized polysaccharides in reversed phase mode

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Enantioselective potential of chiral stationary phases based on immobilized polysaccharides in reversed phase mode

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ABSTRACT

Derivatized polysaccharide-based columns have high enantiodiscrimination potential mainly in normal phase separation mode. In this work chiral recognition ability of four immobilized polysaccharide-derived chiral stationary phases was evaluated under reversed phase conditions. A set of 30 chiral compounds, particularly drugs possessing various functional groups was used for testing. Baseline enantioseparation was achieved for 17 of them. In general, amylose-based chiral stationary phases showed higher enantioselectivity than the cellulose-based ones, mainly for acidic and bifunctional compounds. The influence of the type and pH of the aqueous mobile phase constituents as well as the role of the organic modifier on the enantioselective separation ability of the stationary phases were also investigated and compared. Complementary separations were obtained on the amylose- and cellulose-based columns. The immobilized polysaccharide-based chiral stationary phases were shown to be useful tool for the enantioseparation of a broad spectrum of chiral analytes in reversed phase separation mode.

1. Introduction

Enantioseparations of drug substances have become a very significant topics in analytical chemistry, especially in the pharmaceutical and biomedical fields. The systematic investigation of biological properties of the individual enantiomeric forms is an indispensable part of the development of new chiral drugs. The desired pharmacological activity usually resides mainly in one enantiomer while the other enantiomer may be inactive, less potent, or even toxic [1–3]. The growing trend in the pharmaceutical industry is to produce drugs in enantiomerically pure forms.

Many separation techniques can be used for chiral separations [4–12]. Among them HPLC has become most commonly used for separations of chiral drugs and other biologically active compounds. It can be applied in analytical scale as well as for preparative purposes. Numerous commercially available chiral stationary phases (CSPs) can be utilized [13]. Polysaccharide-based CSPs have proven to be one of the most useful tools for chiral resolution in HPLC [14–19]. They are derived particularly from amylose or cellulose. The native polysaccharides have rather limited ability to serve as chiral selectors. Derivatization substantially improves their recognition ability that depends on both the structure of the polymer chain and on the type of derivative moieties [15,17,20–22]. Aromatic esters and carbamates play the major role for the enantiorecognition profiles of these CSPs and their resolving power enhances significantly when electron-donating or electron withdrawing substituents are introduced in the proper position of the phenyl moiety [17]. Polysaccharide-based CSPs are produced either by physical coating or immobilization of the chiral polymers on a silica support. The coated CSPs could be used either under NP or RP conditions while the immobilized ones are multimodal, i.e. they can be employed in both modes and resist an extended range of solvents [23–25]. Nowadays the immobilized CSPs are mostly used in the normal phase (NP) mode [26–33]. However, reversed phase (RP) separations are preferred in analysis due to better compatibility with MS detection and possibility of direct injection of biological matrices in drug separations. Despite the need for the RP mode separations they have been reported just seldom with the immobilized polysaccharide-based columns [34–37].

This paper is focused on evaluation and comparison of chiral recognition abilities of four commercialized immobilized polysaccharide-based CSPs in RP mode. The objectives of the article are to show the differences of separation behavior among

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0021-9673/

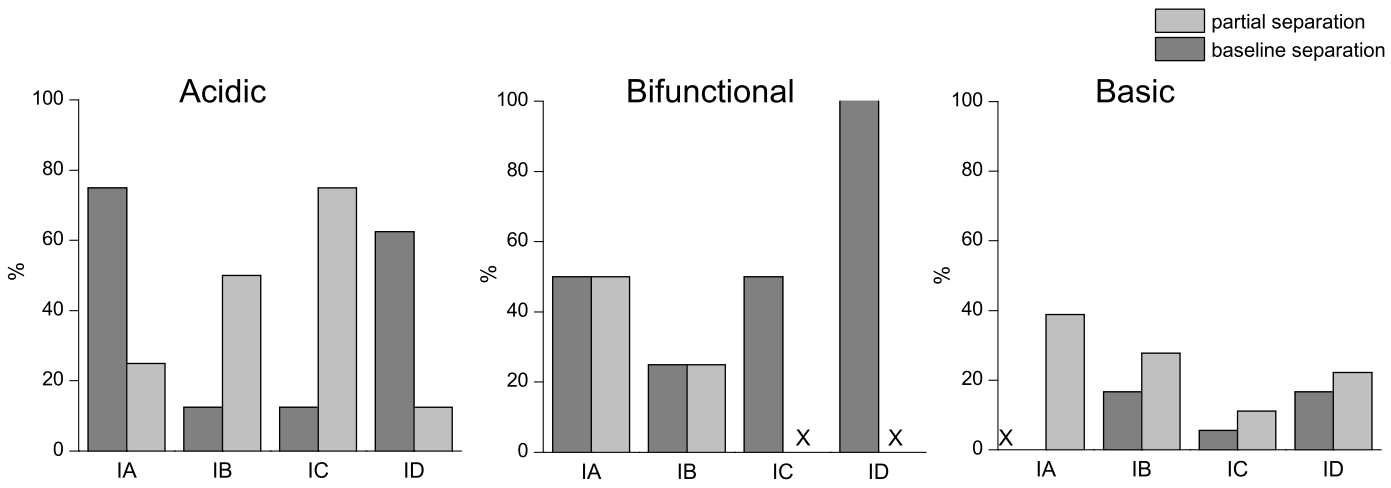

Fig. 1. Separation success rates on different columns (x means no separated compounds).

these CSPs, as they differ both by the nature of the polysaccharide backbone and by the phenyl moiety. The tested columns were CHIRALPAK IA, CHIRALPAK IB, CHIRALPAK IC and CHIRALPAK ID (for their description see Section 2) [25]. In the frame of the column examination various types of the RP mobile phases (MPs) were screened. A set of 30 diverse biological active compounds, including acidic, bifunctional and basic ones, was tested to investigate the enantioselective potential of the individual CSPs.

2. Experimental

2.1. Instrumentation

All chromatographic measurements were performed on Waters Alliance System (Waters, MA, USA) composed of Waters 2695 Separation Module, Waters 2996 Photodiode Array Detector, an autosampler 717 Plus, and Waters Alliance Series column heater, controlled by Empower software, which was used for data acquisition and analyses. The analytical columns and also the guard columns, CHIRALPAK IA, CHIRALPAK IB, CHIRALPAK IC and CHIRALPAK ID were obtained from Chiral Technologies Europe (Illkirch, France). The chiral selectors immobilized on 5 μm silica particles of these CSPs were: tris(3,5-dimethylphenylcarbamate) of amylose, CHIRALPAK IA; tris(3,5-dimethylphenylcarbamate) of cellulose, CHIRALPAK IB; tris(3,5-dichlorophenylcarbamate) of cellulose, CHIRALPAK IC; and tris(3-chlorophenylcarbamate) of amylose, CHIRALPAK ID, respectively. All the tested columns were sized 250 mm × 4.6 mm i.d. and the corresponding guard columns 10 mm × 4.6 mm i.d.

2.2. Chemicals and reagents

Methanol (MeOH, Chromasolv® for HPLC), acetonitrile (ACN, gradient grade), ammonium acetate (purity ≥99%), potassium hexafluorophosphate (KPF_6, 98%), formic acid (HCOOH, reagent grade, ≥95%) and ammonium hydroxide solution (ACS reagent, 28–30% NH_3 basis) were supplied by Sigma-Aldrich (St. Louis, USA). Sodium dihydrogen phosphate anhydrous was purchased from Fluka (Buchs, Switzerland) and *ortho*-phosphoric acid (H_3PO_4, 85%) from Lachema (Neratovice, Czech Republic). The deionized water was purified by a Milli-Q water purification system from Millipore (Bedford, USA). Profens (carprofen, fenoprofen, flurbiprofen, ibuprofen, indoprofen, ketoprofen, suprofen, tiaprofenic acid), β-blockers (acebutolol, alprenolol, atenolol, bopindolol, metipranolol, metoprolol, oxprenolol, pindolol, propranolol), thalidomide and tramadol were purchased from Sigma-Aldrich (St. Louis, USA). Diuretics (bendroflumethiazide, butizide, chlorthalidone, mefruside), amphetamine and its derivatives (2-F-methcathinone, 3-F-amphetamine, 4-F-amphetamine, 4-F-methcathinone) were gifts of Prof. M. G. Schmid from Karl-Franzens University Graz, Austria. BP 766 and its hydrolytic decomposition product BP 34 were synthesized at the University of California, Radiology Research [38]. The structures of the compounds are depicted in Fig. S1 in Supplement.

2.3. Procedures

The various types of RP MPs were tested. MPs were composed of an aqueous part and ACN or MeOH in various volume ratios. Either water acidified with HCOOH or phosphate buffer (20 mM), both pH 2.10, served for the separation of acidic racemates. Either acidified water or aqueous solution of KPF_6 (100 mM) or ammonium acetate buffer (10 mM) adjusted to pH 8.80 with ammonium solution were suitable for separation of bifunctional analytes. MPs containing aqueous solution of KPF_6 (100 mM) or ammonium acetate buffer (10 mM, pH 8.80) were used for separating basic analytes. The MPs were always filtered through a membrane filter with pore size of 0.45 μm and degassed before use.

The chromatographic measurements were performed at a flow rate 1.0 mL/min and at a temperature 25 °C. Detection wavelength was 254 nm. Void volume was determined using the solvent peak. Injection volume was 10 μL. All measurements were performed in triplicates. Stock solutions of samples were prepared in concentration 1 mg/mL using ACN or mixture of ACN and water as solvents.

3. Results and discussion

The chiral selectors based on polysaccharide derivatives are neutral macromolecules and do not offer ionic interactions [37]. Therefore, ionization of chiral compounds negatively affects their enantioseparation. Moreover, solvation of a charged analyte inhibits possible direct interaction between the analyte and the CSP in RP separation system. A set of 30 compounds, mainly drugs (8 acidic, 4 bifunctional and 18 basic) was analyzed in order to evaluate the chiral recognition ability of the tested CSPs. Separation success rates for the individual subgroups of analytes were determined (see Fig. 1). Because of different numbers of compounds in the subgroups the diagram gives just a very rough idea on the separation success in the subsets of analytes. Nevertheless, this

Table 1
Chromatographic data of the studied profens under screening MP conditions. Retention factor of the first eluted enantiomer (k_1), enantioselectivity (α), resolution ($R_{1/2}$).

Compound	MP: ACN/aqueous solution of HCOOH (pH 2.10) 40/60 (v/v)											
	IA	IB	IC	ID	IA	IB	IC	ID	IA	IB	IC	ID
	k_1				α				$R_{1/2}$			
Carprofen	8.70	9.87	3.09	3.37	1.18	1.07	1.00	1.12	2.74	1.59	0.00	1.52
Fenoprofen	4.23	7.05	2.78	3.22	1.00	1.00	1.00	1.00	0.00	0.00	0.00	0.00
Flurbiprofen	7.13	7.65	3.17	3.63	1.22	1.00	1.00	1.27	4.75	0.00	0.00	3.11
Ibuprofen	6.64	6.53	3.37	3.41	1.05	1.00	1.02	1.00	1.19	0.00	0.59	0.00
Indoprofen	5.22	3.91	3.54	2.86	1.07	1.03	1.03	1.14	1.25	0.68	0.61	1.62
Ketoprofen	3.54	4.18	2.49	2.54	1.00	1.00	1.02	1.00	0.00	0.00	0.52	0.00
Surprofen	4.72	4.11	2.72	2.38	1.10	1.00	1.08	1.10	1.08	0.00	1.52	1.07
Tiaprofenic acid	4.73	4.53	3.03	2.90	1.06	1.00	1.02	1.10	1.21	0.00	0.43	1.20
	MP: MeOH/aqueous solution of HCOOH (pH 2.10) 60/40 (v/v)											
	IA	IB	IC	ID	IA	IB	IC	ID	IA	IB	IC	ID
	k_1				α				$R_{1/2}$			
Carprofen	33.49	16.46	4.39	6.28	1.25	1.00	1.04	1.71	1.85	0.00	0.46	3.88
Fenoprofen	12.63	8.10	3.67	5.97	1.07	1.00	1.00	1.00	1.12	0.00	0.00	0.00
Flurbiprofen	3.61	8.56	4.33	6.33	3.06	1.03	1.00	1.83	4.00	0.43	0.00	6.77
Ibuprofen	12.12	7.19	4.46	5.14	1.14	1.05	1.00	1.05	1.72	0.50	0.00	0.35
Indoprofen	26.72	7.12	18.18	15.49	1.17	1.05	1.06	1.45	1.27	0.63	0.63	2.95
Ketoprofen	8.56	4.68	6.16	6.19	1.04	1.00	1.00	1.00	0.86	0.00	0.00	0.00
Surprofen	11.44	4.77	7.62	6.72	1.31	1.00	1.08	1.39	3.42	0.00	0.66	4.33
Tiaprofenic acid	15.45	5.26	8.68	12.08	1.15	1.00	1.00	1.20	1.75	0.00	0.00	2.27

IA, IB, IC, ID mean CHIRALPAK IA, CHIRALPAK IB, CHIRALPAK IC and CHIRALPAK ID columns, respectively.

general view shows that amylose-based columns were more successful in separating the tested analytes, no matter if acidic or bifunctional compounds were tested. Separation of basic analytes was less successful, with the lowest enantioselectivity obtained for cellulose-based CHIRALPAK IC column.

3.1. Enantioseparation of acidic compounds

Profens contain carboxylic group attached to a stereogenic carbon center. For enantioselective separation of the acidic analytes, it is essential to use acidic MPs to suppress dissociation of the analytes. These MPs were composed of ACN or MeOH and acidified water adjusted to pH 2.10 using HCOOH or phosphate buffer (20 mM, pH 2.10). However, no significant differences related to the aqueous parts of the MPs were observed in terms of retentive and enantioselective behavior of the tested CSPs toward separation of profens enantiomers. Therefore, the simple MPs with acidified water were chosen for further study. In combination, appropriate organic modifier (OM) had to be chosen because of its crucial effect on the enantioseparation of the acidic analytes. The difference in hydrogen bonding capability of ACN and MeOH affects the tertiary structure of the polysaccharide-based CSPs and contributes to enantioresolution [39]. The chromatographic data obtained under initial screening conditions are summarized in Table 1. When ACN was used as the OM the phenylcarbamates of cellulose and amylose bearing electron-withdrawing chlorine substituent(s) provided lower retention than tris(3,5-dimethylphenylcarbamate) of amylose or cellulose. However, in the MPs containing MeOH, the same retention trend was not observed. Higher values of enantioresolution were achieved on amylose-based CSPs with non-chlorinated substituents in ACN/aqueous solution of HCOOH (pH 2.10) 40/60 (v/v), except for indoprofen. On the other hand, chlorinated phenylcarbamate of amylose provided better enantioresolution under the screening conditions MeOH/aqueous solution of HCOOH (pH 2.10) 60/40 (v/v) for the majority of the tested profens. In general, both amylose-based CSPs (CHIRALPAK IA and CHIRALPAK ID) provided better separation for the majority of the tested profens with respect to retention and resolution particularly with the MPs composed of MeOH and acidified water. The chromatographic data with optimized MP compositions for achieving baseline separation of the individual profen enantiomers on the four CSPs are shown in Table 2. High amount of MeOH (60–90 vol%) in the MPs resulted in sufficient resolution for the majority of analytes on different columns. We could find conditions that yielded baseline separations for all the acidic analytes except of fenoprofen and ketoprofen, for which just partial enantioseparation was achieved. The complementary effect of the enantioseparation abilities of tris(3,5-dimethylphenylcarbamate) of cellulose

Table 2
Baseline enantiomer resolution of the studied profens. For abbreviations see headings to Table 1.

Compound	Column	k_1	α	$R_{1/2}$	MP composition (v/v)
Carprofen	IA	1.90	1.15	2.16	ACN/A part 60/40
	IB	9.87	1.07	1.59	ACN/A part 40/60
	ID	1.28	1.39	2.56	MeOH/A part 75/25
Flurbiprofen	IA	1.02	1.23	1.84	MeOH/A part 90/10
	ID	1.30	1.50	3.79	MeOH/A part 75/25
Ibuprofen	IA	12.12	1.14	1.72	MeOH/A part 60/40
Indoprofen	IA	4.60	1.14	1.52	MeOH/A part 80/20
	ID	2.86	1.14	1.62	ACN/A part 40/60
Suprofen	IA	2.06	1.23	2.66	MeOH/A part 80/20
	IC	2.72	1.08	1.52	ACN/A part 40/60
	ID	1.71	1.26	2.11	MeOH/A part 75/25
Tiaprofenic acid	IA	2.52	1.12	1.57	MeOH/A part 80/20
	ID	4.17	1.16	1.81	MeOH/A part 70/30

A part: aqueous solution of HCOOH (pH 2.10).

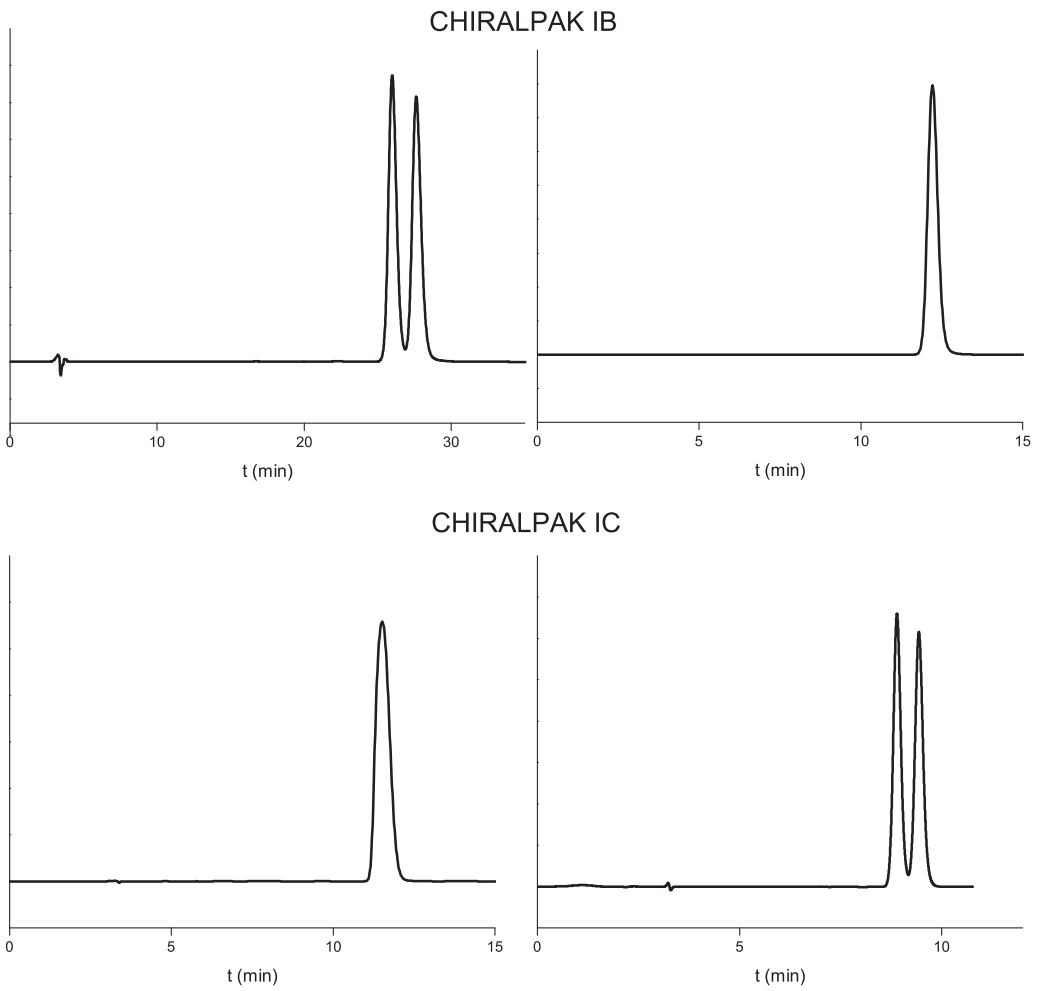

Fig. 2. Chromatograms of separation of carprofen (left) and suprofen (right) on cellulose-based CSPs. MP composition: ACN/aqueous solution of HCOOH (pH 2.10), 40/60 (*v*/*v*).

and tris(3,5-dichlorophenylcarbamate) of cellulose is illustrated on the enantioseparation of carprofen and suprofen in Fig. 2. In summary, the best separation performance (from the viewpoint of baseline resolution) for the acidic analytes was identified for amylose-based CSPs (see also Fig. 1). CHIRALPAK IA was able to resolve 6 compounds to baseline and two partially. On CHIRALPAK ID five of the tested profens were baseline separated and one partially.

3.2. Enantioseparation of bifunctional compounds

Diuretics (bendroflumethiazide, butizide, chlorthalidone, mefruside) are a class of bifunctional analytes, which should be quite insensitive to pH changes, therefore can be separated in acidic, neutral, or basic MPs. For the resolution of bifunctional racemates MPs consisting of acidified water (pH 2.10), 100 mM aqueous solution of KPF_6 or basic 10 mM ammonium acetate (pH 8.80) in all cases with ACN or MeOH as OM were used. In the screening step, the same MPs were used as for the enantioseparation of acidic compounds. Chromatographic data are shown in Table S1 in Supplement. In contrast to the separation of the acidic analytes, no significant differences between the values of retention factors of the bifunctional analytes were found, except for mefruside separated on amylose-based columns (CHIRALPAK IA and CHIRALPAK ID) where the use of MeOH as the organic modifier led to an enormous increase of retention. The weakening of the MP eluting power by replacing ACN for MeOH did not result in any significant improvement of resolution, and additionally the use of MeOH caused substantial worsening of peak shapes. MeOH may disrupt the hydrogen bonding between analyte molecule and CSP and thus worsen enantioseparation [40]. Therefore, a more detailed study focused on the influence of composition of the aqueous part of MPs was carried out with 30 vol% ACN as the OM (see Table 3). The differences in retention and enantioseparation were not significant for the majority of analytes, as they seemed to be insensitive to pH changes (as expected). The retention acquired in the ACN/100 mM aqueous solution of KPF_6 30/70 (*v*/*v*) was in most cases lower than in the corresponding MPs with acidic (pH 2.10) or basic (pH 8.80) solutions. In general, regarding the retention factor, the columns based on tris(3,5-dimethylphenylcarbamate) of amylose or cellulose showed somewhat higher retention for the tested compounds than CSPs based on tris(3,5-dichlorophenylcarbamate) of cellulose and tris(3-chlorophenylcarbamate) of amylose (see Table 3). Comparing the results obtained on amylose- and cellulose-based CSPs, it is evident that the studied analytes exhibited higher (in particular for CHIRALPAK ID) or equal enantioselectivity on amylose-based CSPs. The complementary separation behavior on CHIRALPAK IA and CHIRALPAK IC is shown on the enantioseparation of mefruside and bendroflumethiazide in Fig. 3. Amylose-based CSPs appeared more potent for separation of the bifunctional analytes than cellulose-based columns (compare in Fig. 1). In summary, all the diuretics tested were baseline resolved on CHIRALPAK ID in acidic MP (see Table S2 in Supplement).

Table 3
Chromatographic data of the studied bifunctional compounds in MPs with different aqueous parts. For abbreviations see headings to Table 1.

Compound	MP: ACN/aqueous solution of HCOOH (pH 2.10) 30/70 (*v*/*v*)											
	IA	IB	IC	ID	IA	IB	IC	ID	IA	IB	IC	ID
	k_1				α				$R_{1/2}$			
Bendroflumethiazide	10.81	14.62	4.59	7.05	1.05	1.04	1.11	1.09	0.93	1.04	1.72	1.58
Butizide	6.18	6.06	2.66	3.67	1.16	1.13	1.14	1.23	2.21	2.48	2.57	3.06
Chlorthalidone	2.32	1.96	1.09	1.29	1.06	1.00	1.00	1.32	0.71	0.00	0.00	2.21
Mefruside	11.68	8.28	5.12	11.06	1.70	1.00	1.00	1.17	3.69	0.00	0.00	1.99
	MP: ACN/100 mM aqueous solution of KPF_6 30/70 (*v*/*v*)											
	IA	IB	IC	ID	IA	IB	IC	ID	IA	IB	IC	ID
	k_1				α				$R_{1/2}$			
Bendroflumethiazide	10.64	11.49	4.41	5.99	1.04	1.04	1.11	1.10	0.95	0.61	1.12	1.32
Butizide	5.99	4.89	2.61	3.24	1.13	1.13	1.14	1.24	2.05	2.00	1.77	2.81
Chlorthalidone	2.28	1.57	1.09	1.10	1.09	1.00	1.00	1.40	0.70	0.00	0.00	2.42
Mefruside	11.63	6.76	4.97	9.96	1.63	1.00	1.00	1.18	3.63	0.00	0.00	1.75
	MP: ACN/10 mM ammonium acetate buffer (pH 8.80) 30/70 (*v*/*v*)											
	IA	IB	IC	ID	IA	IB	IC	ID	IA	IB	IC	ID
	k_1				α				$R_{1/2}$			
Bendroflumethiazide	11.39	12.14	4.55	6.23	1.05	1.04	1.08	1.08	1.01	0.70	1.02	1.26
Butizide	6.35	5.48	2.71	3.68	1.15	1.13	1.14	1.22	2.30	2.29	1.72	2.83
Chlorthalidone	2.45	1.72	1.10	1.34	1.00	1.00	1.00	1.00	0.00	0.00	0.00	0.00
Mefruside	11.35	7.07	5.07	11.22	1.70	1.00	1.00	1.17	4.14	0.00	0.00	1.74

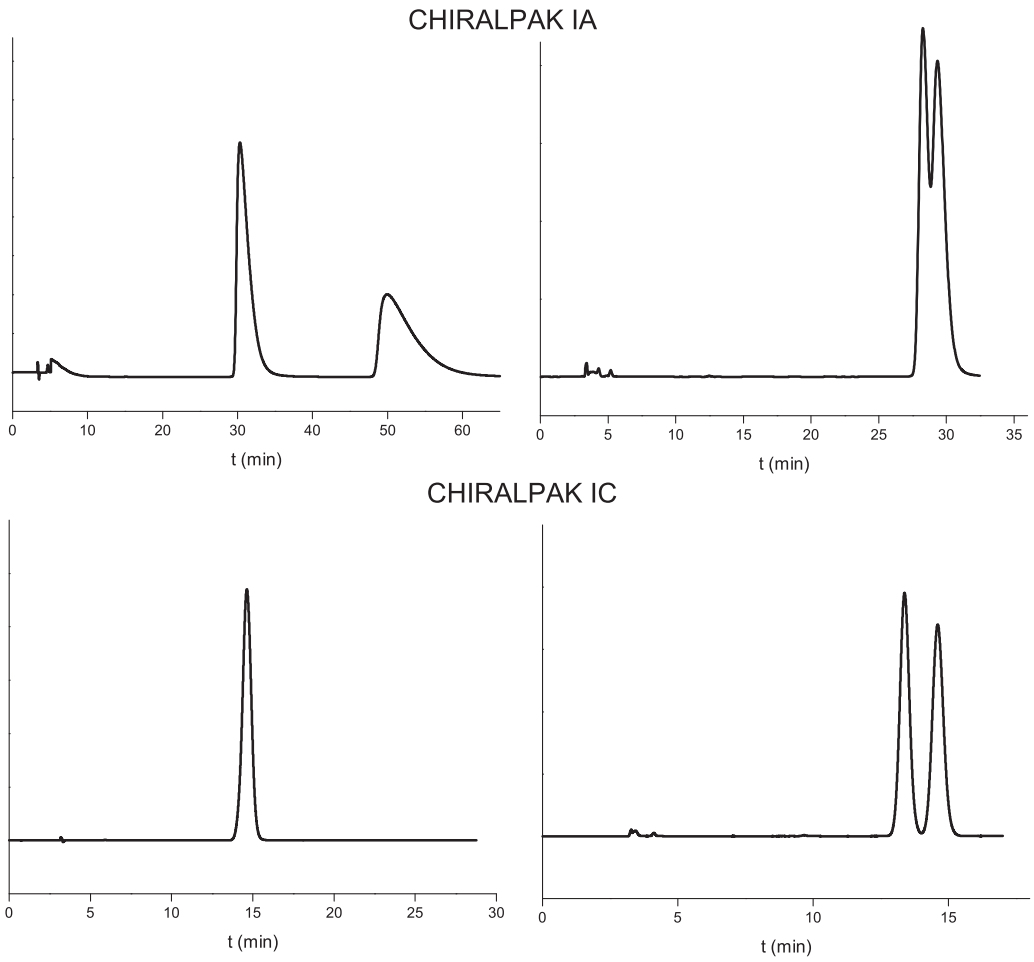

Fig. 3. Chromatograms of separation of mefruside (left) and bendroflumethiazide (right) on amylose- and cellulose-based CSPs. MP composition: ACN/aqueous solution of HCOOH (pH 2.10), 30/70 (*v*/*v*).

Table 4
Baseline enantiomer resolution of the studied basic compounds. For abbreviations see headings to Table 1.

Compound	Columns	k_1	α	$R_{1/2}$	MP composition (v/v)
β-Blockers					
Pindolol	IB	1.57	1.48	2.38	MeOH/B part 55/45
Propranolol	IB	10.81	1.11	1.97	ACN/B part 30/70
Derivatives of amphetamines					
2-F-methcathinone	ID	2.48	1.25	1.68	MeOH/C part 50/50
3-F-amphetamine	ID	2.48	1.25	1.77	MeOH/C part 50/50
Other basic compounds					
BP 34	ID	2.29	1.97	2.00	MeOH/C part 60/40
Thalidomide	IC	16.35	1.20	1.56	MeOH/B part 55/45
Tramadol	IB	12.76	1.12	1.86	ACN/B part 20/80

B part: 100 mM aqueous solution of KPF_6.
C part: 10 mM ammonium acetate buffer (pH 8.80).

3.3. Enantioseparation of basic compounds

A set of 18 basic analytes was used for investigation of enantioselective potential of the CSPs tested. Nine of them were β-blockers, five derivatives of amphetamine, BP 766 and its hydrolytic product BP 34, thalidomide and tramadol. The structures are shown in Fig. S1 in Supplement.

In order to suppress ionization, pH of 10 would be appropriate for some of the basic analytes. However, the use of strong basic conditions (pH > 9) can entirely damage the silica gel matrix. Therefore, the addition of chaotropic salt into the MPs was tested. Hexafluorophosphate anion is characterized by significant delocalization of the charge and forms an ion pair with positively charged analytes [41]. Two types of aqueous media were investigated: 100 mM aqueous solution of KPF_6 and 10 mM ammonium acetate buffer (pH 8.80).

Significant differences in retention, selectivity and enantioresolution were observed in the unbuffered and buffered MPs with the same ACN content (30 vol%) (see Table S3 in Supplement). As follows from Table S2 (also from Fig. 1) the success of enantioresolution of these basic compounds is rather low. There are some exceptions but in binary MP consisting of ACN and 10 mM ammonium acetate buffer, pH 8.80, the majority of the studied analytes exhibited higher retention, unfortunately accompanied by reduced values of enantioselectivity (especially on CHIRALPAK IB), as compared with the corresponding MP with chaotropic reagent. In general, the best separation performance was achieved on CHIRALPAK IB. From the set of nine β-blockers CHIRALPAK IB was able to separate two up to baseline and four partially. However, the rest of the tested columns were not suitable for the enantioseparation of the studied β-blockers. The chromatographic data of the tested basic compounds obtained under optimized separation conditions are shown in Table 4 and more detailed data in Table S2 in Supplement. The results demonstrate that MeOH was the preferred OM. For the less basic β-blockers just addition of the chaotropic agent to MP was sufficient for enantioseparation. More basic analytes (amphetamines, BP 34) required the basic buffer in MP in combination with CHIRALPAK ID column. CHIRALPAK IA column was not suitable for separation of any of the tested basic drug studied.

4. Conclusion

Four immobilized polysaccharide-based CSPs were evaluated and compared under RP separation conditions. Chiral discrimination ability of each CSP was explored using a set of acidic, bifunctional and basic analytes. Typical MPs used in RP HPLC with different compositions of the aqueous and organic parts provided different conditions for enantioseparation of the structurally diverse analytes. Complementary enantioselectivity between the amylose- and cellulose-based CSPs that was observed may be attributed to the difference in their higher order structures. However, the complementary behavior was obvious also on CSPs based on the same polysaccharide but differing in the derivatized moiety. The resolving abilities of the individual columns differed for different MP compositions and separated analytes. Amylose-based CSPs were better choice for the enantioseparation of acidic and bifunctional compounds. On the other hand, CSP based on derivatized cellulose (without chlorine atom) exhibited better chiral recognition ability for basic β-blockers. The paper could serve as a useful tool to analysts searching for enantioseparation conditions on various immobilized polysaccharide-based CSPs in RP mode.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2014.06.040.

References

[1] E.L. Izake, J. Pharm. Sci. 96 (2007) 1659.
[2] F. Liu, M. Qiu, S. Zhai, Curr. Ther. Res. 71 (2010) 1.
[3] H. Tsuchiya, M. Mizogami, Med. Hypotheses 79 (2012) 65.
[4] A.E. Schwaninger, M.R. Meyer, H.H. Maurer, J. Chromatogr. A 1269 (2012) 122.
[5] V. Schurig, Trends Anal. Chem. 21 (2002) 647.
[6] G. Gübitz, M.G. Schmid, J. Chromatogr. A 1204 (2008) 140.
[7] A. Cavazinni, L. Pasti, A. Massi, N. Marchetti, F. Dondi, Anal. Chim. Acta 706 (2011) 205.
[8] M. Lämmerhofer, J. Chromatogr. A 1217 (2010) 814.
[9] J. Vozka, K. Kalíková, E. Tesařová, Anal. Lett. 46 (2013) 2860.
[10] C. West, Curr. Anal. Chem. 10 (2014) 99.
[11] K. Kalíková, T. Šlechtová, J. Vozka, E. Tesařová, Anal. Chim. Acta 821 (2014) 1.
[12] K. De Klerck, Y.V. Heyden, D. Mangelings, J. Chromatogr. A 1328 (2014) 85.
[13] K. Kalíková, M. Riesová, E. Tesařová, Cent. Eur. J. Chem. 10 (2012) 450.
[14] Y. Okamoto, E. Yashima, Angew. Chem. Int. Ed. Engl. 37 (1998) 1020.
[15] E. Yashima, J. Chromatogr. A 906 (2001) 105.
[16] K. Tachibana, A. Ohnishi, J. Chromatogr. A 906 (2001) 127.
[17] B. Chankvetadze, J. Chromatogr. A 1269 (2012) 26.
[18] P. Peluso, S. Cossu, Chirality 25 (2013) 709.
[19] I. Ilisz, Z. Gesce, I. Szatmári, F. Fülöp, A. Péter, Biomed. Chromatogr. 28 (2014) 142.
[20] A.A. Younes, D. Mangelings, Y.V. Heyden, J. Pharm. Biomed. Anal. 55 (2011) 414.
[21] A.A. Younes, H. Ates, D. Mangelings, Y.V. Heyden, J. Pharm. Biomed. Anal. 75 (2013) 74.
[22] I. Ali, K. Saleem, I. Hussain, V.D. Gaitonde, H.Y. Aboul-Enein, Sep. Purif. Rev. 38 (2009) 97.
[23] T. Ikai, C. Yamamoto, M. Kamigaito, Y. Okamoto, J. Chromatogr. B 875 (2008) 2.

[24] I. Ali, H.Y. Aboul-Enein, Curr. Pharm. Anal. 3 (2007) 71.
[25] T. Zhang, P. Franco, D. Nguyen, R. Hamasaki, S. Miyamoto, A. Ohnishi, T. Murakami, J. Chromatogr. A 1269 (2012) 178.
[26] T. Zhang, D. Nguyen, P. Franco, T. Murakami, A. Ohnishi, H. Kurosawa, Anal. Chim. Acta 557 (2006) 221.
[27] T. Zhang, C. Kientzy, P. Franco, A. Ohnishi, Y. Kagamihara, H. Kurosawa, J. Chromatogr. A 1075 (2005) 65.
[28] P. Franco, T. Zhang, J. Chromatogr. B 875 (2008) 48.
[29] J.Y. Jin, W. Lee, C.S. Baek, Chin. J. Anal. Chem. 36 (2008) 1207.
[30] T. Zhang, M. Schaeffer, P. Franco, J. Chromatogr. A 1083 (2005) 96.
[31] T. Zhang, D. Nguyen, P. Franco, J. Chromatogr. A 1191 (2008) 214.
[32] W. Weng, H. Guo, F. Zhan, H. Fang, Q. Wang, B. Yao, S. Li, J. Chromatogr. A 1210 (2008) 178.
[33] A. Ali, Z.A. Al-Othman, H.Y. Aboul-Enein, Methods Mol. Biol. 970 (2013) 127.
[34] T. Zhang, D. Nguyen, P. Franco, Y. Isobe, T. Michishita, T. Murakami, J. Pharm. Biomed. Anal. 46 (2008) 882.
[35] H. Huang, J.Y. Jin, J.H. Hong, J.S. Kang, W. Lee, Bull. Korean Chem. Soc. 30 (2009) 2827.
[36] G. Kučerová, J. Vozka, K. Kalíková, R. Geryk, D. Plecitá, T. Pajpanova, E. Tesařová, Sep. Purif. Technol. 119 (2013) 123.
[37] T. Zhang, D. Nguyen, P. Franco, J. Chromatogr. A 1217 (2010) 1048.
[38] M. Sovák, A.L. Seligson, R. Kučerová, M. Bienová, M. Hajduch, M. Buček, Dermatol. Surg. 28 (2002) 678.
[39] H. Ding, N. Grinberg, R. Thompson, D. Ellison, J. Liq. Chromatogr. Related Technol. 23 (2000) 2641.
[40] L. Peng, S. Jayapalan, B. Chankvetadze, T. Farkas, J. Chromatogr. A 1217 (2010) 6942.
[41] C.M. Phechkrajang, M. U. J. Pharm. 37 (2010) 1.

Publikace IV: Chirální stacionární fáze na bázi imobilizovaných polysacharidů pro enantioseparace v normálním *versus* reversním módu

Sada 31 strukturně odlišných chirálních léčiv byla vybrána pro studium a porovnání enantioselektivního potenciálu dvou imobilizovaných polysacharidových CSF v NP a RP módu. Byly testovány kolony CHIRALPAK IA a CHIRALPAK IB obsahující shodnou derivatizační skupinu *tris*(3,5-dimethylfenylkarbamát), ale navzájem se lišící povahou polymerního řetězce (amylosa *versus* celulosa). Mechanismus separace je odlišný v obou módech. Vodíkové vazby a π-π interakce fenylkarbamátových skupin CSF s aromatickými jádry testovaných analytů jsou preferovány především v NP módu, zatímco hydrofobní interakce s molekulami nedisociovaných analytů representují hlavní příspěvek k retenčnímu mechanismu v RP systému.

MF skládající se z *n*-hexanu a propan-2-olu s malým přídavkem kyselého (TFA) či bazického (TEA) aditiva byly testovány v NP módu. V RP módu byly použity MF obsahující organické modifikátory (acetonitril, methanol) s vodnými roztoky kyseliny mravenčí (pH = 2,10) pro separaci kyselých profenů nebo ve spojení se 100 mM vodným roztokem KPF_6 či octano-amonným pufrem (10 mM, pH = 8,80) pro chirální dělení bazických analytů. Pro enantioseparaci bifunkčních analytů byla testována všechna výše zmíněná separační prostředí.

Ze získaných chromatografických dat vyplývá, že pro chirální dělení profenů je vhodnější SF na bázi amylosy. Z celkového počtu 8 profenů byla kolona CHIRALPAK IA schopna separovat 7 profenů na základní linii v NP módu a 6 v RP módu.

Výsledky enantioseparace bifunkčních diuretik poukazují na komplementární chování mezi testovanými systémy. Ze srovnání separačních systémů vyplývá, že kolona CHIRALPAK IB byla lepší volbou v podmínkách NP módu, zatímco CSF na bázi derivatizované amylosy poskytovala vyšší hodnoty rozlišení a enantioselektivity v RP módu.

Obě polysacharidové kolony poskytovaly dobrou enantioselektivitu pro bazické β-blokátory v NP módu. CSF na bázi *tris*(3,5-dimethylfenylkarbamátu) celulosy byla schopna rozdělit všechny analyty na základní linii. V RP módu vykazovala amylosová CSF nízkou enantioselektivitu a celulosová kolona CHIRALPAK IB poskytovala chirální separaci dvou analytů na základní linii, a zbytek byl separován částečně.

Získaná data ukazují, že NP mód je vhodnější pro enantioseparaci většiny chirálních léčiv na obou imobilizovaných polysacharidových kolonách. V obou separačních módech vykazovala CSF na bázi derivatizované amylosy lepší separační potenciál pro kyselé analyty, zatímco celulosová CSF ukazovala vyšší enantioselektivitu pro chirální báze. Obě imobilizované CSF jsou vzájemně komplementární. Kombinace těchto CSF nabízí mocný nástroj pro enantioseparaci strukturně odlišných chirálních léčiv v NP a RP módu.

Publikace IV

Immobilized polysaccharide‑based stationary phases for enantioseparation in normal *versus* reversed phase HPLC

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SHORT COMMUNICATION

Immobilized Polysaccharide-Based Stationary Phases for Enantioseparation in Normal Versus Reversed Phase HPLC

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Abstract A set of 31 structurally different chiral pharmaceutical compounds was used as model analytes for investigation of the enantioselective potential of two immobilized polysaccharide-based chiral stationary phases under normal and reversed phase separation conditions. These chiral stationary phases differed in the polymeric backbone, amylose or cellulose, but possessed the same derivatization functionality. The results showed that the tris(3,5-dimethylphenylcarbamate) of amylose and cellulose have very broad, and often complementary, enantiorecognition abilities. In general, normal phase separation mode seemed to be more advantageous for separation of the majority of studied pharmaceuticals no matter if amylose- or cellulose-based columns were used. However, in certain cases the reversed phase separation system yielded better results. The combination of these two immobilized chiral stationary phases offers a powerful tool for enantioseparation of different types of pharmaceuticals in the normal and/or reversed phase mode.

Keywords Normal phase mode · Reversed phase mode · Enantioselective potential · Immobilized polysaccharide-based chiral stationary phases · Amylose · Cellulose

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Introduction

Chiral separations have developed into a very important field of analytical chemistry, especially for application in pharmaceutical industry where the demand for enantiomeric purity of drug substances is a significant issue [1]. Various separation techniques can be used for separation of enantiomers [2, 3]. Among them high-performance liquid chromatography (HPLC) is mostly employed [4]. Various types of chiral stationary phases (CSPs) have been developed and extensively used for the enantioselective separation of chiral drugs [5]. Polysaccharide derivative-based CSPs have proved to be one of the most useful tools for direct enantioseparation in HPLC. Particularly, tris(3,5-dimethylphenylcarbamate) of amylose and tris(3,5-dimethylphenylcarbamate) of cellulose have shown wide application possibilities [6]. Considering the way of depositing the polysaccharide derivatives on silica support, coated or immobilized CSPs are available. The columns with immobilized phases are multimodal, i.e. can be used in any separation modes, while coated ones are designed just for the given mode (NP or RP) [6, 7]. The coated CSPs exhibit better enantioseparation ability for certain analytes [6, 8]. However, the advantage of the immobilized CSPs lies in their universal use due to compatibility with many different solvents and one column gives the possibility of changes among different separation modes. The immobilized CSPs have been mostly employed in the NP and PO modes [7, 9, 10]. However, a few application reports under RP conditions can be found in the literature, too [11–13].

In this work, a set of 31 structurally different pharmaceuticals, covering acidic, basic and bifunctional analytes, was used as model compounds for comparison of enantioseparation abilities of two immobilized polysaccharide-based CSPs under NP and RP separation conditions. The

tested columns were CHIRALPAK IA and CHIRALPAK IB, i.e. CSPs based on tris(3,5-dimethylphenylcarbamate) of amylose and cellulose, respectively [7]. The enantioselective recognition abilities of these CSPs in the RP mode have been already investigated in our laboratory [13]. This article is focused on the evaluation and comparison of the enantioselective performance of these CSPs in the NP and RP separation modes. The influence of mobile phase (MP) composition and the effect of the acidic and basic additives on enantioselectivity are also discussed. This work can provide a useful guide for the use of CSPs based on derivatized polysaccharide for analyzing chiral pharmaceutical compounds in the both separation modes.

Materials and Methods

Instrumentation

All chromatographic measurements were carried out on Waters Alliance System (Waters, MA, USA) composed of Waters 2695 Separation Module, Waters 2996 Photodiode Array Detector, an autosampler 717 Plus, and Waters Alliance Series column heater, controlled by Empower software, which was used for data acquisition and analyses. The columns, CHIRALPAK IA, CHIRALPAK IB sized 250 mm × 4.6 mm i.d. were obtained from Chiral Technologies Europe (Illkirch, France). The polysaccharide derivatives are chemically immobilized on 5 μm silica particles. The corresponding guard columns (10 mm × 4.6 mm i.d.) from the same company were used.

Chemicals and Reagents

n-Hexane (HEX, Chromasolv® for HPLC, ≥97 %), propane-2-ol (IPA, Chromasolv® for HPLC, ≥99.8 %), acetonitrile (ACN, Chromasolv®, gradient grade, for HPLC, ≥99.9 %), methanol (MeOH, Chromasolv®, gradient grade, for HPLC, ≥99.9 %), triethylamine (TEA, minimum 99 %), trifluoroacetic acid (TFA, 99 %), ammonium acetate (purity ≥99 %), potassium hexafluorophosphate (KPF_6, 98 %), formic acid (HCOOH, reagent grade, ≥95 %) and ammonium hydroxide solution (ACS reagent, 28–30 % NH_3 basis) were supplied by Sigma-Aldrich (St. Louis, USA). The deionized water was purified by a Milli-Q water purification system from Millipore (Bedford, USA). The set of chiral compounds consisted of 31 racemates. Profens (carprofen, fenoprofen, flurbiprofen, ibuprofen, indoprofen, ketoprofen, suprofen, tiaprofenic acid), calcium channel blockers (CaCB; nicardipine, nimodipine, nisoldipine, nitrendipine, verapamil), β-blockers (alprenolol, metoprolol, oxprenolol, pindolol, propranolol), phenothiazines (promethazine, thioridazine), thalidomide and tramadol, were purchased from Sigma-Aldrich (St. Louis, USA). Diuretics (bendroflumethiazide, butizide, chlorthalidone, mefruside) and derivatives of amphetamine (2-F-methcathinone, 4-F-amphetamine, 4-F-methcathinone, epinephrine, norepinephrine) were gifts of Prof. M. G. Schmid from Karl-Franzens University Graz, Austria. The structures of the analytes are depicted in Supplement, Fig. S1.

Chromatographic Conditions

All measurements were performed in triplicates. The injection volume was 10 μL. The void volume was determined using the solvent peak. Stock solutions of samples were prepared in concentration of 1 mg/mL using MeOH as a solvent. The chromatographic measurements were performed at a flow rate 1.0 mL/min. Temperature of the columns was 25 °C. The UV detection was performed at the wavelength of 220 and 254 nm. MPs were always filtered through a membrane filter with pore size of 0.45 μm. Sonication for 30 min was used for degassing HEX and aqueous components of the MPs.

Normal Phase Separation Mode

MPs consisted of HEX mixed with IPA at various volume ratios. Small amounts of TFA and TEA served as MP additives for optimizing separations and improving peak shape. The additives were used at the proportion 0.10–0.30 % by volume.

Reversed Phase Separation Mode

MPs containing ACN or MeOH as the organic modifiers (OMs) and acidified water (HCOOH, pH 2.10) were used for enantioseparation of acidic compounds. The aqueous parts of the MPs composed of 100 mM aqueous solution of KPF_6 or 10 mM ammonium acetate buffer (pH 8.80) were used for separating basic analytes. Either acidified water (pH 2.10) or aqueous solution of 100 mM KPF_6 or 10 mM ammonium acetate buffer (pH 8.80) was suitable for separation of bifunctional analytes. The pH of the basic buffer was adjusted with ammonium solution to the required pH value prior the addition of the OM.

Results and Discussion

The chromatographic data of all the studied pharmaceuticals on the both columns under the best separation conditions in the NP and RP systems are summarized in Table 1. Then the results of the individual groups of structurally related compounds are discussed separately in the following subchapters.

Table 1 The best results achieved in the NP and RP modes. Retention factor of the first eluted enantiomer (k_1), enantioselectivity (α), resolution ($R_{1/2}$); In the case of baseline separation, the optimized chromatographic data were chosen with respect to the shortest separation time (the lowest values of retention factor)

Compound	NP mode					RP mode [13]			
	CSP	k_1	α	$R_{1/2}$	MP composition (*v*/*v*/*v*)	k_1	α	$R_{1/2}$	MP composition (*v*/*v*)
Profens									
Carprofen	IA	0.29	1.63	**2.92**	Hex/IPA/TFA/40/60/0.1	1.90	1.15	**2.16**	ACN/A part 60/40
	IB	0.76	1.26	**2.25**	Hex/IPA/TFA/80/20/0.1	9.87	1.07	**1.59**	ACN/A part 40/60
Fenoprofen	IA	0.16	1.49	**1.53**	Hex/IPA/TFA/60/40/0.3	12.63	1.07	1.12	MeOH/A part 60/40
	IB	2.06	1.06	1.18	Hex/IPA/TFA/98/2/0.1	27.65	1.04	0.56	MeOH/A part 50/50
Flurbiprofen	IA	0.11	1.93	**2.03**	Hex/IPA/TFA/50/50/0.1	1.02	1.23	**1.84**	MeOH/A part 90/10
	IB	1.86	1.09	1.43	Hex/IPA/TFA/98/2/0.1	29.99	1.05	0.61	MeOH/A part 40/60
Ibuprofen	IA	n.s.				12.12	1.14	**1.72**	MeOH/A part 60/40
	IB	0.83	1.21	1.15	Hex/IPA/TFA/98/2/0.3	7.19	1.05	0.50	MeOH/A part 60/40
Indoprofen	IA	1.90	1.15	**1.98**	Hex/IPA/TFA/60/40/0.3	4.60	1.14	**1.52**	MeOH/A part 80/20
	IB	0.86	1.22	**1.86**	Hex/IPA/TFA/60/40/0.1	11.32	1.04	1.02	ACN/A part 30/70
Ketoprofen	IA	2.16	1.13	**1.77**	Hex/IPA/TFA/90/10/0.1	8.56	1.04	0.86	MeOH/A part 60/40
	IB	17.64	1.04	0.91	Hex/IPA/TFA/99/1/0.1	n.s.			
Suprofen	IA	1.15	1.13	**1.64**	Hex/IPA/TFA/80/20/0.1	2.06	1.23	**2.66**	MeOH/A part 80/20
	IB	15.55	1.07	1.37	Hex/IPA/TFA/98/2/0.3	n.s.			
Tiaprofenic acid	IA	1.06	1.19	**1.95**	Hex/IPA/TFA/80/20/0.1	2.52	1.12	**1.57**	MeOH/A part 80/20
	IB	1.34	1.13	**1.66**	Hex/IPA/TFA/90/10/0.3	n.s.			
Diuretics									
Bendroflumethiazide	IA	2.67	1.10	0.91	Hex/IPA/TFA/80/20/0.1	11.39	1.05	1.01	ACN/C part 30/70
	IB	6.72	1.15	1.15	Hex/IPA/TFA/80/20/0.1	14.62	1.04	1.04	ACN/A part 30/70
Butizide	IA	n.s.				2.27	1.09	**1.58**	ACN/B part 40/60
	IB	0.86	1.40	**1.59**	Hex/IPA/TFA/60/40/0.1	2.54	1.09	**1.53**	ACN/A part 40/60
Chlorthalidone	IA	0.42	1.47	**1.79**	Hex/IPA/TFA/40/60/0.1	6.57	1.09	0.86	ACN/A part 20/80
	IB	7.98	1.20	1.16	Hex/IPA/TFA/80/20/0.1	n.s.			
Mefruside	IA	19.53	1.07	0.55	Hex/IPA/TEA/90/10/0.1	0.67	1.37	**2.57**	ACN/C part 80/20
	IB	13.92	1.04	0.78	Hex/IPA/TFA/90/10/0.1	n.s.			
β-Blockers									
Alprenolol	IA	0.39	1.63	**2.28**	Hex/IPA/TEA/80/20/0.1	3.59	1.08	0.80	MeOH/B part 50/50
	IB	0.73	1.44	**1.99**	Hex/IPA/TEA/80/20/0.3	27.80	1.04	0.80	ACN/B part 20/80
Metoprolol	IA	5.14	1.09	1.03	Hex/IPA/TEA/95/5/0.3	n.s.			
	IB	9.75	1.31	**1.65**	Hex/IPA/TEA/95/5/0.3	8.71	1.06	0.93	ACN/B part 20/80
Oxprenolol	IA	1.71	1.30	**1.62**	Hex/IPA/TEA/90/10/0.3	n.s.			
	IB	0.75	1.59	**1.83**	Hex/IPA/TEA/40/60/0.1	18.77	1.21	1.39	ACN/B part 20/80
Pindolol	IA	n.s.				n.s.			
	IB	0.93	1.98	**2.61**	Hex/IPA/TEA/40/60/0.1	1.57	1.48	**2.38**	MeOH/B part 55/45
Propranolol	IA	n.s.				n.s.			
	IB	4.50	1.31	**1.53**	Hex/IPA/TEA/90/10/0.3	10.81	1.11	**1.97**	ACN/B part 30/70
Derivates of amphetamine									
2-F-methcathinone	IA	2.73	1.15	**2.67**	Hex/IPA/TFA/90/10/0.1	14.02	1.06	0.73	MeOH/C part 40/60
	IB	n.s.				n.s.			
4-F-methcathinone	IA	2.40	1.11	1.23	Hex/IPA/TEA/95/5/0.3	n.s.			
	IB	1.87	1.11	**1.82**	Hex/IPA/TFA/98/2/0.1	n.s.			
4-F-amphetamine	IA	n.s.				17.76	1.07	1.10	MeOH/C part 40/60
	IB	n.s.				n.s.			
Epinephrine	IA	n.s.				n.s.			
	IB	7.45	1.26	**3.62**	Hex/IPA/TFA/90/10/0.3	n.s.			

Table 1 continued

Compound	NP mode					RP mode [13]			
	CSP	k_1	α	$R_{1/2}$	MP composition (*v*/*v*/*v*)	k_1	α	$R_{1/2}$	MP composition (*v*/*v*)
Norepinephrine	IA	6.00	1.12	0.87	Hex/IPA/TEA/95/5/0.3	n.s.			
	IB	2.55	1.05	0.80	Hex/IPA/TFA/90/10/0.3	n.s.			
CaCB									
Nicardipine	IA	12.02	1.15	1.28	Hex/IPA/TEA/98/2/0.1	n.s.			
	IB	10.18	1.05	0.85	Hex/IPA/TEA/95/5/0.1	n.s.			
Nimodipine	IA	6.76	1.05	1.11	Hex/IPA/TFA/95/5/0.3	n.s.			
	IB	7.21	1.04	0.85	Hex/IPA/TEA/95/5/0.3	n.s.			
Nisoldipine	IA	1.44	1.14	0.46	Hex/IPA/TEA/98/2/0.3	n.s.			
	IB	22.86	1.08	1.26	Hex/IPA/TEA/98/2/0.1	n.s.			
Nitrendipine	IA	5.20	1.06	1.42	Hex/IPA/TEA/95/5/0.3	n.s.			
	IB	n.s.				n.s.			
Verapamil	IA	1.87	1.16	**1.70**	Hex/IPA/TEA/90/10/0.3	n.s.			
	IB	7.41	1.14	**1.90**	Hex/IPA/TFA/80/20/0.1	n.s.			
Others									
Promethazine	IA	1.14	1.15	**1.71**	Hex/IPA/TEA/95/5/0.3	n.s.			
	IB	n.s.				n.s.			
Thalidomide	IA	3.10	1.82	**7.69**	Hex/IPA/TFA/40/60/0.1	n.s.			
	IB	n.s.				n.s.			
Thioridazine	IA	4.35	1.12	0.69	Hex/IPA/TFA/60/40/0.1	n.s.			
	IB	n.s.				n.s.			
Tramadol	IA	0.12	2.00	**1.87**	Hex/IPA/TEA/50/50/0.1	4.33	1.10	1.12	ACN/B part 30/70
	IB	8.09	1.57	**2.32**	Hex/IPA/TFA/80/20/0.1	12.76	1.12	**1.86**	ACN/B part 20/80

IA, IB mean CHIRALPAK IA, CHIRALPAK IB columns, respectively. A part: aqueous solution of HCOOH (pH 2.10); B part: 100 mM aqueous solution of KPF_6; C part: 10 mM ammonium acetate buffer (pH 8.80). Results in bold accentuate baseline separations

n.s. no separation in any MPs studied

Enantioseparation of Profens

Both the polysaccharide-based CSPs could be used for enantioseparation of the majority of profens in the NP mode. It is obvious from the comparison of the both columns that the amylose-based CSP showed significantly higher enantioselectivity and enantioresolution values for the tested profens (Table 1). As shown in Table 1, from the total number of eight profens seven were baseline separated on CHIRALPAK IA column. Cellulose-based CSP allowed baseline enantioseparation of three profens and the others were resolved partially in the NP mode.

The amylose-based column was advantageous for separation of profen enantiomers also in the RP separation mode. In summary, the amylose-based CSP was able to resolve six compounds to baseline and two partially while the cellulose-based column was less effective for chiral resolution of the tested profens in the RP mode.

In general, the enantioselectivities on these two CSPs for profens under RP conditions are in most cases lower than those under NP conditions. Moreover, it is evident that in the NP mode the studied analytes exhibited sufficient resolution at lower retention (see Table 1). Comparing amylose- and cellulose-based CSPs, better results—enantioselectivity, resolution values, were obtained on CHIRALPAK IA column in the both separation modes. The enantioseparation behavior was often complementary between the separation modes on one CSP or in the same mode but on different polysaccharide-based columns.

Enantioseparation of Diuretics

Complementary separation behavior of diuretics was obtained on the amylose- and cellulose-based columns in the NP mode (compare enantioseparation of butizide and chlorthalidone on these two CSPs in Table 1). Nevertheless, better enantioresolution of diuretics was mostly achieved on CHIRALPAK IB column in the NP mode.

Both polysaccharide-based CSPs showed certain separation potential also in the RP mode for the tested diuretics. Higher values of enantioselectivity were achieved on CHIRALPAK IA column.

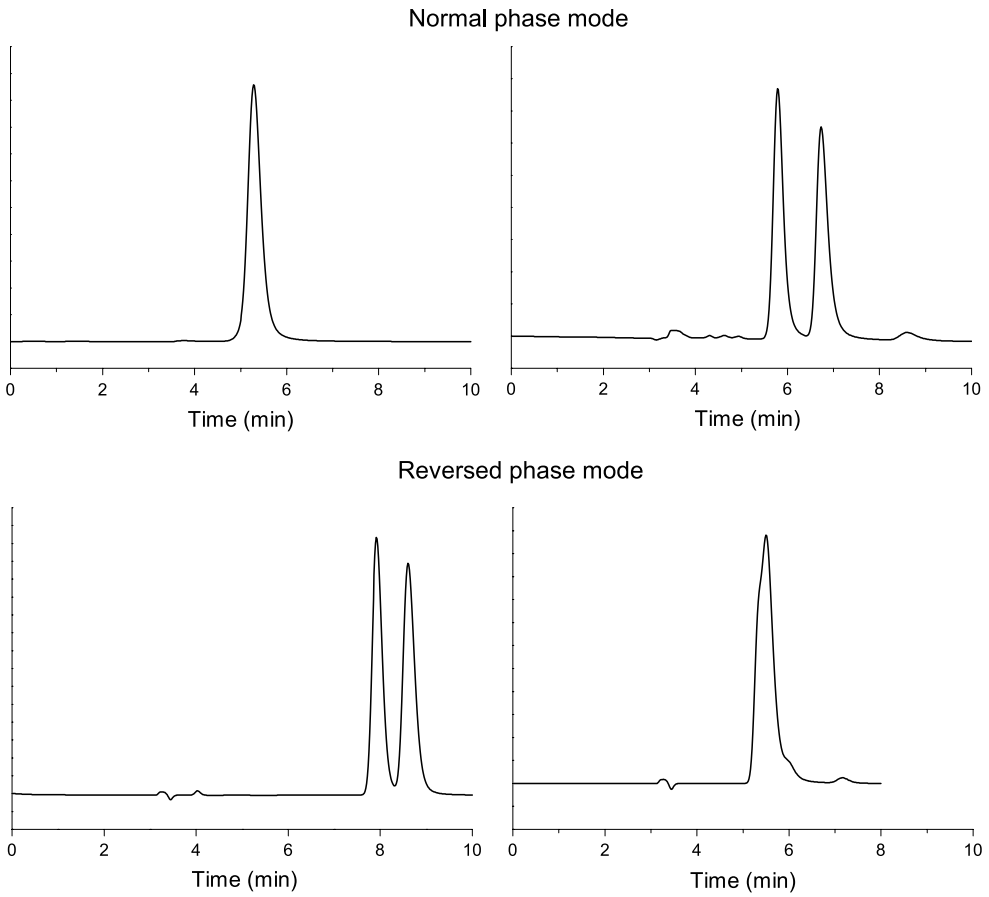

Fig. 1 Chromatograms of enantioseparation of butizide (*left*) and chlorthalidone (*right*) on amylose-based CSP in the both separation modes. NP MP composition: HEX/IPA/TFA 50/50/0.1 (*v*/*v*/*v*), RP MP composition: ACN/aqueous solution of HCOOH (pH 2.10), 40/60 (*v*/*v*)

Comparing the two separation modes, CHIRALPAK IB column seemed to be better choice for the separation of the studied diuretics (except chlorthalidone) under NP conditions. On the other hand, the amylose-based CSP appeared to be more potent for separation of the tested compounds in the RP mode. The complementary effect of the enantioseparation abilities of tris(3,5-dimethylphenylcarbamate) of amylose in two separation modes is illustrated on the enantioresolution of butizide and chlorthalidone in Figs. 1 and 2.

Enantioseparation of β-Blockers

Both CSPs showed good separation ability for β-blockers in the NP mode. The CSP based on derivatized cellulose gave somewhat better results because the obtained enantioselectivities achieved for β-blockers on the cellulose-based CSP were higher than on the amylose-based CSP except for alprenolol (see Table 1). As is shown in Table 1, in the NP mode all the tested β-blockers were baseline separated on CHIRALPAK IB column while CHIRALPAK IA column was able to resolve two analytes up to baseline and one partially.

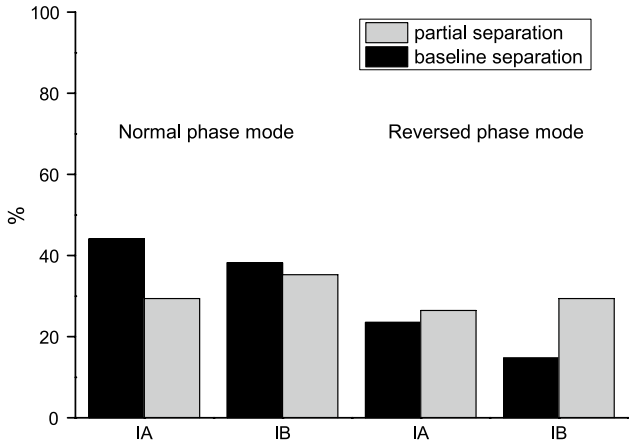

Fig. 2 Success rates on amylose- and cellulose-based CSPs in the both separation modes

Neither, in the RP mode the amylose-based CSP was suitable for enantioseparation of the tested β-blockers. The cellulose-based CSP exhibited mostly better enantioselectivity and resolution values. From the set of five β-blockers

two were baseline separated under RP conditions on CHIRALPAK IB column and the others were resolved partially. No sufficient enantioseparation of any analyte of this group could be achieved on the corresponding amylose-based CSP.

Comparing the results obtained on the both polysaccharide-based CSPs under NP and RP separation conditions, it is obvious that the chiral recognition abilities of these two CSPs for enantioseparation of β-blockers are higher under NP conditions than in the RP mode. Moreover, the studied β-blockers were baseline separated under NP conditions in shorter analysis time than if analyzed in the RP mode. In summary, the cellulose-based column was significantly more successful in separating the tested β-blockers than the amylose-based CSP, no matter if NP or RP mode was used.

Enantioseparation of Derivatives of Amphetamine

From the results in Table 1 it is hard to judge what CSP was better for enantioseparation of amphetamine derivatives in the NP mode. Complementary behavior of tris(3,5-dimethylphenylcarbamate) of amylose and cellulose was observed for the separation of methcathinone derivatives in the NP mode. The highest enantioresolution values of this set of drugs could be achieved on the both polysaccharide-based CSPs only if IPA content in the MP did not exceed 10 vol %.

On these polysaccharide-based CSPs suitable RP separation conditions were not found for this group of analytes. The best results (partial separation of two derivatives) were obtained with the amylose-based column in the MP composed of MeOH and 10 mM ammonium acetate buffer (pH 8.80). The cellulose-based CSP did not provide even partial separation for the tested derivatives of amphetamines. In general, better enantioseparation performance for derivatives of amphetamine (with exception of 2-F-methcathinone) exhibited CSP based on derivatized cellulose in the NP mode.

Enantioseparation of Calcium Channel Blockers

In the NP mode, the amylose-based column provided higher enantioselectivity values for the tested CaCB. All the examined analytes were at least partially separated on CHIRALPAK IA column. However, the retention values were unacceptable high for practical applications. Addition of TEA (even 0.30 vol %) yielded improved separation. CHIRALPAK IB column was slightly less effective in separation of these analytes, but the results do not allow any generalization.

The RP mode was not suitable for the enantioseparation of any analyte from this group on any of these CSPs.

Enantioseparation of Others Compounds

In the NP mode, the amylose-based column showed better separation performance with the highest enantioresolution value for thalidomide. Concerning the CSPs and also MP additives complementary behavior was observed for the enantioseparation of tramadol in the NP mode. Baseline separation of tramadol enantiomers could be achieved only with TEA on the amylose-based CSP while TFA was more promising MP additive for enantioseparation on CHIRALPAK IB column.

The RP mode was not suitable for enantioseparation of these analytes. The only exception was tramadol that could be baseline resolved on cellulose- and partially separated on amylose-based CSPs.

As follows from Table 1 significantly better results were obtained in the NP mode on CHIRALPAK IA column where all the studied drugs were baseline separated (except of thioridazine) while in the RP mode only partial enantioseparation of tramadol was achieved.

Conclusion

The enantiodiscrimination ability of two CSPs based on derivatized polysaccharides, tris(3,5-dimethylphenylcarbamate) of amylose and/or cellulose, was evaluated in the NP and RP modes. The success rates of enantioseparation results for the tested columns and diverse groups of chiral pharmaceuticals are clearly demonstrated in Fig. 2. The results show that the NP mode was found to be more advantageous for the separation of the majority of the studied compounds on the both columns. The chiral recognition mechanism is clearly different in the both separation modes [14]. Hydrogen bonding and π-π interactions of the phenylcarbamate moieties of the CSPs with the aromatic parts of the analytes are preferred in the NP separation system. Hydrophobic interactions with non-dissociated or non-protonated analytes represent the main contribution to interaction mechanism in RP separation mode. Conditions for the hydrophobic interactions were established by addition of formic acid and ammonium acetate buffer to the MPs for separation of acidic and basic compounds, respectively. Another possibility is use of chaotropic agent as MP additive for separation of basic analytes. The distribution of interactions in both modes can explain much better results generally obtained in the NP mode. In the both separation modes, the CSP based on derivatized amylose was more convenient for the enantioseparation of acidic profens while cellulose-based CSP exhibited higher resolving power for the set of β-blockers. The studied polysaccharide-based CSPs often exhibit complementary separation properties. In summary, the combination of these two

immobilized CSPs in the NP and RP modes offers a powerful tool for enantioresolution of a great variety of drugs. By a proper optimization, chromatographic conditions can be found that allow separation of the majority of analytes of interest.

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Conflict of interest The authors declare no conflict of interest.

References

1. Mohan SJ, Mohan EC, Yamsani MR (2009) Int J Pharm Sci Nanotechnol 1:309–316
2. Schwaninger AE, Meyer MR, Maurer HH (2012) J Chromatogr A 1269:122–135
3. Kalíková K, Šlechtová T, Vozka J, Tesařová E (2014) Anal Chim Acta 821:1–33
4. Ribeiro AR, Maia AS, Cass QB, Tiritan ME (2014) J Chromatogr B 968:8–21
5. Kalíková K, Riesová M, Tesařová E (2012) Cent Eur J Chem 10:450–471
6. Chankvetadze B (2012) J Chromatogr A 1269:26–51
7. Zhang T, Franco P, Nguyen D, Hamasaki R, Miyamoto S, Ohnishi A, Murakami T (2012) J Chromatogr A 1269:178–188
8. Geryk R, Vozka J, Kalíková K, Tesařová E (2013) Chromatographia 76:483–489
9. Shen J, Ikai T, Okamoto Y (2014) J Chromatogr A 1363:51–61
10. Rahou I, Belboukhari N, Sekkoum K, Cheriti A, Aboul-Enein HY (2014) Chromatographia 77:1195–1201
11. Zhang T, Nguyen D, Franco P (2010) J Chromatogr A 1217:1048–1055
12. Kučerová G, Vozka J, Kalíková K, Geryk R, Plecitá D, Pajpanova T, Tesařová E (2013) Sep Purif Technol 119:123–128
13. Geryk R, Kalíková K, Vozka J, Plecitá D, Schmid MG, Tesařová E (2014) J Chromatogr A 1363:155–161
14. Lämmerhofer M (2010) J Chromatogr A 1217:814–856

Publikace V: Charakterizace a porovnání kolon CHIRALPAK IA a CHIRALPAK AD-RH obsahující CSF na bázi amylosy v reversním módu vysokoúčinné kapalinové chromatografie

CSF na bázi amylosy mohou být na silikagelovém nosiči zakotvené (pokrývají nosič pomocí fyzikálních interakcí) či imobilizované prostřednictvím kovalentní vazby. V rámci **Publikace V** jsme se soustředili na evaluaci a srovnání separačního potenciálu pokryté kolony CHIRALPAK AD-RH a imobilizované kolony CHIRALPAK IA v RP módu HPLC.

Testované CSF byly charakterizovány a porovnávány prostřednictvím jejich enantioselektivity pro sadu 37 chirálních biologicky aktivních látek obsahující kyselé, bifunkční/neutrální a bazické analyty. Navzdory faktu, že obě kolony obsahují totožný CS na bázi *tris*(3,5-dimethylfenylkarbamátu) amylosy, docházelo k výrazným rozdílům v retenčním a enantiodiskriminačním chování. Kyselé profeny a *N*-blokované AMK byly separovány v MF skládající se z acetonitrilu a vodného roztoku kyseliny mravenčí (pH = 2,10). Pro enantioseparaci profenů vykazovala kolona CHIRALPAK AD-RH vyšší separační potenciál. Pro chirální separaci blokovaných AMK s benzyloxykarbonylovou skupinou se ukázala lepší volbou imobilizovaná kolona CHIRALPAK IA, zatímco AMK s *terc*-butoxykarbonylovou skupinou poskytovaly vyšší hodnoty rozlišení na pokryté CSF. Bifunkční analyty byly separovány s vyšší enantioselektivitou na imobilizované CSF. Chirální separace bazických látek byly prováděny v MF složené ze směsi acetonitrilu a octano-amonného pufru (10 mM, pH = 8,80). Z porovnání chromatografických dat vyplývá, že vyšších hodnot rozlišení bylo dosaženo na koloně CHIRALPAK AD-RH.

V rámci této práce byl také použit LFER model, který umožňuje charakterizovat separační systémy z hlediska jejich interakčních možností a kvalitativně i kvantitativně popsat příspěvky jednotlivých interakcí k celkové retenci (viz kapitola 4.1). Testovací sada pro LFER obsahovala 42 achirálních analytů charakterizovaných Abrahamovými deskriptory [40]. CSF byly testovány ve dvou MF: acetonitril/vodný roztok kyseliny mravenčí (pH = 2,10) 30/70 (*v*/*v*) a acetonitril/octano-amonný pufr (10 mM, pH = 8,80) 30/70 (*v*/*v*). LFER měření byla prováděna a srovnávána pro obě kolony ve shodném složení MF, proto mohou být získané regresní koeficienty přímo využity pro porovnání jednotlivých interakčních příspěvků kolon. Regresní koeficient *v* (příspěvek hydrofobicity) vykazoval nejvyšší kladné hodnoty a v obou MF kolona CHIRALPAK AD-RH vykazovala vyšší hodnoty ve srovnání s kolonou imobilizovanou. Hodnoty koeficientu *e* byly také kladné a příspěvky interakcí

prostřednictvím *n*- a π-elektronových párů byly opět silnější na koloně se zakotveným chirálním polymerem na silikagelový nosič. Regresní koeficienty *s*, *a*, *b* vykazovaly negativní hodnoty a vyšší absolutní hodnoty byly získány pro CHIRALPAK AD-RH s výjimkou koeficientu *s* (dipól-dipól interakce) v bazickém prostředí.

Pro popis iontových interakcí, které nemohou být stanoveny pomocí základního LFER modelu, byla využita sada silných achirálních kyselin (kyselina benzensulfonová, toluensulfonová) a zásad (fenylethylamin, trimethylfenylamonium chlorid). Tyto testovací analyty byly analyzovány v MF: acetonitril/vodný roztok kyseliny mravenčí (pH = 2,10) 30/70 (*v*/*v*), acetonitril/octano-amonný pufr (10 mM, pH = 4,50) 30/70 (*v*/*v*) a acetonitril/octano-amonný pufr (10 mM, pH = 8,80) 30/70 (*v*/*v*). Výsledky ukázaly, že iontové interakce jsou silnější na imobilizované koloně CHIRALPAK IA. Vyšší příspěvek iontových interakcí může být způsoben přítomností většího počtu nederivatizovaných silanolových skupin oproti pokryté CSF.

Srovnání enantioselektivního potenciálu ukázalo, že obě polysacharidové kolony mají podobné chirální rozlišovací schopnosti pro kyselé analyty, zatímco pro enantioseparaci bazických látek je vhodnější pokrytá CSF. LFER model odhalil, že dispersní interakce a interakce prostřednictvím *n*- a π-elektronových párů poskytovaly hlavní příspěvek k celkové retenci v RP systému.

Publikace V

Evaluation of differences between Chiralpak IA and Chiralpak AD-RH amylose-based chiral stationary phases in reversed-phase high-performance liquid chromatography

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Research Article

Evaluation of differences between Chiralpak IA and Chiralpak AD-RH amylose-based chiral stationary phases in reversed-phase high-performance liquid chromatography

Polysaccharide-based chiral stationary phases can be used for the enantioselective separation of a wide range of structurally different compounds. These phases are available with chiral selectors coated or immobilized on silica gel support. The means of attachment of the chiral selector to the carrier can influence the separation performance of these stationary phases. This paper deals with evaluation of differences in the separation abilities of coated Chiralpak AD-RH versus immobilized Chiralpak IA amylose-based stationary phases in the reversed–phase mode of high–performance liquid chromatography. A set of chiral analytes was separated under acidic and basic conditions. Differences were observed in the enantioseparation potential of the tested phases. The linear-free energy relationship and additional evaluation of ionic interactions were used to ascertain whether the interactions that participate in retention and enantioseparation are affected by the means of preparation of these phases. All the interactions covered by the linear-free energy relationship were significant for the studied phases and their absolute values were almost always higher for the coated phase. Ionic interactions were found to be more important on the immobilized stationary phase but did not contribute to any improvement in the enantioselective separation performance.

Keywords: Amylose / Chiral stationary phases / Enantioselective separations / Interaction mechanisms
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Additional supporting information may be found in the online version of this article at the publisher's web-site

1 Introduction

HPLC employing chiral stationary phases (CSPs) is a widely used enantioselective separation technique on both analytical and preparative scales [1–3]. New or modified CSPs are still being developed and improved in response to constantly growing demands from customers in both the commercial and academic spheres. CSPs based on derivatized polysaccharides can serve as an example. At the present time, these CSPs, which mostly contain derivatized cellulose or amylose, belong among the most frequently employed CSPs on the market [4]. The older generation of these CSPs was prepared by physical coating of a polysaccharide chiral selector (CS) onto silica support [5, 6]. This means of preparing the stationary phase strictly limits the use of particular solvents. For instance, chloroform, ethyl acetate or tetrahydrofuran cause swelling or dissolving of this type of CSP and therefore cannot be used [6–8]. Moreover, these CSPs are not multimodal. Each chromatographic mode has a compatible polysaccharide-based CSP designed for typical solvents of this mode.

To overcome these limitations, a new generation of polysaccharide-based CSPs was developed [9–12]. These innovated polysaccharide-based CSPs contain a chiral selector covalently bonded to the silica support. This CSP preparation significantly broadens the variety of applicable solvents and also enables switching between HPLC modes [4, 5, 12].

Both previously mentioned types of polysaccharide-based CSPs are commercially available. Since the preparation procedure of immobilized and coated CSPs differs, enantioselectivity of these CSPs could also differ despite the fact that both contain a chiral selector originating from the same derivatized polysaccharide. Chemical bonding can change the steric arrangement of CS, which could have a great impact on the retention and enantioselective properties.

The aim of our work is to compare the enantioselective performance of immobilized CSP, Chiralpak IA, and coated

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Abbreviations: **AA**, ammonium acetate; **ACN**, acetonitrile; **CSP**, chiral stationary phase; **LFER**, linear-free energy relationship; **MeOH**, methanol

Table 1. List of descriptors and regression coefficients of the LFER model

Descriptor	Described characteristic of solute
E	excess molar refraction
S	dipolarity/polarizibility
A	effective or overall hydrogen bond acidity
B	effective or overall hydrogen bond basicity
V	McGowan's characteristic molecular volume
Regression coefficient	**Reflected type of interaction**
e	interaction with *n*- and π-electron pairs
s	dipolarity/polarizibility
a	hydrogen bond basicity
b	hydrogen bond acidity
v	dispersion interactions (hydrophobicity)

CSP, Chiralpak AD-RH, both containing amylose tris(3,5-dimethylphenylcarbamate) as a CS (see Supporting Information Fig. S1A for the structure). Some attempts to describe differences in the enantioselectivity of immobilized and coated polysaccharide-based CSPs can be found in the literature. These CSPs have already been compared using various small or extensive sets of analytes in normal-phase [13, 14] or polar-organic HPLC modes [15] and in SFC [16]. The interaction possibilities in systems with polysaccharide-based CSPs were also characterized under SFC conditions [17–19]. Here we are focused on RP-HPLC, since this mode is suitable for many types of assays. Furthermore, polysaccharide-based CSPs have already shown considerable enantioselective potential for RP separations [20–22].

Three different approaches were chosen for the comparison: (i) A set of 37 racemates, including acidic, bifunctional/neutral and basic ones, were analyzed, since our main field of interest was to study chromatographic behavior of these CSPs in mobile phases with pH near the critical limits recommended by the producer. A mobile phase with pH 2.10 was used for acidic racemates and a phase with pH 8.80 was used for basic racemates. Bifunctional/neutral racemates were separated in both types of mobile phases. (ii) The linear-free energy relationship (LFER) model (using 42 achiral solutes) was employed to classify fundamental retention interactions taking place in the studied chromatographic systems. (iii) A set of strong achiral acids and bases was used for evaluation of possible ionic interactions that cannot be revealed by the basic LFER model. To our knowledge, this type of analytical and physicochemical comparison of the mentioned CSPs in RP mode has not been performed so far.

2 Materials and methods

2.1 Instrumentation

All the chromatographic measurements were carried out on the Waters Alliance System (Waters, MA, USA) composed of Waters 2695 Separation Module, Waters 2996 Photodiode Array Detector, an 717 Plus autosampler, and Waters Alliance Series column heater, controlled by Empower software, which was used for data acquisition and analysis. The Chiralpak IA and Chiralpak AD-RH columns purchased from Chiral Technologies Europe (Illkirch, France) had dimensions of 250 mm × 4.6 mm id with particle size of 5 μm. The corresponding guard columns (10 mm × 4.6 mm id) from the same company were used.

2.2 Chemicals and reagents

Acetonitrile (ACN, Chromasolv®, gradient grade, for HPLC, ≥ 99.9%), methanol (MeOH, Chromasolv®, gradient grade, for HPLC, ≥ 99.9%), ammonium acetate (AA, purity ≥ 99%), formic acid (HCOOH, reagent grade, ≥ 95%) and ammonium hydroxide solution (ACS reagent, 28–30% NH_3 basis) were supplied by Sigma–Aldrich (Steinheim, Germany). The deionized water was purified by a Milli-Q water purification system from Millipore (Bedford, USA). The set of chiral compounds consisted of 37 racemates. Profens (carprofen, fenoprofen, flurbiprofen, ibuprofen, indoprofen, ketoprofen, suprofen, tiaprofenic acid), derivatized amino acids (*N*-benzyloxycarbonyl-2F-phenylalanine (*Z*-2F-phenylalanine), *N*-benzyloxycarbonyl-3F-phenylalanine (*Z*-3F-phenylalanine), *N*-benzyloxycarbonyl-4F-phenylalanine (*Z*-4F-phenylalanine), *N*-(*tert*-butoxy)carbonyl-phenylalanine (*N*-BOC phenylalanine), *N*-(*tert*-butoxy)carbonyl-tyrosine (*N*-BOC tyrosine), *N*-(*tert*-butoxy)carbonyl-tryptophan (*N*-BOC tryptophan)), β-blockers (alprenolol, acebutolol, metipranolol, metoprolol, oxprenolol, pindolol, propranolol, atenolol), esters of tryptophan (tryptophan octylester, tryptophan butylester, tryptophan methylester, tryptophan benzylester), binaphthol, 1,1′-binaphthalene-2,2′-diamine (OBIN-NH_2), and tramadol were purchased from Sigma–Aldrich. Amphetamine and its derivatives (2-F-methcathinone, 3-F-amphetamine, 4-F-amphetamine, 4-F-methcathinone), thalidomide, chlorthalidone, bendroflumethiazide, butizide, and mefruside were supplied by Prof. M. G. Schmid from Karl-Franzens University Graz, Austria. The structures of the analytes are depicted in the Supporting Information Fig. S1B. Forty-two LFER solutes, which were purchased from Sigma–Aldrich, are listed together with their descriptors in the Supporting Information Table S1. Solutes for evaluation of the ionic interactions (benzenesulfonic acid, toluenesulfonic acid, phenylethylamine, and trimethylphenylammonium chloride) were purchased from the same company.

2.3 Procedures

2.3.1 Chromatographic measurements

All the measurements were performed in triplicate. The void volume was determined using the solvent peak. The injection

Table 2. Chromatographic data for acidic and bifunctional/neutral analytes under acidic pH conditions; k_1 is retention factor of the first eluted enantiomer, α is enantioselectivity, and *Rs* is enantioresolution

Analyte	k_1		α		*Rs*	
	IA	AD-RH	IA	AD-RH	IA	AD-RH
Mobile phase HCOOH (pH 2.10)/ACN 60:40 v/v						
Indoprofen	5.22	5.73	1.07	1.12	1.25	1.35
Tiaprofenic acid	4.73	4.48	1.06	1.09	1.21	1.35
Ketoprofen	3.54	2.98	1.00	1.00	0.00	0.00
Fenoprofen	4.23	4.74	1.00	S.I.	0.00	S.I.
Suprofen	4.72	4.63	1.10	1.13	1.07	1.09
Carprofen	8.70	11.98	1.18	1.23	2.74	2.62
Ibuprofen	6.64	5.94	1.05	1.10	1.19	1.79
Flurbiprofen	7.13	6.53	1.22	1.35	4.75	5.54
Z-DL-4F-phenylalanine	3.92	2.55	1.08	1.09	1.31	0.63
Z-DL-3F-phenylalanine	3.46	2.50	1.05	1.00	1.05	0.00
Z-DL-2F-phenylalanine	3.09	2.36	1.04	1.00	0.76	0.00
N-BOC tryptophane	1.45	0.91	1.00	1.00	0.00	0.00
N-BOC phenylalanine	1.48	1.05	1.14	1.36	1.35	1.36
N-BOC tyrosine	0.50	0.29	1.00	1.00	0.00	0.00
Binaphthol	5.95	4.90	1.05	1.09	0.90	0.63
Chlorthalidone	1.31	0.68	1.00	1.00	0.00	0.00
Bendroflumethiazide	3.34	1.29	1.00	1.00	0.00	0.00
Butizide	2.33	0.94	1.12	1.37	1.57	1.22
Mefruside	4.42	3.53	1.61	2.28	4.02	3.59
Mobile phase HCOOH (pH 2.10)/ACN 70:30 v/v						
Indoprofen	16.03	18.60	1.08	1.13	1.20	1.54
Tiaprofenic acid	14.59	15.14	1.05	1.09	1.08	1.51
Ketoprofen	10.48	9.73	1.00	1.00	0.00	0.00
Fenoprofen	19.35	18.57	1.00	1.04	0.00	0.96
Suprofen	14.18	15.12	1.10	1.13	1.07	1.17
Carprofen	46.05	N.M.	1.17	N.M.	2.47	N.M.
Ibuprofen	22.69	N.M.	1.06	N.M.	1.36	N.M.
Flurbiprofen	27.75	N.M.	1.23	N.M.	4.35	N.M.
Z-DL-4F-phenylalanine	12.20	11.00	1.08	1.10	1.22	1.02
Z-DL-3F-phenylalanine	11.79	10.56	1.06	1.02	1.04	0.39
Z-DL-2F-phenylalanine	10.55	9.79	1.04	1.00	0.77	0.00
N-BOC tryptophane	4.68	3.03	1.00	1.08	0.00	0.47
N-BOC phenylalanine	4.17	3.29	1.15	1.38	1.86	2.27
N-BOC tyrosine	1.41	0.66	1.00	1.00	0.00	0.00
Binaphthol	25.86	23.02	1.06	1.10	1.08	1.01
Chlorthalidone	2.32	1.58	1.06	1.00	0.71	0.00
Bendroflumethiazide	10.81	5.49	1.05	1.00	0.93	0.00
Butizide	6.18	3.01	1.16	1.41	2.21	3.01
Mefruside	11.68	N.M	1.70	N.M	3.69	N.M

S.I., slight indication of enantioseparation; N. M., not measured.

volume was 10 μL. The chromatographic measurements were performed at a flow rate 1.0 mL/min. The temperature of the columns was maintained at 25°C. The UV detection was performed at wavelengths of 220 and 254 nm. Sonication for 30 min was used for degassing the aqueous part of the mobile phases. ACN was chosen as an organic modifier. The buffer solutions, namely 10, 50, and 100 mM AA-buffers (pH 4.50 and 8.80) and an aqueous solution of HCOOH (pH 2.10), were prepared according to the calculation by PeakMaster software

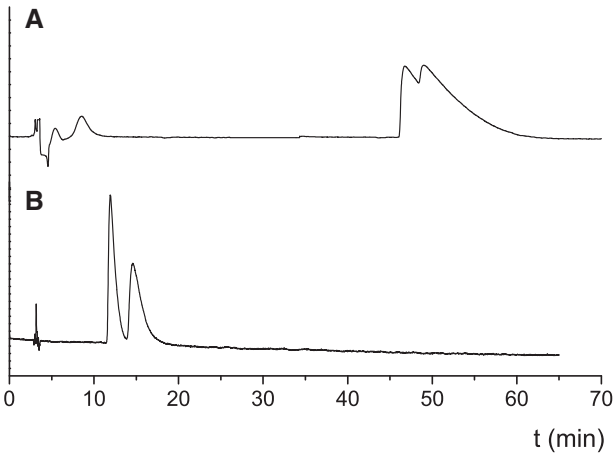

Figure 1. Chromatograms of enantioseparation of tramadol on Chiralpak IA (A) and Chiralpak AD-RH (B) columns. Mobile phase composition: AA/buffer (10 mM, pH 8.80)/ACN 70:30 v/v.

[23]. The buffers were filtered before use. Stock solutions of samples were prepared in concentrations of 1 mg/mL using MeOH as a solvent.

2.3.2 LFER model

LFER is an extensively used tool that provides insight into the retention mechanisms of various stationary phases [[17–19], [24–30]] and of separation systems in general. This model considers retention (a thermodynamic process) to be a summation of contributions originating from several independent interaction types [31–34]. It is worth noting that the LFER model does not take into account chirality and thus it cannot be employed for prediction of enantioselective interactions. It can only indicate which types of interactions participate in the retention and separation processes. The most common LFER equation comprises five types of retention interactions (see Eq. (1)).

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

where k is the retention factor, terms e, s, a, b, v are the regression coefficients, E, S, A, B, V are solute descriptors and term c is the intercept.

Each of independent contributions is the product of the regression coefficient (e–v) and the solute descriptor (E–V). Solute descriptors (in our study Abraham's descriptors [33]) reflect certain characteristic of the solute. Table 1 contains explanations for the employed descriptors. The regression coefficients were obtained from multivariate linear regression (log k versus descriptors). Each coefficient expresses the difference between the ability of the stationary and mobile phases to interact with analytes through the corresponding interaction. This is why the regression coefficients are termed also system constants. A positive value of the coefficient means that the interaction is stronger with stationary phase, and therefore, increases retention. If the interaction is

Table 3. Chromatographic data for basic and bifunctional/neutral analytes under basic pH conditions; k_1 is retention factor of the first eluted enantiomer, α is enantioselectivity, and *Rs* is enantioresolution

Analyte	k_1		α		*Rs*	
	IA	AD-RH	IA	AD-RH	IA	AD-RH
Mobile phase AA-buffer(10 mM, pH 8.80)/ACN 60:40 v/v						
Alprenolol	9.34	1.41	1.00	1.13	0.00	0.95
Acebutolol	3.90	0.70	1.00	1.00	0.00	0.00
Pindolol	3.60	0.76	1.00	S.I.	0.00	S.I.
Metipranolol	6.59	0.98	1.00	1.00	0.00	0.00
Atenolol	1.26	0.43	1.00	1.00	0.00	0.00
Metoprolol	4.52	1.35	1.00	1.00	0.00	0.00
Propranolol	11.60	1.68	1.00	S.I.	0.00	S.I.
Oxprenolol	6.21	0.92	1.00	S.I.	0.00	S.I.
Amphetamine	3.97	0.78	1.00	1.00	0.00	0.00
3-F-amphetamine	4.74	0.93	1.00	S.I.	0.00	S.I.
2-F-metcathinone	4.54	0.93	1.00	S.I.	0.00	S.I.
4-F-metcathinone	3.50	1.26	1.00	1.09	0.00	0.92
4-F-amphetamine	4.60	0.88	1.00	1.00	0.00	0.00
Tryptophan octylester	25.64	27.53	1.05	1.05	0.94	0.81
Tryptophan butylester	3.47	3.00	1.00	1.00	0.00	0.00
Tryptophan methylester	0.99	0.77	1.00	1.00	0.00	0.00
Tryptophan benzylester	0.99	0.77	1.00	1.00	0.00	0.00
Thalidomide	3.82	3.24	1.00	1.00	0.00	0.00
Tramadol	7.77	1.15	1.06	1.25	0.52	1.20
OBIN-NH_2	13.23	11.70	1.09	1.16	1.80	2.06
Binaphthol	6.23	4.74	1.05	1.09	0.90	0.67
Butizide	2.33	0.94	1.12	1.36	1.57	1.27
Bendroflumethiazide	3.34	1.28	1.00	1.00	0.00	0.00
Mefruside	4.42	3.38	1.61	2.25	4.02	3.63
Chlortalidon	1.31	0.67	1.00	1.00	0.00	0.00
Mobile phase AA-buffer(10 mM, pH 8.80)/ACN 70:30 v/v						
Alprenolol	14.22	3.52	1.00	1.18	0.00	1.27
Acebutolol	10.02	1.22	1.00	1.00	0.00	0.00
Pindolol	11.21	1.33	1.00	1.15	0.00	0.88
Metipranolol	10.50	2.15	1.00	1.00	0.00	0.00
Atenolol	2.62	0.51	1.00	1.00	0.00	0.00
Metoprolol	11.92	2.64	1.00	S.I.	0.00	S.I.
Propranolol	18.96	4.41	1.00	1.07	0.00	0.52
Oxprenolol	18.98	1.79	1.00	1.12	0.00	0.88
Amphetamine	5.58	1.09	1.00	S.I.	0.00	S.I.
3-F-amphetamine	8.72	1.48	1.00	1.12	0.00	1.25
2-F-metcathinone	8.46	1.48	1.00	1.12	0.00	1.18
4-F-metcathinone	8.57	2.27	1.00	1.10	0.00	1.27
4-F-amphetamine	6.71	1.33	1.00	S.I.	0.00	S.I.
Tryptophan octylester	N.M.	N.M.	N.M.	N.M	N.M.	N.M
Tryptophan butylester	11.17	10.00	1.00	1.00	0.00	0.00
Tryptophan methylester	2.14	1.73	1.00	1.00	0.00	0.00
Tryptophan benzylester	2.15	1.73	1.00	1.00	0.00	0.00
Thalidomide	7.50	6.72	1.00	1.07	0.00	0.39
Tramadol	18.35	2.97	1.06	1.29	0.55	1.39
OBIN-NH_2	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.
Binaphthol	27.41	22.72	1.06	1.11	1.23	1.08
Butizide	6.35	2.81	1.15	1.38	2.30	1.35
Bendroflumethiazide	11.39	4.73	1.05	1.00	1.01	0.00
Mefruside	11.35	N.M.	1.70	N.M.	4.14	N.M.
Chlortalidon	2.45	1.43	1.00	1.00	0.00	0.00

S.I., slight indication of enantioseparation; N. M., not measured.

Table 4. Chromatographic data of basic and bifunctional analytes obtained under the optimized conditions; k_1 is retention factor of the first eluted enantiomer, α is enantioselectivity, and *Rs* is enantioresolution

Analyte	CSP	Mobile phase	k_1	α	*Rs*
Alprenolol	AD-RH	AA-buffer (10 mM, pH 8.80)/ACN 80:20 v/v	13.85	1.21	1.57
Pindolol	AD-RH	AA-buffer (10 mM, pH 8.80)/ACN 85:15 v/v	8.78	1.21	0.60
Metoprolol	AD-RH	AA-buffer (10 mM, pH 8.80)/ACN 85:15 v/v	23.38	1.03	0.22
Propranolol	AD-RH	AA-buffer (10 mM, pH 8.80)/ACN 80:20 v/v	19.71	1.10	0.52
Oxprenolol	AD-RH	AA-buffer (10 mM, pH 8.80)/ACN 80:20 v/v	5.62	1.18	1.07
Amphetamine	AD-RH	AA-buffer (10 mM, pH 8.80)/ACN 90:10 v/v	5.83	1.17	1.27
3-F-amphetamine	AD-RH	AA-buffer (10 mM, pH 8.80)/ACN 80:20 v/v	3.20	1.15	1.61
2-F-metcathinone	AD-RH	AA-buffer (10 mM, pH 8.80)/ACN 80:20 v/v	3.17	1.16	1.70
4-F-metcathinone	AD-RH	AA-buffer (10 mM, pH 8.80)/ACN 80:20 v/v	5.95	1.10	1.47
4-F-amphetamine	AD-RH	AA-buffer (10 mM, pH 8.80)/ACN 90:10 v/v	9.19	1.17	1.03
Tryptophane octylester	AD-RH	AA-buffer (10 mM, pH 8.80)/ACN 60:40 v/v	27.53	1.05	0.81
	IA	AA-buffer (10 mM, pH 8.80)/ACN 60:40 v/v	25.64	1.05	0.94
Tryptophane methylester	AD-RH	AA-buffer (10 mM, pH 8.80)/ACN 80:20 v/v	5.90	1.06	0.65
Tryptophane benzylester	AD-RH	AA-buffer (10 mM, pH 8.80)/ACN 85:15 v/v	12.20	1.09	0.74
Thalidomide	AD-RH	AA-buffer (10 mM, pH 8.80)/ACN 80:20 v/v	24.02	1.09	0.57
Tramadol	AD-RH	AA-buffer (10 mM, pH 8.80)/ACN 70:30 v/v	2.97	1.29	1.39
	IA	AA-buffer (10 mM, pH 8.80)/ACN 70:30 v/v	18.35	1.06	0.55
OBIN-NH_2	AD-RH	AA-buffer (10 mM, pH 8.80)/ACN 60:40 v/v	11.70	1.16	2.06
	IA	AA-buffer (10 mM, pH 8.80)/ACN 60:40 v/v	13.23	1.09	1.80
Binaphtol	AD-RH	AA-buffer (10 mM, pH 8.80)/ACN 70:30 v/v	22.72	1.11	1.08
	IA	AA-buffer (10 mM, pH 8.80)/ACN 70:30 v/v	27.41	1.06	1.23
Butizide	AD-RH	AA-buffer (10 mM, pH 8.80)/ACN 75:25 v/v	5.92	1.40	1.57
	IA	AA-buffer (10 mM, pH 8.80)/ACN 70:30 v/v	6.35	1.15	2.30
Bendroflumethiazide	IA	AA-buffer (10 mM, pH 8.80)/ACN 70:30 v/v	11.39	1.05	1.01
Mefruside	AD-RH	AA-buffer (10 mM, pH 8.80)/ACN 60:40 v/v	3.38	2.25	3.63
	IA	AA-buffer (10 mM, pH 8.80)/ACN 60:40 v/v	4.42	1.61	4.02

stronger with the mobile phase, the corresponding regression coefficient has a negative value. The regression coefficients are explained in Table 1. Term *c* is characteristic for given system; however, it has no physicochemical interpretation.

Our LFER testing set was composed of 42 achiral solutes (see Supporting Information Table S1). The descriptors of the chosen solutes cover a wide range of values, so that no interaction is preferred. The statistical calculations were processed using NCSS software [35].

3 Results and discussion

3.1 Chiral separations

The initial mobile phase for enantioselective separation of acidic and bifunctional/neutral analytes was an aqueous solution of HCOOH (pH 2.10)/ACN 60:40 v/v. A mobile phase composed of aqueous solution of HCOOH (pH 2.10)/ACN 70:30 v/v was also tested for racemates that were separated partially or not at all. The content of the aqueous part of the mobile phase was not further increased because of the unacceptably high retention of the analytes. The obtained results are summarized in Table 2.

Despite the fact that both studied CSPs contain a chiral selector based on the same derivatized polysaccharide, their retention and enantiodiscrimination behaviors differ significantly. Some acidic analytes are more strongly retained on Chiralpak IA than on Chiralpak AD-RH and vice versa. Higher separation potential in terms of enantioresolution values for analytes in the profen family was observed on Chiralpak AD-RH.

For N-blocked amino acids, Chiralpak IA seemed to be a better choice for enantioseparation of benzyloxycarbonyl amino acids (*Z*-substituent), while Chiralpak AD-RH yielded better separation of amino acids with a *tert*-butoxycarbonyl group (*N*-BOC substituent).

Comparable results were obtained for binaphthol atropisomers on both the CSPs. Bifunctional racemates of butizide and mefruside could be baseline-separated on both CSPs. Enantiomers of chlorthalidone and bendroflumethiazide could be separated only partly on Chiralpak IA, while no enantioseparation of these analytes was observed on Chiralpak AD-RH in acidic mobile phases.

In basic pH an initial mobile phase composed of AA-buffer (10 mM, pH 8.80)/ACN, 60:40 v/v was chosen for enantioselective evaluation of the studied CSPs. The results are shown in Table 3. In general, better separations were obtained on the Chiralpak AD-RH column. Tramadol can serve as an illustrative example (see Fig. 1). However, better resolution values for binaphthol, butizide, and bendroflumethiazide were achieved on Chiralpak IA. Baseline separations

of OBIN-NH_2 and mefruside racemates were obtained on both columns.

Mobile phases with larger amounts of the aqueous part were used to increase the retention. The results obtained in optimized mobile phases are summarized in Table 4. Overall, 19 of 25 basic or bifunctional/neutral racemates were at least partially resolved on Chiralpak AD-RH and 7 on Chiralpak IA. Interestingly, Chiralpak IA retained all the basic analytes much better than Chiralpak AD-RH in all the mobile phases tested. High retention of basic analytes on Chiralpak IA cannot be explained by the LFER model. However, since the pH of the mobile phase is close to the pK_a of the analytes, they are at least partly present in the protonized forms. Additional measurements focused on ionic interactions (see Section 3.3) revealed that this type of interaction takes place predominantly on Chiralpak IA. The cation exchange activity of Chiralpak IA in the basic pH region therefore resulted in stronger retention of basic analytes but no observable impact on the enantioselectivity. See Section 3.2 for a detailed description of the retention interactions of the studied CSPs. The ionic interactions are discussed in Section 3.3.

3.2 Comparison based on the LFER model

LFER results were obtained based on the chromatographic data for 42 achiral solutes with known descriptors (see the Supporting Information Table S1). The CSPs were tested in two mobile phases, namely, an aqueous solution of HCOOH (pH 2.10)/ACN 70:30 v/v and AA-buffer (10 mM, pH 8.80)/ACN 70:30 v/v. These mobile phases were chosen because the enantioselectivity of both the studied CSPs substantially differed in the mentioned mobile phases and also significant differences in the retention were observed. Since the LFER measurements for Chiralpak IA and Chiralpak AD-RH were performed for the same mobile phase compositions, the obtained coefficients can be used directly for comparison of the interactions participating in the retention and enantioseparation mechanisms on these CSPs.

The calculated regression coefficients are summarized in Table 5. All the LFER models fit an R^2 value equal to or higher than 0.95, which indicates strong correlation between the predicted and experimental retention data. All five types of interactions were found to be statistically significant for the retention mechanism on both the CSPs in acidic and basic environments.

The regression coefficients v reached the most positive values in all the systems. This is in agreement with general knowledge, since this term reflects hydrophobicity in RP systems (the stationary phase is less polar than the mobile phase). In both mobile phase compositions, systems with Chiralpak AD-RH yielded slightly higher values compared to systems with Chiralpak IA. These results indicate that the means of preparation of CSP can influence its hydrophobicity. The values of the regression coefficient e were also positive in all the systems. The interactions of n or π electron pairs are somewhat stronger on Chiralpak AD-RH. Contributions of this type of interactions to the retention are higher in mobile phases with basic pH values.

The values of coefficients s, a, and b were negative, so the corresponding interactions do not contribute to an increase in the retention regardless of the pH of the mobile phase. Interestingly, higher absolute values of these coefficients were also obtained for Chiralpak AD-RH with the exception of coefficient s in basic buffers. The values of coefficient a remain unchanged with respect to the confidence interval for both CSPs. The values of coefficients a and b are affected by sorption of mobile phase constituents on the CSPs. High absolute values of b (hydrogen bond acidity), about twice as high as those of a, in all compared systems denote typical RP behavior in separation systems with silica-based stationary phases [36]. If the silica gel is better accessible for the aqueous part of the mobile phase, the absolute b value decreases, as in the case of the Chiralpak IA column. Coefficient s, reflecting the dipolarity/polarizability, reached significantly higher absolute values for both CSPs in basic pH environments. Thus, the corresponding interaction lowers the retention more in basic mobile phases.

3.3 Evaluation of ionic interactions

Since the basic LFER model does not take into account ionic interactions, an additional approach was used. To reveal the ionic interactions caused by a silica support (it is believed that a polysaccharide-based chiral selector itself cannot participate in these interactions [37]) a set of suitable achiral analytes was chosen, i.e. the strong acids benzenesulfonic acid (pK_a = –2.80 [38]) and toluenesulfonic acid (pK_a = –2.54 [38]) for acidic pH and phenylethylamine (pK_a = 9.83 [39]) and trimethylphenylammonium chloride for basic pH (see Table 6). These compounds were initially analyzed in the following mobile phases: aqueous solution of HCOOH (pH 2.10)/ACN 70:30 v/v, AA-buffer (10 mM, pH 4.50)/ACN 70:30 v/v and AA-buffer (10 mM, pH 8.80)/ACN 70:30 v/v. To minimize other effects (mainly hydrophobicity), their retention factors were related to the retention factor of benzene. It is obvious from Table 6 that the acidic analytes are retained much more in the acidic mobile phase on Chiralpak IA. Basic analytes show the same behavior in the basic mobile phase. Additional measurements were performed in mobile phases composed of AA-buffer (50 mM, pH 4.50)/ACN 70:30 v/v, AA-buffer (50 mM, pH 8.80)/ACN 70:30 v/v, AA-buffer (100 mM, pH 4.50)/ACN 70:30 v/v, and AA-buffer (100 mM, pH 8.80)/ACN 70:30 v/v to study the effect of the ionic strength. Analogous mobile phases (of the same ionic strength) as those at pH 2.10 were not used because of the unacceptably high consumption of HCOOH. The results are presented in Table 7. Some reduction of the retention values of basic compounds with increasing ionic strength of the aqueous mobile phase constituent could be observed only on the immobilized column with pH 8.80 buffer. These results suggest that the ionic interactions are more likely present on Chiralpak IA, for which cation and anion exchange ability

Table 5. Regression coefficients of the LFER equation

CSP	Mobile phase		*e*	*s*	*a*	*b*	*v*	*c*	R^2
Chiralpak IA	aq. sol. HCOOH (pH 2.10)/ACN		0.245	–0.179	–0.606	–1.587	1.865	–0.584	0.99
	70:30	±95%CI	0.102	0.106	0.068	0.105	0.135	0.132	
	v/v	*p*	0.000	0.002	0.000	0.000	0.000	0.000	
Chiralpak AD-RH	aq. sol. HCOOH (pH 2.10)/ACN		0.293	–0.196	–0.700	–1.625	1.956	–0.639	0.99
	70:30	±95%CI	0.177	0.131	0.084	0.129	0.167	0.163	
	v/v	*p*	0.000	0.005	0.000	0.000	0.000	0.000	
Chiralpak IA	AA-buffer (10 mM, pH 8.80)/ACN		0.445	–0.761	–0.574	–1.273	1.927	–0.420	0.95
	70:30	±95%CI	0.289	0.299	0.191	0.259	0.381	0.372	
	v/v	*p*	0.004	0.000	0.000	0.000	0.000	0.028	
Chiralpak AD-RH	AA-buffer (10 mM, pH 8.80)/ACN		0.463	–0.646	–0.665	–1.395	2.001	–0.539	0.97
	70:30	±95%CI	0.239	0.247	0.158	0.244	0.315	0.307	
	v/v	*p*	0.000	0.000	0.000	0.000	0.000	0.001	

CI represents ±95% confidence interval, i.e. the values determining the interval, in which a measurement or trial falls corresponding to a given probability; *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.

Table 6. Retention factors *k* of the solutes used for the evaluation of ionic interactions

CSPs	Solute	pH 2.10		pH 4.50		pH 8.80	
		k	$k/k_{benzene}$	*k*	$k/k_{benzene}$	*k*	$k/k_{benzene}$
Chiralpak IA	Benzenesulfonic acid	1.02	0.24	0.22	0.05	X	X
	Toluenesulfonic acid	1.81	0.43	0.33	0.07	X	X
	Benzene	4.20		4.50		4.54	
Chiralpak AD-RH	Benzenesulfonic acid	0.02	0.00	0.02	0.00	X	X
	Toluenesulfonic acid	0.10	0.02	X	X	X	X
	Benzene	4.41		4.64		4.69	
Chiralpak IA	Phenylethylamine	0.01	0.00	0.18	0.04	2.95	0.65
	Trimethylphenylammonium chloride	0.01	0.00	0.18	0.04	3.75	0.83
	Benzene	4.20		4.50		4.54	
Chiralpak AD-RH	Phenylethylamine	0.02	0.00	0.14	0.03	0.47	0.10
	Trimethylphenylammonium chloride	0.02	0.00	0.13	0.03	0.20	0.04
	Benzene	4.41		4.64		4.69	

X, solute was not retained.
Mobile phases were composed of aqueous solution of HCOOH (pH 2.10)/ACN 70/30 v/v, AA-buffer (10 mM, pH 4.50)/ACN, 70/30 v/v, and AA-buffer (10 mM, pH 8.80)/ACN, 70/30 v/v.

of the silica support was observed. A possible explanation can be found in the structural differences of the CSPs. For the CSP prepared by coating (Chiralpak AD-RH), a relatively thicker and compact layer of chiral selector on the silica particles can be expected, while immobilized CSP (Chiralpak IA) can form a thinner layer due to localized chemical bonds. Consequently, the underivatized silanol groups responsible for ionic interactions are more readily accessible on Chiralpak IA. This hypothesis can also be supported by the LFER results. The values of coefficient *v*, which reflect the hydrophobicity, are lower for Chiralpak IA in both acidic and basic systems due to the larger number of assumed accessible silanol groups. As mentioned above, systems with Chiralpak AD-RH yielded higher values of all the positive regression coefficients. Since the mobile phases were the same for both Chiralpak columns, this observation indicates a larger amount of functioning chiral selector on Chiralpak AD-RH. The same explanation has already been proposed for cellulose-based CSPs evaluated in SFC [19].

4 Concluding remarks

Two commercially available polysaccharide-based CSPs containing amylose tris(3,5-dimethylphenylcarbamate) chiral selector, coated or immobilized on silica support, were compared under different separation conditions.

Table 7. Retention factors *k* of the solutes used for the evaluation of ionic interactions

CSPs	Solute	50 mM			
		pH 4.50		pH 8.80	
		k	$k/k_{benzene}$	*k*	$k/k_{benzene}$
Chiralpak IA	Benzenesulfonic acid	0.18	0.04	X	X
	Toluenesulfonic acid	0.19	0.04	X	X
	Benzene	4.35		4.39	
Chiralpak AD-RH	Benzenesulfonic acid	0.04	0.01	0.05	0.01
	Toluenesulfonic acid	0.04	0.01	0.01	0.00
	Benzene	4.80		4.85	
Chiralpak IA	Phenylethylamine	0.18	0.04	1.68	0.38
	Trimethylphenylammonium chloride	0.17	0.04	1.92	0.44
	Benzene	4.35		4.39	
Chiralpak AD-RH	Phenylethylamine	0.17	0.04	0.36	0.08
	Trimethylphenylammonium chloride	0.16	0.03	0.19	0.04
	Benzene	4.80		4.85	
CSPs	Solute	100 mM			
		pH 4.50		pH 8.80	
		k	$k/k_{benzene}$	*k*	$k/k_{benzene}$
Chiralpak IA	Benzenesulfonic acid	0.19	0.04	X	X
	Toluenesulfonic acid	0.18	0.04	X	X
	Benzene	4.27		4.40	
Chiralpak AD-RH	Benzenesulfonic acid	0.04	0.01	0.03	0.01
	Toluenesulfonic acid	0.04	0.01	0.01	0.00
	Benzene	4.65		4.82	
Chiralpak IA	Phenylethylamine	0.19	0.04	1.28	0.29
	Trimethylphenylammonium chloride	0.19	0.04	1.38	0.31
	Benzene	4.27		4.40	
Chiralpak AD-RH	Phenylethylamine	0.13	0.00	0.33	0.03
	Trimethylphenylammonium chloride	0.13	0.00	0.19	0.03
	Benzene	4.65		4.82	

X, solute was not retained.
Mobile phases were composed of aqueous solution of AA-buffer (50 or 100 mM, pH 4.50)/ACN, 70:30 v/v, and AA-buffer (50 or 100 mM, pH 8.80)/ACN, 70:30 v/v.

Based on the enantioselective screening, the immobilized CSP, Chiralpak IA, and coated CSP, Chiralpak AD-RH, had comparable enantiorecognition potential for acidic analytes in the RP mode. Chiralpak AD-RH seemed to be a better choice for the separation of basic analytes, despite the fact that Chiralpak IA exhibited higher retention for the majority of analytes.

The LFER model yielded fundamental characteristics of the retention interactions that were in general quite similar for both the columns. Dispersion interactions (hydrophobicity) and interactions by *n* and π electron pairs were proven to be the main retention-supporting interactions in the studied systems. Ionic interactions, which are not involved in LFER, were evaluated using appropriate solutes, strong organic acids, and bases. The ionic interactions were found to contribute to the retention on Chiralpak IA but have no enantioselective character.

In conclusion, significant differences in the retention and enantioselective behavior of polysaccharide-based CSPs, coated versus immobilized, were observed. However, it is not possible to prefer any of them in general. Considering the lower price of the coated stationary phase its application could be the first choice.

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5 References

[1] Ribeiro, A. R., Maia, A. S., Cass, Q. B., Tiritan, M. E., *J. Chromatogr. B* 2014, *968*, 8–21.

[2] Kalíková, K., Riesová, M., Tesařová, E., *Cent. Eur. J. Chem.* 2012, *10*, 450–471.

[3] Lämmerhofer, M., *J. Chromatogr. A* 2010, *1217*, 814–856.

[4] Chankvetadze, B., *J. Chromatogr. A* 2012, *1269*, 26–51.

[5] Chankvetadze, B., *Methods Mol. Biol.* 2013, *970*, 81–111.

[6] Okamoto, Y., Kawashima, M., Hatada, K., *J. Am. Chem. Soc.* 1984, *106*, 5357–5359.

[7] Younes, A. A., Ates, H., Mangelings, D., Vander Heyden, Y., *J. Pharm. Biomed. Anal.* 2013, *75*, 74–85.

[8] Matarashvili, I., Chankvetadze, L., Fanali, S., Farkas, T., Chankvetadze, B., *J. Sep. Sci.* 2013, *36*, 140–147.

[9] Shen, J., Ikai, T., Okamoto, Y., *J. Chromatogr. A* 2014, *1363*, 51–61.

[10] Zhang, T., Franco, P., Nguyen, D., Hamasaki, R., Miyamoto, S., Ohnishi, A., Murakami, T., *J. Chromatogr. A* 2012, *1269*, 178–188.

[11] Franco, P., Zhang, T., *J. Chromatogr. B* 2008, *875*, 48–56.

[12] Ali, I., Aboul-Enein, H. Y., *J. Sep. Sci.* 2006, *29*, 762–769.

[13] Thunberg, L., Hashemi, J., Andersson, S., *J. Chromatogr. B* 2008, *875*, 72–80.

[14] Ghanem, A., Naim, L., *J. Chromatogr. A* 2006, *1101*, 171–178.

[15] Ghanem, A., Hoenen, H., Aboul-Enein, H. Y., *Talanta* 2006, *68*, 602–609.

[16] De Klerck, K., Vander Heyden, Y., Mangelings, D., *J. Chromatogr. A* 2014, *1328*, 85–97.

[17] West, C., Zhang, Y., Morin-Allory, L., *J. Chromatogr. A* 2011, *1218*, 2019–2032.

[18] West, C., Guenegou, G., Zhang, Y., Morin-Allory, L., *J. Chromatogr. A* 2011, *1218*, 2033–2057.

[19] Khater, S., Zhang, Y., West, C., *J. Chromatogr. A* 2013, *1303*, 83–93.

[20] Younes, A. A., Mangelings, D., Vander Heyden, Y., *J. Chromatogr. A* 2012, *1269*, 154–167.

[21] Kučerová, G., Vozka, J., Kalíková, K., Geryk, R., Plecitá, D., Pajpanova, T., Tesařová, E., *Sep. Purif. Technol.* 2013, *119*, 123–128.

[22] Geryk, R., Kalíková, K., Vozka, J., Plecitá, D., Schmid, M. G., Tesařová, E., *J. Chromatogr. A* 2014, *1363*, 155–161.

[23] Jaroš, M., Hruška, V., Štědrý, M., Zusková, I., Gaš, B., *Electrophoresis* 2004, *25*, 3080–3085.

[24] Kalíková, K., Lokajová, J., Tesařová, E., *J. Sep. Sci.* 2006, *29*, 1476–1485.

[25] Mitchell, C. R., Benz, N. J., Zhang, S., *J. Chromatogr. B* 2008, *875*, 65–71.

[26] Mitchell, C. R., Benz, N. J., Zhang, S., *J. Sep. Sci.* 2010, *33*, 3060–3067.

[27] Janečková, L., Kalíková, K., Vozka, J., Armstrong, D. W., Bosáková, Z., Tesařová, E., *J. Sep. Sci.* 2011, *34*, 2639–2644.

[28] Vozka, J., Kalíková, K., Roussel, C., Armstrong, D. W., Tesařová, E., *J. Sep. Sci.* 2013, *36*, 1711–1719.

[29] Schuster, G., Lindner, W., *J. Chromatogr. A* 2013, *1273*, 73–94.

[30] Schuster, G., Lindner, W., *J. Chromatogr. A* 2013, *1301*, 98–110.

[31] Kamlet, M. J., Doherty, R. M., Abboud, J. L. M., Abraham, M. H., Taft, R. W., *Chemtech* 1986, *16*, 566–576.

[32] Poole, C. F., Poole, S. K., *J. Chromatogr. A* 2002, *965*, 263–299.

[33] Abraham, M. H., Ibrahim, A., Zissimos, A. M., *J. Chromatogr. A* 2004, *1037*, 29–47.

[34] Vitha, M., Carr, P. W., *J. Chromatogr. A* 2006, *1126*, 143–194.

[35] Hintze, J. L., NCSS User's Guide II, NCSS, Kaysville, UT, USA 2007.

[36] Sándi, Á., Nagy, M., Szepesy, L., *J. Chromatogr. A* 2000, *893*, 215–234.

[37] Zhang, T., Nguyen, D., Franco, P., *J. Chromatogr. A* 2010, *1217*, 1048–1055.

[38] Guthrie, J. P., *Can. J. Chem.* 1978, *56*, 2342–2354.

[39] Tuckerman, M. M., Mayer, J. R., Nachod, F. C., *J. Am. Chem. Soc.* 1959, *81*, 92–94.

Publikace VI: Enantioselektivní separace biologicky aktivních bazických sloučenin pomocí ultra účinné superkritické fluidní chromatografie

Chirální separace biologicky aktivních bazických sloučenin pomocí SFC často vykazují nízký enantioselektivní potenciál a jejich analýza se může projevovat zhoršenou symetrií píků. Navíc chirální báze mají tendenci být zadržovány na koloně kvůli sekundárním iontovým interakcím mezi záporně nabitými silanolovými skupinami CSF a protonizovanými skupinami bazických sloučenin. V rámci **Publikace VI** byly vyvinuty rychlé SFC metody vhodné pro enantioseparaci a možnou purifikaci 27 chirálních bází na amylosové CSF. Sada analytů se skládala z derivátů amfetaminu a kathinonu, derivátů benzofuranu a syntetických analogů aminonaftolu (Betti báze). Byl zkoumán vliv složení MF (typ organického modifikátoru, efekt přídavku kyselých nebo bazických aditiv či jejich směsi), teploty a zpětného tlaku na enantioseparace.

MF se skládaly ze směsi CO_2, organického modifikátoru (methanol, propan-2-ol či methanol/propan-2-ol 50/50 (*v*/*v*) v rozmezí 2-10 objemových %) a přídavku aditiv (0,05-0,10 objemových %). Chromatografická data jasně ukázala, že přítomnost aditiva má příznivý účinek na symetrii píků a enantioselektivitu. Navíc bylo zjištěno, že primární IPA jako aditivum do MF poskytoval vyšší hodnoty rozlišení a enantioselektivity ve srovnání s běžně používaným sekundárním DEA či terciárním TEA. Obecně lze říci, že MF skládající se z CO_2 a směsi methanol/propan-2-ol 50/50 (*v*/*v*) či propan-2-ol (všechny MF s přídavkem 0,10 objemových % IPA) byly vhodnější pro chirální dělení bazických analytů.

Pro enantioseparaci derivátů amfetaminu a kathinonu bylo nejlepších výsledků s ohledem na retenci a rozlišení dosaženo v MF obsahující CO_2, propan-2-ol a přídavek aditiv TFA/IPA 50/50 (*v*/*v*). Kombinace kyselého a bazického aditiva způsobila výrazné zvýšení enantioselektivity a úspěšnosti separace. Z celkového počtu 14 derivátů amfetaminu a kathinonu byly separovány za počátečních chromatografických podmínek (138 bar, 35 °C) bez optimalizace zpětného tlaku a teploty všechny analyty až na základní linii s výjimkou 3,4-dimethylmethkathinonu. Následná optimalizace chromatografického systému (vyšší zpětný tlak, 150 bar; vyšší teplota 40 °C) vedla k chirálnímu dělení všech psychostimulantů.

Nejlepší enantioseparace derivátů benzofuranu bylo dosaženo v MF: CO_2/methanol/propan-2-ol/IPA 95/2,5/2,5/0,10 (*v*/*v*/*v*/*v*). Všechny deriváty byly chirálně rozděleny až na základní linii a čas analýzy nepřekročil 5 minut.

Pro chirální dělení Betti bází byl dosaženo nejvyšších hodnot rozlišení a enantioselektivity v MF obsahující propan-2-ol jako organický modifikátor (5 objemových %) s přídavkem 0,10 objemových % IPA. Nicméně hodnoty retence byly nepřijatelně vysoké pro praktické SFC aplikace (více než 30 minut). Jako kompromis mezi retenčním časem analytů a rozlišením byla vybrána MF obsahující: CO_2/methanol/propanol-2-ol/IPA (*v*/*v*/*v*/*v*). Všechny testované analogy aminonaftolu byly chirálně rozděleny až na základní linii a čas analýzy nepřesáhnul 7 minut.

CSF na bázi imobilizované derivatizované amylosy, tj. *tris*(3,5-dimethylfenylkarbamát) amylosy se projevila jako vhodný nástroj pro enantioseparaci širokého spektra bazických analytů. **Publikace VI** může sloužit jako vodítko pro vývoj enantioselektivních metod pro separaci bazických racemátů v podmínkách SFC.

Publikace VI

Enantioselective separation of biologically active basic compounds in ultra-performance supercritical fluid chromatography

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Enantioselective separation of biologically active basic compounds in ultra-performance supercritical fluid chromatography

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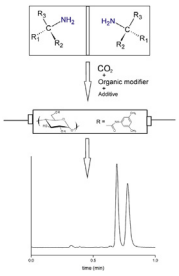
HIGHLIGHTS

- Enantioseparation of 27 biologically active basic compounds was tested in SFC.
- Effects of various co-solvents and additives on enantioseparation were evaluated.
- Influences of temperature and back pressure were investigated.
- CSP based on amylose derivative was used.
- All racemates were baseline separated.

GRAPHICAL ABSTRACT



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ABSTRACT

The enantioseparation of basic compounds represent a challenging task in modern SFC. Therefore this work is focused on development and optimization of fast SFC methods suitable for enantioseparation of 27 biologically active basic compounds of various structures. The influences of the co-solvent type as well as different mobile phase additives on retention, enantioselectivity and enantioresolution were investigated. Obtained results confirmed that the mobile phase additives, especially bases (or the mixture of base and acid), improve peak shape and enhance enantioresolution. The best results were achieved with isopropylamine or the mixture of isopropylamine and trifluoroacetic acid as additives. In addition, the effect of temperature and back pressure were evaluated to optimize the enantioseparation process. The immobilized amylose-based chiral stationary phase, *i.e.* tris(3,5-dimethylphenylcarbamate) derivative of amylose proved to be useful tool for the enantioseparation of a broad spectrum of chiral bases. The chromatographic conditions that yielded baseline enantioseparations of all tested compounds were discovered. The presented work can serve as a guide for simplifying the method development for enantioseparation of basic racemates in SFC.

1. Introduction

SFC is a powerful technique for enantioselective separation of a wide range of analytes including non-polar, polar or ionizable ones. It is progressively becoming the first choice in enantioseparation and purification in pharmaceutical and biomedical applications [1,2]. SFC may offer many benefits, *i.e.* improved resolution, faster

Abbreviations: AA, ammonium acetate; BP, back pressure; CSP, chiral stationary phase; DEA, diethylamine; IPA, isopropylamine; MeOH, methanol; MP, mobile phase; OM, organic modifier; PrOH, propane-2-ol; SF, supercritical fluid; TEA, triethylamine.

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separations or higher throughput in comparison with HPLC [3–5]. These advantages arise from the characteristics of supercritical fluids (SFs). SFs display diffusivity and viscosity features similar to those of gas. On the other hand, density and solvating power of SFs are closer to those of a liquid [6]. Supercritical CO_2 is used as a major part of mobile phase (MP) which is considered green MP because of its limited environmental impact [1]. Many published studies address only neutral, acidic or weakly basic compounds [3,7,8]. However, bases are of a particular interest because of their frequent use as catalysts, pharmaceuticals *etc*. [9]. Therefore, the separations of bases have become a challenging task among modern SFC applications because of the strong secondary ionic interactions that can occur between the negatively charged residual silanol groups of stationary phase carrier and the positively charged bases [3,10]. Many pharmaceutical compounds are chiral bases. The desired pharmacological activity usually resides preferentially from one enantiomer while the other enantiomer may be inactive, less potent, or even toxic [6]. The chiral analyses of basic compounds by SFC provided poor enantioselective recognition and may suffer from strong peak shape distortions [11]. The set of 27 separated analytes in this work contained three different groups of chiral basic biologically active compounds: (i) amphetamine- and cathinone-related psychostimulants (group A), (ii) benzofury derivatives (group B) and (iii) the synthesized aminonaphthols known in the literature as "Betti bases" (group C) [12]. The structures of the analytes are depicted in Fig. 1. Amphetamine and cathinone derivatives gained popularity on the global drug market in the recent years [13]. Cathinones represent β-keto analogues of amphetamines, *i.e*. cathinone relates to amphetamine and methcathinone to methamphetamine, respectively. The *S*-enantiomers of methcathinone and amphetamine exhibit stronger psychotropic effect than their *R*-isomers [14]. Benzofury derivatives are relatively new psychoactive substances which have similar stimulating effect as amphetamines. The main difference lies in the presence of the benzofuran ring in the molecule [15]. At present, the reported enantioselective methods for chiral recognition of amphetamine, cathinone and benzofury derivatives are primarily based on GC [14–16], CE [17,18], HPLC [19–21] and CEC [22]. Albals et al. [23] presented a comparative study of CEC, SFC and HPLC methods for enantioselective separation of some amphetamine and cathinone derivatives. Aminonaphthol derivatives have received attention because of their interesting pharmacological properties (antituberculous activity) and also because they display good enantioselectivity in asymmetric catalytic reactions [24]. Different enantioselective methods for the enantioseparation of some aminonaphthol analogues have been developed using different analytical techniques, such as CE [25], HPLC [26–30] or SFC [31]. Many chiral stationary phases (CSPs) can be used for chiral separation of basic compounds under SFC conditions [4,32–34]. Among them polysaccharide-based CSPs have proven to be one of the most useful tools for chiral resolution in SFC [35,36]. The aim of our work was to develop fast SFC methods suitable for enantioseparation and possible purification of 27 biologically active basic compounds using amylose-based CSP. An investigation of the influence of the type of organic modifier (OM) and a detailed study of the effect of additives (acids, bases or salts) were explored. In addition, the effect of temperature and back pressure (BP) was evaluated for optimizing the enantioseparation process.

2. Experimental

2.1. Chemicals and reagents

Methanol (MeOH, Chromasolv®, gradient grade, for HPLC, ≥ 99.9%), propane-2-ol (PrOH, Chromasolv® for HPLC, ≥ 99.8%), ammonium acetate (AA, purity ≥ 99%), triethylamine (TEA, minimum 99%), diethylamine (DEA, ≥ 99.5%), isopropylamine (IPA, ≥ 99.5%), trifluoroacetic acid (TFA, 99%) and tetrahydrofuran (THF, Chromasolv® for HPLC) were supplied by Sigma–Aldrich (St. Louis, USA). Pressurized liquid CO_2 4.5 grade (99.995%) was purchased from Messer (Prague, Czech Republic). Amphetamine and cathinone derivatives (A1, 4-bromomethcathinone; A2, buphedrone; A3, butylone; A4, 3,4-dimethylmethcathinone; A5, ethylbuphedrone; A6, ethylone; A7, mephedrone; A8, 4-fluoromethcathinone; A9, methylendioxypyrovalerone; A10, 4-methylethcathinone; A11, amphetamine; A12, 3-fluoroamphetamine; A13, 4-fluoroamphetamine; A14, 2-fluoromethcathinone) and benzofury derivatives (B1, 5-(2-aminopropyl)benzofuran; B2, 6-(2-aminopropyl)benzofuran; B3, 5-(2-aminopropyl)-2,3-dihydrobenzofuran; B4, 6-(2-aminopropyl)-2,3-dihydrobenzofuran; B5, 1-(benzofuran-5-yl)-*N*-ethylpropan-2-amine; B6, 1-(benzofuran-6-yl)-*N*-ethylpropan-2-amine; B7, 1-(benzofuran-5-yl)-*N*-methylpropan-2-amine) were bought in relevant online stores. Because of their novelty most analytes were not available from official suppliers. Prior to use, the compounds were characterized by GC–ESI–MS and NMR if necessary. Aminonaphthol analogues (C1, 1-[amino-(4-methylphenyl)methyl]-2-naphthol; C2, 1-[amino-(4-methoxyphenyl)methyl]-2-naphthol; C3, 1-[amino-(4-fluorophenyl)methyl]-2-naphthol); C4, 2-[amino-(4-methylphenyl)methyl]-1-naphthol; C5, 2-[amino-(4-methoxyphenyl)methyl]-1-naphthol; C6, 2-[amino-(4-fluorophenyl)methyl]-1-naphthol) were obtained from Prof. A. Péter from University of Szeged, Hungary (see Fig. 1 for the structures of the analytes).

2.2. Standard solutions

The stock solutions of amphetamine and cathinone derivatives as well as aminonaphthol analogues were prepared in MeOH at concentration 0.50 mg mL^{-1}. The stock solutions of benzofury derivatives were prepared at concentration 0.25 mg mL^{-1} in MeOH/THF (50/50 (*v*/*v*)).

2.3. SFC instrumentation and analysis

The Waters Acquity Ultra Performance Convergence Chromatography™ (UPC^2) system was equipped with a binary solvent delivery pump compatible with MP flow rates up to 4 mL min^{-1} and pressures up to 400 bar, an autosampler which included partial loop volume injection system, a BP regulator, a column oven (compatible with 250 mm length columns) and a photodiode array detector (Waters, Milford, USA). The Empower 3 software was used for system control and data acquisition. The analytical column CHIRAL ART Amylose SA was obtained from YMC Europe GmbH (Dinslaken, Germany). The chiral selector immobilized on 3 μm silica particles was tris(3,5-dimethylphenylcarbamate) derivative of amylose. The column sized 150 mm × 3.0 mm i.d. MPs composed of CO_2 and OM(s) with the addition of different additives in various volume ratios. The initial chromatographic measurements were performed at a flow rate 2.5 mL min^{-1}, at a temperature 35 °C, BP of 138 bar and UV detection at wavelengths of 230 and 254 nm. In the frame of method optimization the influence of temperature (30 °C, 40 °C) and BP (124 bar, 152 bar) were investigated for the selected MPs. Void volume was determined using the solvent peak. Injection volume was 1 μL. All measurements were performed in triplicates.

3. Results and discussion

Amylose-based CSP (CHIRAL ART Amylose SA column) was used

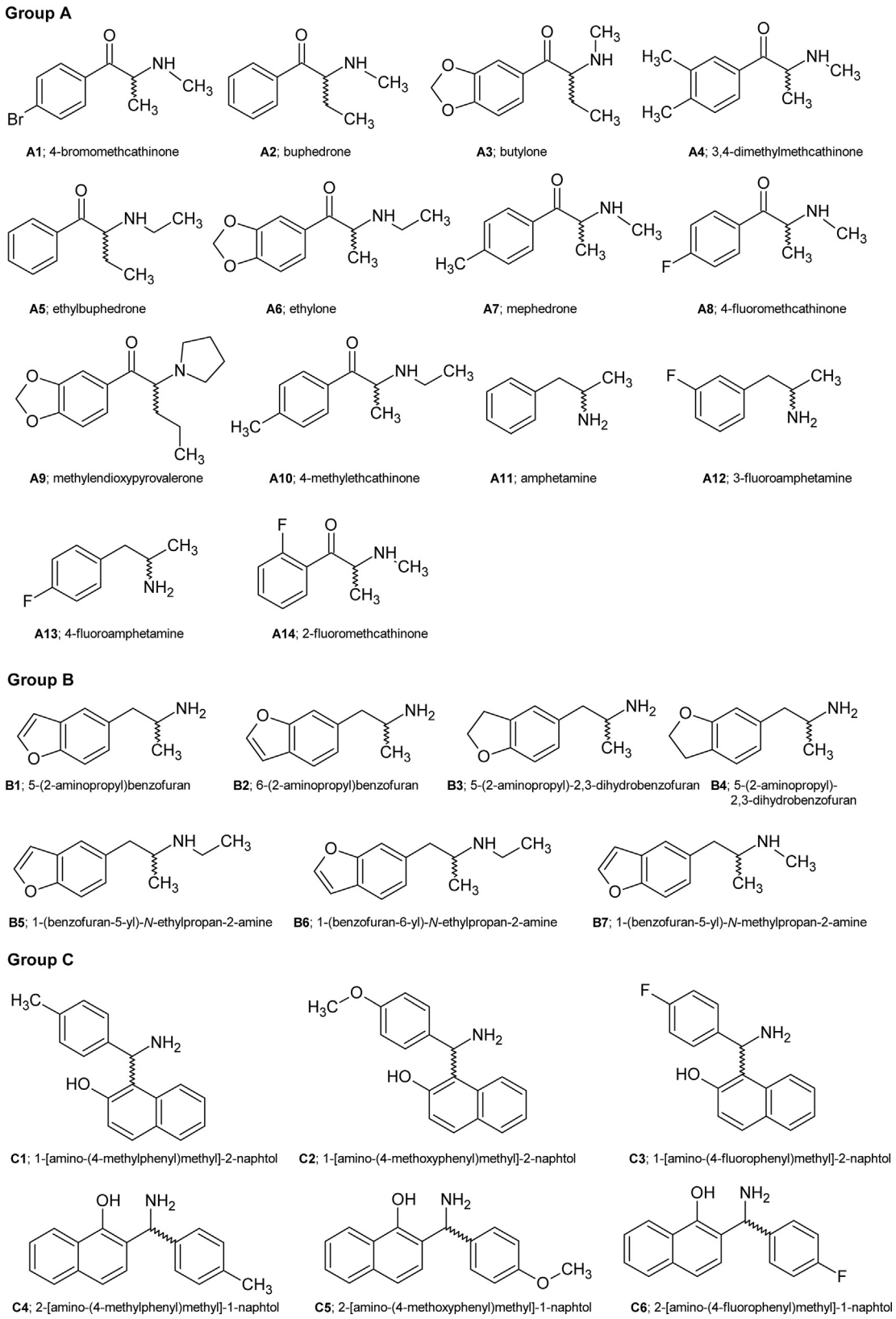

Fig. 1. Structures of basic compounds.

for all measurements. The tested MPs for enantioselective separation of the basic analytes was composed of CO_2 mixed with OM(s) (co-solvent) and small amount of additive(s). OMs are widely used to increase the eluting strength of the MP in SFC [37]. We tested the influence of three different co-solvents - MeOH, MeOH/PrOH (50/50 (*v*/*v*)) and PrOH - to examine the effect of OM(s) on the retention

and enantioseparation. The analyses were performed with varying amount of each co-solvent ranging from 2 to 10 vol%. It is well known that MP components can adsorb on the surface of CSP in various extent [38]. CO_2 reacts with alcohol molecules to form alkylcarbonic acid. The formation of acid with sterically hindered PrOH is disfavored as compared to sterically accessible MeOH. PrOH should suppress the formation of any transient complexes. Moreover, the use of branched PrOH can possibly alter the glycosidic linkage between the glucose units in the chiral selector [37,39]. The addition of small amounts of basic (TEA, DEA, IPA), acidic (TFA) or the combination of both additives (TFA/IPA, 50/50 (*v*/*v*)) served for enantioseparation and peak shape improvements. The additives were used in the volume proportions 0.05 and 0.10%. The use of volatile salt (AA) as additive was also tested but it did not show any beneficial effect on enantioseparation (data not shown). Amine-based additives are able to mask residual silanol groups present on silica-based CSP and suppress the interactions of basic analytes with these groups [3,32,40]. In general, primary amines were more effective additives than secondary or tertiary amines [41]. The best chromatographic data of all 27 basic compounds obtained under the initial separation conditions (see Experimental) are summarized in Table 1. While small individual differences were observed for certain analytes, the following comments should be applied to the majority of tested racemates.

At first, MPs consisting of CO_2/MeOH and 0.10 vol% of the basic additives (TEA, DEA, IPA) were investigated. The results showed that IPA was a more effective additive than more commonly used DEA and TEA. The effect of amine-based additives in MPs with MeOH as co-solvent on chromatographic data is summarized in Table S1 in Supplement. When IPA was used as MP additive, prolonged retentions were observed for the majority of analytes (compared to addition of TEA or DEA). This may be due to that TEA cannot act as hydrogen bond donor and DEA is considerably weaker donor than IPA [42]. The presence of additive has also a beneficial effect on the peak shape and enantioselectivity. There are some exceptions but in general in MP consisting of CO_2/MeOH and 0.10 vol% of the basic additive, the majority of the analytes exhibited higher values of enantioselectivity and resolution as compared to the corresponding MP with the addition of only 0.05 vol% (data not shown). General observation confirmed that IPA was the most promising MP additive for enantioseparation of the tested analytes. Since co-solvent can substantially affect retention/enantioselectivity/enantioseparation, the influence of type and amount of co-solvent was also tested (see Table 2). For this study the same type and amount of basic additive, *i.e.* 0.10 vol % IPA was added into MP. As expected, the retention times increased in the following order: MeOH < MeOH/PrOH (50/50 (*v*/*v*)) < PrOH. It is obvious from the comparison of the tested OMs that MPs with PrOH as co-solvent (and 0.10 vol% IPA) exhibited significantly higher enantioselectivity and enantioresolution values for the majority of the basic compounds (see Table 2). It can be generalized that MPs consisting of CO_2 and the mixture of MeOH/PrOH (50/50 (*v*/*v*)) or PrOH itself as OM(s) (all MPs with 0.10 vol% IPA) were more advantageous for enantioseparation of the tested analytes, regardless of the group (A, B or C) tested (see Table 1). However, the results in Table 1 also revealed that substantial increase in enantioresolution can occur in MPs of the following composition: CO_2/PrOH/TFA/IPA. We observed broader enantioselectivity on amylose-based CSP when using simultaneously IPA and TFA, *i.e.* basic and acidic additives (primarily for amphetamine and cathinone derivatives) as compared to the use of individual additives in the MPs.

The success rates of enantioseparation results for the tested basic compounds are clearly presented in Fig. 2. The results showed that CSP based on tris(3,5-dimethylphenylcarbamate) derivative of

Table 1
The best results achieved under SFC conditions on CHIRAL ART Amylose SA column. Retention time of the first eluted enantiomer (t_{R1}), retention factor of the first eluted enantiomer (k_1), enantioselectivity (α), resolution (R_S); flow rate 2.5 mL min^{-1}; column temperature 35 °C; 138 bar as BP; detection 230 and 254 nm; injection volume 1 μL. *Note*: In the case of baseline separation, the optimized chromatographic data were chosen with respect to the shortest analysis time.

Compounds	t_{R1} [min]	k_1	α	R_S	MP composition (volume ratio)
Group A					
A1	0.97	2.13	1.30	**2.54**	CO_2/PrOH/TFA/IPA/90/10/0.05/0.05
A2	5.16	15.64	1.40	**1.73**	CO_2/MeOH/PrOH/IPA/98/1/1/0.1
A3	1.31	3.24	1.10	**1.58**	CO_2/MeOH/IPA/95/5/0.1
A4	9.32	29.07	1.25	1.42	CO_2/PrOH/TFA/IPA/98/2/0.05/0.05
A5	0.63	1.04	1.22	**1.64**	CO_2/PrOH/TFA/IPA/90/10/0.05/0.05
A6	3.37	9.86	1.45	**2.65**	CO_2/PrOH/TFA/IPA/95/5/0.05/0.05
A7	2.66	7.59	1.26	**1.82**	CO_2/PrOH/TFA/IPA/95/5/0.05/0.05
A8	0.65	1.08	1.31	**2.23**	CO_2/PrOH/TFA/IPA/90/10/0.05/0.05
A9	0.69	1.24	1.23	**1.89**	CO_2/MeOH/IPA/90/10/0.1
A10	0.71	1.27	1.21	**1.56**	CO_2/PrOH/TFA/IPA/90/10/0.05/0.05
A11	1.35	3.34	1.17	**2.04**	CO_2/PrOH/IPA/90/10/0.1
A12	1.19	2.84	1.19	**1.85**	CO_2/PrOH/IPA/90/10/0.1
A13	14.75	46.57	1.16	**2.19**	CO_2/PrOH/TFA/IPA/98/2/0.05/0.05
A14	1.19	2.85	1.19	**2.00**	CO_2/PrOH/IPA/90/10/0.1
Group B					
B1	1.48	3.77	1.12	**1.72**	CO_2/MeOH/PrOH/IPA/90/5/5/0.1
B2	1.48	3.76	1.12	**1.65**	CO_2/MeOH/PrOH/IPA/90/5/5/0.1
B3	4.88	14.74	1.15	**2.47**	CO_2/MeOH/PrOH/IPA/95/2.5/2.5/0.1
B4	1.49	3.82	1.22	**1.80**	CO_2/MeOH/TFA/IPA/95/5/0.05/0.05
B5	1.27	3.11	1.17	**2.08**	CO_2/PrOH/IPA/90/10/0.1
B6	1.29	3.16	1.14	**1.71**	CO_2/PrOH/IPA/90/10/0.1
B7	3.15	9.17	1.12	**2.00**	CO_2/MeOH/PrOH/IPA/95/2.5/2.5/0.1
Group C					
C1	2.28	6.36	1.29	**2.83**	CO_2/MeOH/DEA/90/10/0.1
C2	2.75	7.88	1.40	**3.68**	CO_2/MeOH/DEA/90/10/0.1
C3	5.91	18.07	1.27	**1.83**	CO_2/MeOH/PrOH/IPA/95/2.5/2.5/0.1
C4	4.55	13.67	1.32	**1.64**	CO_2/MeOH/PrOH/IPA/90/5/5/0.1
C5	5.82	17.76	1.30	**1.83**	CO_2/MeOH/PrOH/IPA/90/5/5/0.1
C6	3.60	10.63	1.35	**1.65**	CO_2/MeOH/PrOH/IPA/90/5/5/0.1

Results in bold accentuate baseline separations.

Table 2
The effect of the type and amount of OMs on chromatographic data of the basic compounds; flow rate 2.5 mL min^{-1}; column temperature 35 °C; 138 bar as BP; detection 230 and 254 nm; injection volume 1 μL. For abbreviations see headings to Table 1.

Compound	CO_2/x/IPA/95/5/0.1 (volume ratio)									CO_2/x/IPA/98/2/0.1 (volume ratio)								
Group A	MeOH			MeOH/PrOH (1:1)			PrOH			MeOH			MeOH/PrOH (1:1)			PrOH		
	k_1	α	R_S	k_1	α	R_S	k_1	α	R_S	k_1	α	R_S	k_1	α	R_S	k_1	α	R_S
A1	3.97	1.07	0.79	7.78	1.00	0.00	16.03	1.09	0.72	12.48	1.12	1.01	47.13	1.11	0.83	51.79	1.06	0.47
A2	1.70	1.00	0.00	2.82	1.17	0.89	7.02	1.20	0.84	5.13	1.11	0.79	15.64	1.40	**1.73**	19.44	1.36	**1.90**
A3	3.24	1.10	**1.58**	5.75	1.12	1.06	13.58	1.17	1.16	10.03	1.20	**1.55**	34.25	1.21	1.30	40.09	1.22	**1.75**
A4	2.32	1.09	0.80	4.25	1.13	1.26	10.16	1.13	1.09	7.11	1.12	0.79	12.55	1.15	1.19	27.77	1.16	1.28
A5	0.97	1.00	0.00	1.29	1.00	0.00	2.34	1.00	0.00	1.86	1.00	0.00	2.63	1.00	0.00	4.57	0.00	0.00
A6	2.78	1.12	1.08	4.07	1.09	0.44	6.31	1.00	0.00	6.66	1.16	1.16	11.93	1.11	0.71	15.03	1.25	**1.54**
A7	2.47	1.00	0.00	4.32	1.12	1.14	9.83	1.31	**2.45**	7.37	1.08	0.67	13.91	1.16	1.31	27.14	1.30	**2.31**
A8	1.54	1.00	0.00	2.62	1.08	0.43	6.53	1.11	0.82	5.09	1.00	0.00	9.13	1.00	0.00	19.60	1.05	0.36
A9	2.05	1.28	**2.59**	2.13	1.48	**3.47**	2.22	1.36	**3.22**	3.21	1.57	**4.01**	3.36	1.62	**3.12**	3.67	1.72	**3.62**
A10	1.75	1.00	0.00	2.61	1.00	0.00	4.07	1.16	0.52	4.21	1.00	0.00	5.60	1.00	0.00	9.42	1.00	0.00
A11	2.69	1.47	1.11	7.37	1.13	**2.18**	15.84	1.22	**2.78**	11.44	1.08	0.98	26.45	1.15	**1.73**	51.87	1.19	**2.25**
A12	2.96	1.00	0.00	5.86	1.10	**1.52**	13.64	1.22	**2.15**	11.86	1.08	1.02	21.57	1.12	1.28	45.65	1.19	**1.88**
A13	3.04	1.00	0.00	6.11	1.09	1.21	13.70	1.10	1.22	11.84	1.07	1.03	22.67	1.08	0.66	46.64	1.11	0.89
A14	3.05	1.00	0.00	5.90	1.10	**1.56**	13.60	1.23	**2.29**	11.88	1.08	1.06	21.55	1.13	1.26	45.54	1.19	**1.64**
Compound	CO_2/x/IPA/90/10/0.1 (volume ratio)									CO_2/x/IPA/95/5/0.1 (volume ratio)								
Group B	MeOH			MeOH/PrOH (1:1)			PrOH			MeOH			MeOH/PrOH (1:1)			PrOH		
	k_1	α	R_S	k_1	α	R_S	k_1	α	R_S	k_1	α	R_S	k_1	α	R_S	k_1	α	R_S
B1	2.05	1.07	0.70	3.77	1.12	**1.72**	5.47	1.18	**2.63**	6.78	1.12	1.32	13.10	1.22	**3.71**	21.61	1.17	**2.76**
B2	2.04	1.07	0.77	3.76	1.12	**1.65**	5.42	1.14	**2.59**	6.81	1.22	**1.52**	13.77	1.21	**3.58**	21.76	1.17	**2.78**
B3	2.03	1.00	0.00	3.77	1.08	1.14	5.93	1.06	1.02	7.24	1.11	1.44	14.74	1.15	**2.47**	23.80	1.06	1.00
B4	2.56	1.10	1.19	4.92	1.15	**2.12**	7.51	1.17	**2.73**	8.94	1.12	**1.96**	18.55	1.20	**3.32**	24.12	1.17	**2.77**
B5	1.50	1.00	0.00	2.58	1.08	1.01	3.11	1.17	**2.08**	4.44	1.00	0.00	7.64	1.13	**1.99**	9.66	1.20	**2.97**
B6	1.58	1.00	0.00	2.72	1.09	1.15	3.16	1.14	**1.71**	4.58	1.00	0.00	7.88	1.14	**2.05**	9.33	1.15	**1.94**
B7	1.72	1.00	0.00	3.01	1.08	1.05	3.98	1.10	1.49	5.17	1.00	0.00	9.17	1.12	**2.00**	13.29	1.14	**2.28**
Compound	CO_2/x/IPA/90/10/0.1 (volume ratio)									CO_2/x/IPA/95/5/0.1 (volume ratio)								
Group C	MeOH			MeOH/PrOH (1:1)			PrOH			MeOH			MeOH/PrOH (1:1)			PrOH		
	k_1	α	R_S	k_1	α	R_S	k_1	α	R_S	k_1	α	R_S	k_1	α	R_S	k_1	α	R_S
C1	6.64	1.28	**2.77**	10.78	1.00	0.00	16.70	2.07	**3.85**	16.28	1.22	**2.07**	29.44	1.35	**2.60**	46.41	1.86	**5.62**
C2	8.20	1.39	**3.67**	13.65	1.31	**1.61**	21.15	2.12	**4.98**	21.34	1.24	**2.56**	36.61	1.47	**2.04**	60.83	1.49	**6.59**
C3	5.22	1.08	0.73	7.45	1.17	1.44	14.48	2.48	**4.56**	13.32	1.00	0.00	18.07	1.27	**1.83**	39.04	2.30	**6.72**
C4	9.39	1.08	0.74	13.67	1.32	**1.64**	47.53	1.23	0.92	23.56	1.26	**1.52**	36.65	1.45	**2.15**	x	x	x
C5	11.93	1.06	0.42	17.76	1.30	**1.83**	56.15	1.18	1.07	32.88	1.00	0.00	74.34	1.63	**4.93**	x	x	x
C6	7.73	1.00	0.00	10.63	1.35	**1.65**	32.50	1.40	**1.69**	17.77	1.11	0.42	32.06	1.48	**1.97**	100.30	1.58	**6.46**

Results in bold accentuate baseline separations. *Note*: x – no elution within 60 min.

amylose in the SFC mode is convenient for enantioseparation of the majority of the basic compounds. It is important to combine the versatility of the CSP with variety of MPs. MPs composed of CO_2/MeOH/PrOH/IPA and CO_2/PrOH/IPA provided 78% of baseline resolutions and 19% of partial resolutions under initial conditions (see Experimental). It means that 21 analytes were baseline resolved and 5 exhibited partial resolution (see Fig. 2A). The only racemate A5 out of 27 basic analytes did not show any resolution in MPs mentioned above. Using of combination of acidic and basic MP additives (MP: CO_2/PrOH/TFA/IPA) increased the number of baseline resolutions to 26 analytes (success rate 96%) and only for A4 analyte partial resolution (4%) was achieved. Separation success rates for the individual subgroups of analytes in different MPs were also determined (see Fig. 2B). It is important to note that the success rate could be further increased by investigating other chromatographic conditions (temperature, BP). The method parameters optimization was performed by subtle tuning of temperature and BP. The changes of column temperature (30 °C, 40 °C) and BP (124 bar, 152 bar) were tested for three selected MPs including CO_2/MeOH/IPA, CO_2/MeOH/PrOH/IPA and CO_2/PrOH/IPA. Zou et al. reported that under constant pressure an increase of temperature first increased retention due to the reduction of the MP density. After reaching maximum, the retention values dropped at higher temperatures, while at constant temperature retention of solutes decreased with an increase of pressure [43]. In addition, an increase of temperature in SFC contributes to stronger interactions between the analyte and the CSP [44]. BP can greatly influence many significant attributes in SFC, for example MP density or solvation power. At a higher back pressure, MP density becomes higher which results in greater solvating power and reduces the solute retention [45]. In some cases the certain tuning of temperature and/or BP provided enantioselectivity and resolution improvement (see Table S2 in Supplement). Comparing the results obtained under optimized separation conditions, it is obvious that under higher BP (152 bar) or temperature (40 °C) the majority of tested analytes exhibited baseline resolution at lower retention. It is worth mentioning that the chromatograms obtained under optimized separation conditions did not show any peak distortions. In summary, the optimized chromatographic conditions enabled baseline enantioseparation of all basic compounds (see Table S2 in Supplement). The obtained results for individual groups of basic analytes are described and discussed in the following subchapters.

3.1. Enantioseparation of amphetamine and cathinone derivatives

Based on the results in Table 1 and Table S2 in Supplement, PrOH would be recommended as the polar OM to yield enantioseparation of analytes of group A. The results described above indicate that the

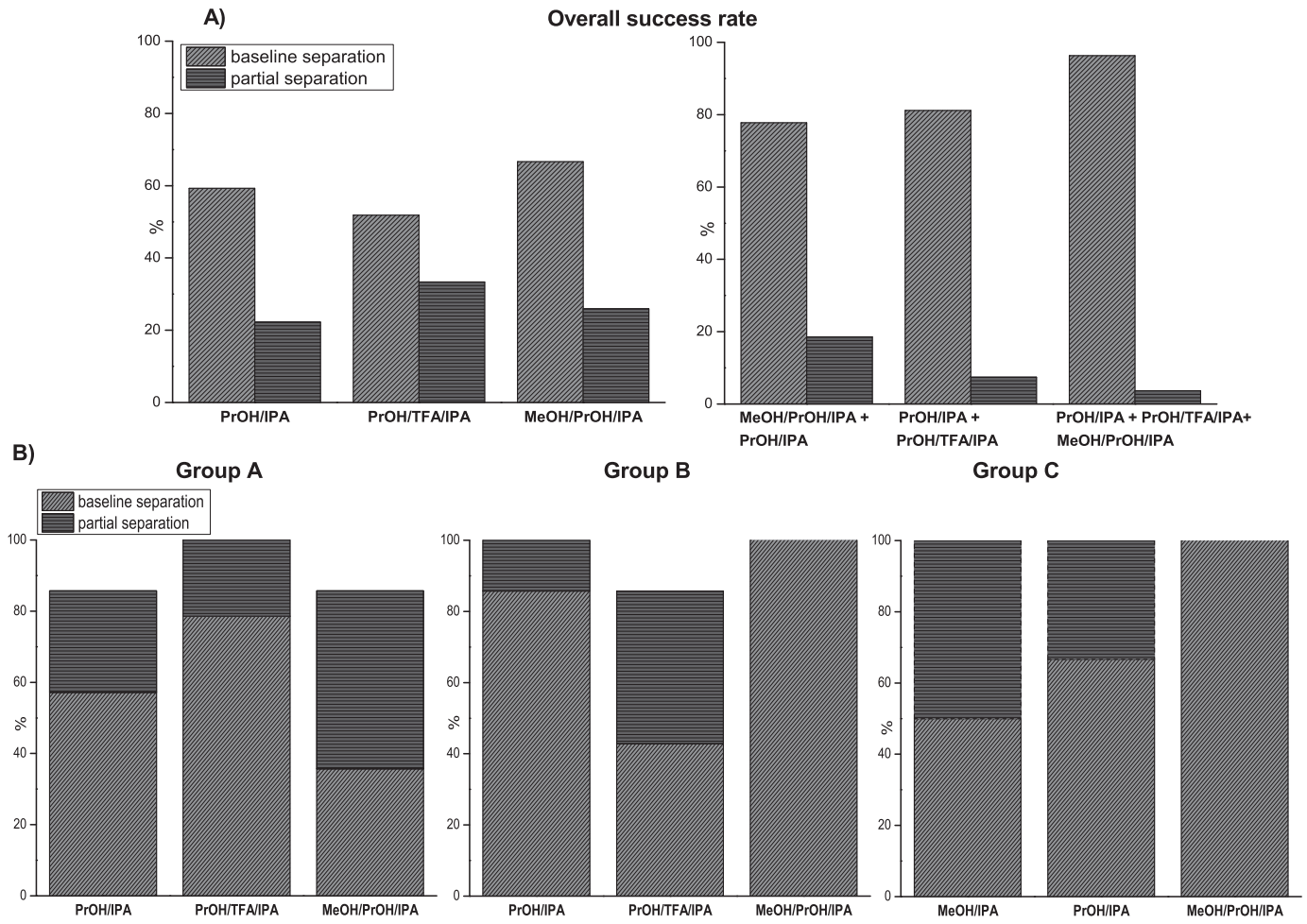

Fig. 2. Success rates of enantioseparation results in different MPs and their summations. A) Overall success rate; B) Success rate of individual groups of compounds using the most promising MPs. *Note*: The composition of MPs is indicated concerning the co-solvent and additives types but it does not concern the amount of individual OM and additives. The success rates were evaluated under following SFC conditions: column CHIRAL ART Amylose SA (150 mm × 3.0 mm i.d., 3 μm); flow rate 2.5 mL min^{-1}; column temperature 35 °C; 138 bar as BP; injection volume 1 μL.

use of MeOH as co-solvent did not provide the required enantioselectivity (with the exception of analytes A3 and A9). As shown in Table 2 adding low amount (5 and 2 vol%) of MeOH (and 0.10 vol% IPA) to CO_2 led to significant increase of retention but did not result in achieving baseline resolution except of two analytes. On the other hand, low amount (2 vol%) of PrOH (and 0.10 vol% IPA) in the MP provided conditions for baseline resolution of eight basic compounds. An example of enantioseparation of three cathinone derivatives differing in the substituent at the *para* position is depicted in Fig. 3. Two similar compounds A1 and A8 both with halogen substituents in *para* position bromo and fluoro atom, respectively, showed similar retention and separation under equal MP composition. Compound A7 with methyl group in *para* position behaved differently and baseline separation could be achieved but with MP of different composition allowed longer retention. As mentioned above, the use of TEA or DEA as MP basic additives provided worse results than IPA, even at increased retention. The results in Table 1 showed that combining TFA/IPA (50/50 (*v*/*v*)) in the MP can substantially increase enantioselectivity, compared to the use of individual basic additives. The combination of these additives led to higher enantioseparation success rates shown in Fig. 2B. We have discovered conditions that yielded baseline separations of all amphetamine and cathinone derivatives except of analyte A4, for which just partial enantioseparation was achieved (under initial chromatographic conditions). Further optimization of chromatographic conditions, *i.e.* higher BP or temperature resulted in baseline separation of all amphetamine and cathinone derivatives. The retention times of the first eluted enantiomer ranged between 0.63 and 14.32 min under the conditions for baseline separation with respect to the shortest analysis time (see Table S2 in Supplement).

3.2. Enantioseparation of benzofury derivatives

Comparing the results summarized in Table 1 and Table S2, it is evident that 4 benzofury derivatives exhibited the best enantioseparation with respect to retention and resolution using the mixture of MeOH/PrOH (50/50 (*v*/*v*)) as co-solvents in the MP. The highest resolution values for the majority of these drugs could be achieved if (MeOH/PrOH (50/50 (*v*/*v*)), with IPA) content in the MP is 5 vol % (see Table 2). MP composed of CO_2/PrOH/IPA 90/10/0.1 (*v*/*v*/*v*) exhibited baseline resolution (with exception of analytes B3 and B7) at lower retention. On the other hand, no sufficient enantioseparation of any analyte was achieved using 10 vol% of MeOH (and 0.10 vol% IPA) as co-solvent. The effect of additive type on enantioseparation of analyte B4 is shown in Fig. 4. No enantioseparation was obtained with TEA while using DEA led to partial enantioseparation. The addition of IPA or even better of mixture IPA/TFA (50/

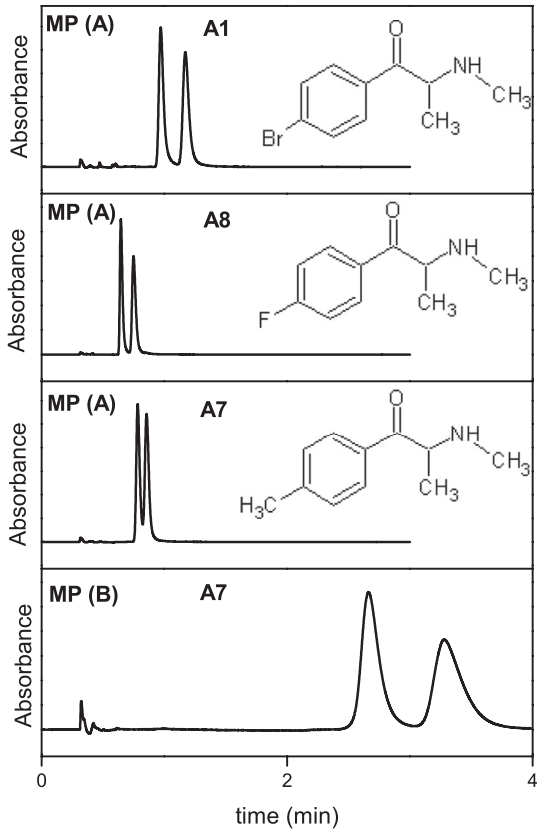

Fig. 3. SFC chromatograms of enantioseparation of analytes A1, A8 and A7. Conditions: column CHIRAL ART Amylose SA (150 mm × 3.0 mm i.d., 3 μm); MP (A): CO_2/PrOH/TFA/IPA 90/10/0.05/0.05 (*v*/*v*/*v*/*v*); MP (B): CO_2/PrOH/TFA/IPA 95/5/0.05/0.05 (*v*/*v*/*v*/*v*); flow rate 2.5 mL min^{1}; column temperature 35 °C; 138 bar as BP; detection 254 nm; injection volume 1 μL.

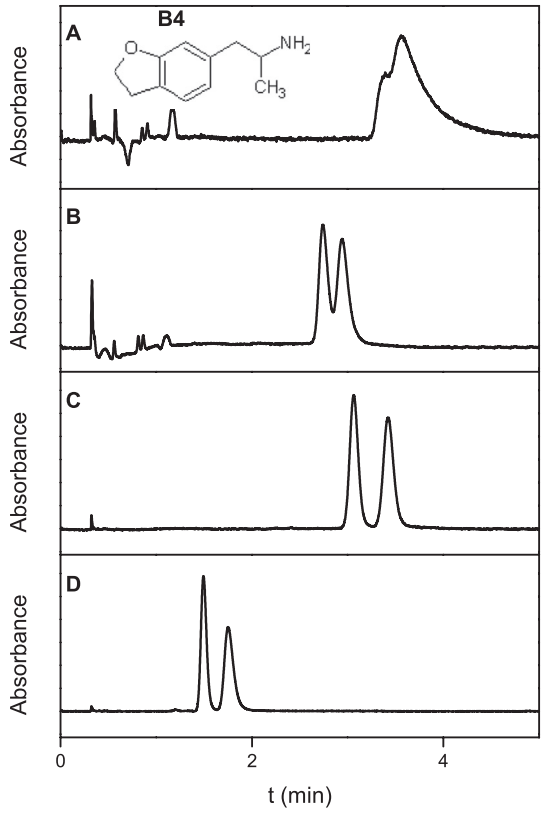

Fig. 4. The effect of the type of additive on enantioseparation of compound B4: A) TEA, B) DEA, C) IPA D) TFA/IPA (50/50 (*v*/*v*)). Conditions: column CHIRAL ART Amylose SA (150 mm × 3.0 mm i.d., 3 μm); MP: CO_2/MeOH/x 95/5/0.1 (*v*/*v*/*v*); flow rate 2.5 mL min^{-1}; temperature 35 °C; 138 bar as BP; detection 230 nm; injection volume 1 μL.

50 (*v*/*v*)) led to baseline enantioseparation. Especially the mixture of IPA/TFA (50/50 (*v*/*v*)) is beneficial because of the baseline separation at the shortest analysis time. In summary, all benzofury derivatives were baseline separated and the analysis time did not exceed 5 min.

3.3. Enantioseparation of aminonaphthol analogues

Based on the results in Table 2, a decrease of the MP eluting power by replacing 10 vol% of MeOH with PrOH (with IPA in the MP in both cases) resulted in increased retention and improved enantioresolution of the majority of the basic aminonaphthol derivatives. Addition of 5 vol% of MeOH/PrOH (50/50 (*v*/*v*)), with IPA) in the MP yielded baseline resolution of all enantiomers. The highest enantioselectivity and enantioresolution values were achieved in the MP with 5 vol% PrOH (and IPA) content (see Table 2). There are some exceptions but the retention values were unacceptably high for practical applications in SFC (more than 30 min). MP composed of CO_2/MeOH/PrOH/IPA appeared more potent for separation of the aminonaphthols than MP consisting of MeOH or PrOH as co-solvent (compare in Fig. 2B). As follows from Table 1 and Table S2 in Supplement all the tested aminonaphthol analogues were baseline enantioresolved and the analysis times under the optimized conditions did not exceed 7 min.

4. Conclusion

SFC is rapidly increasing technique in many pharmaceutical or forensic applications. Still, the analysis of basic compounds remains a challenging task. In this work we developed and optimized SFC methods suitable for chiral separation of biologically active basic compounds. The influences of the OM type (co-solvent) as well as different MP additives on retention and enantioresolution were evaluated. These results confirmed that the additives, usually acids or bases, in the OM improve peak shape and enhance enantioresolution. For the enantioseparation of amphetamine and cathinone derivatives the MP composed of CO_2/PrOH/TFA/IPA was the most promising even if the volume ratio differed for individual analytes. On the other hand, MP consisting of CO_2 and the mixture of MeOH/PrOH (50/50 (*v*/*v*)) with 0.10 vol% IPA as the additive exhibited the best chiral recognition ability for the majority of benzofury and aminonaphthol derivatives. More than 96% of baseline resolutions were obtained with no further optimization on Chiral ART Amylose SA column. After additional optimization of temperature and BP, chromatographic conditions that allow baseline separation of all basic analytes of interest can be found.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.aca.2016.04.044.

References

[1] B. Andri, A. Dispas, R.D. Marini, P. Hubert, Overview of the analytical lifecycle of supercritical fluid chromatography methods, Am. J. Anal. Chem. 7 (2016) 75–91.

[2] R. Geryk, M. Švidrnoch, A. Přibylka, K. Kalíková, V. Maier, E. Tesařová, Supercritical fluid chromatography method for the systematic toxicology analysis of cannabinoids and their metabolites, Anal. Methods 7 (2015) 6056–6059.

[3] L. Nováková, A.G.G. Perrenoud, I. Francois, C. West, E. Lesellier, D. Guillarme, Modern analytical supercritical fluid chromatography using columns packed with sub-2mm particles: A tutorial, Anal. Chim. Acta 824 (2014) 18–35.

[4] K. Kalíková, T. Šlechtová, J. Vozka, E. Tesařová, Supercritical fluid chromatography as a tool for enantioselective separation; A review, Anal. Chim. Acta 821 (2014) 1–33.

[5] C. West, Enantioselective separations with supercritical fluids - review, Curr. Anal. Chem. 10 (2014) 99–120.

[6] J.M. Płotka, M. Biziuk, C. Morrison, J. Namiesnik, Pharmaceutical and forensic drug applications of chiral supercritical fluid chromatography, Trends Anal. Chem. 56 (2014) 74–89.

[7] F. Chou, W. Wang, G. Wei, Using subcritical/supercritical fluid chromatography to separate acidic, basic, and neutral compounds over an ionic liquid-functionalized stationary phase, J. Chromatogr. A 1216 (2009) 3594–3599.

[8] E. Lemasson, S. Bertin, C. West, The use and practice of achiral and chiral supercritical fluid chromatography in pharmaceutical analysis and purification, J. Sep. Sci. 39 (2016) 212–233.

[9] M. Ventura, B. Murphy, W. Goetzinger, Ammonia as a preferred additive in chiral and achiral applications of supercritical fluid chromatography for small, drug-like molecules, J. Chromatogr. A 1220 (2012) 147–155.

[10] A.G.G. Perrenoud, J. Boccard, J.L. Veuthey, D. Guillarme, Analysis of basic compounds by supercritical fluid chromatography: Attempts to improve peak shape and maintain mass spectrometry compatibility, J. Chromatogr. A 1262 (2012) 205–213.

[11] C. Hamman, D.E. Schmidt Jr., M. Wong, M. Hayes, The use of ammonium hydroxide as an additive in supercritical fluid chromatography for achiral and chiral separations and purifications of small, basic medicinal molecules, J. Chromatogr. A 1218 (2011) 7886–7894.

[12] C. Cardellicchio, M.A.M. Capozzi, F. Naso, The Betti base: the awakening of a sleeping beauty, Tetrahedron Assymetry 21 (2010) 507–517.

[13] M. Taschwer, J.A. Weiß, O. Kunert, M.G. Schmid, Analysis and characterization of the novel psychoactive drug 4-chloromethcathinone (clephedrone), Forensic Sci. Int. 244 (2014) 56–59.

[14] S. Mohr, J.A. Weiß, J. Spreitz, M.G. Schmid, Chiral separation of new cathinone- and amphetamine-related designer drugs by gas chromatography–mass spectrometry using trifluoroacetyl-l-prolyl chloride as chiral derivatization reagent, J. Chromatogr. A 1269 (2012) 352–359.

[15] J.A. Weiß, S. Mohr, M.G. Schmid, Indirect chiral separation of new recreational drugs by gas chromatography-mass spectrometry using trifluoroacetyl-l-prolyl chloride as chiral derivatization reagent, Chirality 27 (2015) 211–215.

[16] C. Morrison, F.J. Smith, T. Tomaszewski, K. Stawiarska, M. Biziuk, Chiral gas chromatography as a tool for investigations into illicitly manufactured methylamphetamine, Chirality 23 (2011) 519–522.

[17] S. Mohr, S. Pilaj, M.G. Schmid, Chiral separation of cathinone derivatives used as recreational drugs by cyclodextrin-modified capillary electrophoresis, Electrophoresis 33 (2012) 1624–1630.

[18] L. Burrai, M. Nieddu, M.A. Pirisi, A. Carta, I. Briguglio, G. Boatto, Enantiomeric separation of 13 new amphetamine-like designer drugs by capillary electrophoresis, using modified-β-cyclodextrins, Chirality 25 (2013) 617–621.

[19] S. Mohr, M. Taschwer, M.G. Schmid, Chiral separation of cathinone derivatives used as recreational drugs by HPLC-UV using a CHIRALPAK® AS-H column as stationary phase, Chirality 24 (2012) 486–492.

[20] M. Taschwer, Y. Seidl, S. Mohr, M.G. Schmid, Chiral separation of cathinone and amphetamine derivatives by HPLC/UV using sulfated ß-cyclodextrin as chiral mobile phase additive, Chirality 26 (2014) 411–418.

[21] R.W.H. Perera, I. Abraham, S. Gupta, P. Kowalska, D. Lightsey, C. Marathaki, N.S. Singh, W.J. Lough, Screening approach, optimisation and scale-up for chiral liquid chromatography of cathinones, J. Chromatogr. A 1269 (2012) 189–197.

[22] Z. Aturki, M.G. Schmid, B. Chankvetadze, S. Fanali, Enantiomeric separation of new cathinone derivatives designer drugs by capillary electrochromatography using a chiral stationary phase, based on amylose tris(5-chloro-2-methylphenylcarbamate), Electrophoresis 35 (2014) 3242–3249.

[23] D. Albals, Y. Vander Heyden, M.G. Schmid, B. Chankvetadze, D. Mangelings, Chiral separations of cathinone and amphetamine-derivatives: Comparative study between capillary electrochromatography, supercritical fluid chromatography and three liquid chromatographic modes, J. Pharm. Biomed. Anal. 121 (2016) 232–243.

[24] M. Heydenreich, A. Koch, S. Klod, I. Szatmári, F. Fülöp, E. Kleinpeter, Synthesis and conformational analysis of naphth[1′,2′:5,6][1,3]oxazino[3,2-c][1,3]benzoxazine and naphth[1′,2′:5,6][1,3]oxazino[3,4-c][1,3]benzoxazine derivatives, Tetrahedron 62 (2006) 11081–11089.

[25] I. Ilisz, R. Iványi, Z. Pataj, J. Kupai, P. Huszthy, I. Szatmári, F. Fülöp, A. Péter, CE enantioseparation of Betti bases with cyclodextrins and crown ether as chiral selectors, Chromatographia 71 (2010) 115–119.

[26] A. Sztoikov-Ivanov, I. Szatmári, A. Péter, F. Fülöp, Structural and temperature effects in the high performance liquid chromatographic enantioseparation of 1-(a-aminobenzyl)-2-naphthol and 2-(a-aminobenzyl)-1-naphthol analogs, J. Sep. Sci. 28 (2005) 2505–2510.

[27] R. Berkezc, I. Ilisz, A. Sztojkov-Ivanov, I. Szatmári, F. Fülöp, D.W. Armstrong, A. Péter, HPLC enantioseparation of 1-(a-aminobenzyl)-2-naphthol and 2-(a-aminobenzyl)-1-naphthol analogs on a β-cyclodextrin-based chiral stationary phase, Chirality 65 (2007) 337–341.

[28] A. Sztoikov-Ivanov, D. Tóth, I. Szatmári, F. Fülöp, A. Péter, High-performance liquid chromatographic enantioseparation of 1-(aminoalkyl)-2-naphthol analogs on polysaccharide-based chiral stationary phases, Chirality 19 (2007) 374–379.

[29] I. Ilisz, Z. Pataj, R. Berkecz, I. Szatmári, F. Fülöp, A. Péter, Comparison of separation performances of cellulose-based chiral stationary phases in LC enantioseparation of aminonaphthol analogues, Chromatographia 70 (2009) 723–729.

[30] A. Aranyi, I. Ilisz, Z. Pataj, I. Szatmári, F. Fülöp, D.W. Armstrong, A. Péter, High-performance liquid chromatographic enantioseparation of Betti base analogs on a newly developed isopropyl carbamate-cyclofructan6-based chiral stationary phase, Chirality 23 (2011) 549–556.

[31] A. Aranyi, I. Ilisz, A. Péter, F. Fülöp, C. West, Exploring the enantioseparation of amino-naphthol analogues by supercritical fluid chromatography, J. Chromatogr. A 1387 (2015) 123–133.

[32] W. Ren-Qi, O. Teng-Teng, T. Weihua, N. Siu-Choon, Recent advances in pharmaceutical separations with supercritical fluid chromatography using chiral stationary phases, Trends Anal. Chem. 37 (2012) 83–100.

[33] D. Wolrab, M. Kohout, M. Boras, W. Lindner, Strong cation exchange-type chiral stationary phase for enantioseparation of chiral amines in subcritical fluid chromatography, J. Chromatogr. A 1289 (2013) 94–104.

[34] J. Vozka, K. Kalíková, E. Tesařová, Rapid supercritical fluid chromatography method for separation of chlorthalidone enantiomers, Anal. Lett. 46 (2013) 2860–2869.

[35] K. De Klerck, Y. Vander Hayden, D. Mangelings, Pharmaceutical-enantiomers resolution using immobilized polysaccharide-based chiral stationary phases in supercritical fluid chromatography, J. Chromatogr. A 1328 (2014) 85–97.

[36] P. Franco, T. Zhang, Common screening approaches for efficient analytical method development in LC and SFC on columns packed with immobilized polysaccharide-derived chiral stationary phases, Methods Mol. Biol. 970 (2013) 113–126.

[37] S. Khater, C. West, Insights into chiral recognition mechanisms in supercritical fluid chromatography V. Effect of the nature and proportion of alcohol mobile phase modifier with amylose and cellulose tris-(3,5-dimethylphenylcarbamate) stationary phases, J. Chromatogr. A 1373 (2014) 197–210.

[38] L. Toribio, J.L. Bernal, M.T. Martín, J. Bernal, M.J. del Nozal, Effects of organic modifier and temperature on the enantiomeric separation of several azole drugs using supercritical fluid chromatography and the Chiralpak AD column, Biomed. Chromatogr. 28 (2014) 152–158.

[39] T. Wang, R.M. Wenslow Jr., Effects of alcohol mobile-phase modifiers on the structure and chiral selectivity of amylose tris(3,5-dimethylphenylcarbamate) chiral stationary phase, J. Chromatogr. A 1015 (2003) 99–110.

[40] Y.K. Ye, K.G. Lynam, R.W. Stringham, Chiral separation of amines in subcritical fluid chromatography using polysaccharide stationary phases and acidic additives, J. Chromatogr. A 1070 (2005) 163–170.

[41] Y.K. Ye, K.G. Lynam, R.W. Stringham, Effect of amine mobile phase additives on chiral subcritical fluid chromatography using polysaccharide stationary phases, J. Chromatogr. A 1041 (2004) 211–217.

[42] Y.K. Ye, R.W. Stringham, Effect of mobile phase amine additives on enantioselectivity for phenylalanine analogs, J. Chromatogr. A 927 (2001) 53–60.

[43] W. Zou, J.G. Dorsey, Modifier effects on column efficiency in packed-column supercritical fluid chromatography, Anal. Chem. 72 (2000) 3620–3626.

[44] C. West, A. Bouet, S. Routier, E. Lesellier, Effects of mobile phase composition and temperature on the supercritical fluid chromatography enantioseparation of chiral fluoro-oxoindole-type compounds with chlorinated polysaccharide stationary phases, J. Chromatogr. A 1269 (2012) 325–335.

[45] C. Wang, Y. Zhang, Effects of column back pressure on supercritical fluid chromatography separations of enantiomers using binary mobile phases on 10 chiral stationary phases, J. Chromatogr. A 1281 (2013) 127–134.

Publikace VII: SFC metoda pro systematickou toxikologickou analýzu kanabinoidů a jejich metabolitů

Syntetické kanabinoidy (SC) jsou skupinou strukturně různorodých látek, které byly vyvinuty jako farmaka pro léčbu širokého spektra nemocí a pro zvýšení farmakologického účinku Δ^9 tetrahydrokanabinolu (THC). Jejich velká část se však objevuje na drogové scéně vzhledem k jejich relativně snadné dostupnosti a psychoaktivním účinkům. V rámci **Publikace VII** byla vyvinuta a validována jednoduchá a rychlá SFC metoda pro simultánní analýzu přírodních kanabinoidů a široké skupiny SC a jejich metabolitů v lidské moči.

Optimalizovaný chromatografický systém byl tvořen kolonou Zorbax Rx-SIL, MF: CO_2/acetonitril 93/7 (*v*/*v*), průtok 2,5 ml/min, teplota 40 °C, zpětný tlak 95 barů a vlnová délka 210 nm. Tři různé extrakční metody zahrnující extrakci pevným sorbentem, extrakci kapaliny-kapalinou a vysolovací extrakci kapaliny-kapalinou byly použity pro stanovení studovaných analytů v moči. Procentuální hodnoty výtěžnosti byly nejvyšší pro extrakci vysolováním a pohybovaly se v rozmezí 71,2 - 124,3%.

Vyvinutá metoda byla validována s ohledem na přesnost, preciznost, selektivitu, citlivost, linearitu, mez detekce, mez stanovitelnosti a robustnost. Validovaná metoda byla použita pro screeningovou analýzu studovaných sloučenin ve vzorcích moči u osob podezřelých z nelegálního užívání látky naftalen-1-yl-(1-butylindol-3-yl)methanon (JWH 073).

Navrhovaná metoda prokázala, že je vhodná pro toxikologické analýzy důležitých SC a jejich metabolitů v lidské moči.

Publikace VII

A supercritical fluid chromatography method for the systematic toxicology analysis of cannabinoids and their metabolites

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A supercritical fluid chromatography method for the systematic toxicology analysis of cannabinoids and their metabolites†

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A novel, simple and rapid supercritical fluid chromatography method was developed as a screening tool for natural and synthetic cannabinoids and their metabolites in biological samples.

Numerous narcotic analogs have been widely distributed as psychotropic substances in recent years.[1] Synthetic cannabinoids (SCs) are a diverse group of compounds that are derived from indole, indene and pyrrole structures and bind to one or both cannabinoid receptors with different affinities. They were chemically designed for enhancing the pharmacological potency of Δ^9-tetrahydrocannabinol (THC, the active component of cannabis). Many of them are not structurally related to the naturally occurring cannabinoids based on dibenzopyran. SCs have code names (*e.g.* JWH-, AM-) which are mostly derived from the initials of the name of the scientist who first synthesized them or the given names can be also derived from their long chemical names. The European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) registered 134 SCs until December 2014 and the number of detected SCs on illegal drug scene grows year on year.[2] SCs are available in the market as smoking blends or collectibles and gained popularity due to their easy accessibility and psychoactive effects.[3,4] Cases of abuse of SCs with threat to life or even fatal were reported in the last decade.[5] Some methods were reported for determination of SCs in herbal products and in different biological matrices including whole blood, serum, plasma, hair, oral fluids or urine.[6–8] The analysis of urine samples is further complicated by the fact that SCs are rapidly biotransformed into a large number of metabolites.[9] Moreover, the conventional cannabinoid immunochemical screening tests are ineffective in detecting this class of compounds with sufficient specificity. For systematic toxicology analysis of SCs, fast and efficient analytical methods are required. Determination of original, unchanged drugs as well as their metabolites in biological fluids is a requisite. At present, the reported methods are primarily based on GC or LC coupled with MS or high resolution accurate MS.[10–15] Few studies are focused on analyzing SCs using MEKC or LC connected with UV detection, respectively.[16,17] However, until now no SFC method for determination of SCs has been published. In different laboratory applications, SFC could be used because of its high separation efficiency, short analysis time and last but not least lower contribution to environmental pollution.[18,19] The aim of this work was to develop and validate a simple and fast SFC method for the simultaneous analysis of natural cannabinoids and a wide group of SCs and their metabolites in human urine. The influence of mobile phase (MP) composition and the effect of temperature, back pressure (BP) and flow rate were evaluated for optimizing the separation process. In addition, the performance of various extraction techniques for extraction from urine samples was examined and compared with respect to recovery and simplicity of extraction. The potential of the developed method was demonstrated by analysis of real urine samples obtained from patients after herbal blend abuse containing JWH-073 (naphthalen-1-yl-(1-butylindol-3-yl)methanone). The structures of the studied analytes are depicted in Fig. S1.† The chosen analytes contained the most abused SCs in the European Union and their main metabolites and also two natural cannabinoids (THC and CBD) for comparison.

Detailed information about instrumentation, experimental conditions, standards, calibration and sample preparation is stated in the Experimental part in ESI S1.†

At first, various MPs differing in the type and amount of organic modifier (OM), acetonitrile (ACN), methanol (MeOH), and propan-2-ol (IPA) added to supercritical CO_2 were evaluated. No significant differences among the values of retention factors and resolutions of the studied analytes in the MPs with the same OM contents were found. Nevertheless, MPs

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composed of CO_2 and ACN showed better peak shapes and a higher response. As expected, the retention factors and resolution values decreased with increasing ACN concentration in the MPs (data not shown), as the MP polarity increased. The most promising MP contained 7% of ACN in CO_2, therefore, this MP composition was studied in detail. The flow rate of the optimized MP was examined first. As a compromise between resolution values and analysis time a flow rate of 2.5 mL min^{-1} was used. The effect of column temperature was studied under the optimized MP conditions in the range of 25–40 °C. Concerning retention and resolution values, the optimum temperature for the analysis was 40 °C (data not shown). The influence of the BP was tested in the range of 80–150 bars. Higher BP resulted in lower retention that was accompanied by a certain decrease of resolution values whereas lower BP provided conditions for better separation. Therefore, a BP of 95 bars was chosen.

Three extraction techniques including solid-phase extraction (SPE), liquid–liquid extraction (LLE)[11] and salting out assisted liquid–liquid extraction (SALLE)[20] were employed for the determination of natural cannabinoids, SCs and their metabolites in urine samples. Sample preparation by SPE with a Supel™-Select SCX SPE Tube (1 mL × 30 mg) and LLE was associated with lower recovery values (data not shown), compared to SALLE. Therefore, the SALLE procedure was applied in further study. Detailed description of this procedure is given in ESI S1.†

Validation of the method was carried out under the optimized separation conditions. The newly developed method was validated in terms of precision, selectivity, sensitivity, linearity, extraction recovery and robustness according to forensic analysis standards published by Peters *et al.*[21] Intra-day precision was evaluated by extracting and analyzing eight urine samples spiked at three concentration levels using target analytes (3.0 μg mL^{-1}, 5.0 μg mL^{-1}, and 10.0 μg mL^{-1}). Inter-day precision was evaluated by preparing and analyzing eight urine samples spiked with the analytes at 5.0 μg mL^{-1} final concentration within three consecutive days. The values expressed as RSD of retention times and peak areas are summarized in Table S1.† The RSD values for retention time were ≤0.16% and 0.19% for intra-day and inter-day precision, respectively. Satisfactory results were also achieved for peak areas with RSD ≤5.12% and 6.58% for intra- and inter-day measurements. The selectivity was assessed by comparing the chromatographic data of eight different blank human urines with the corresponding spiked urine. Fig. 1 shows the typical chromatograms of blank urine and blank urine spiked with 5 μg mL^{-1} of the mixture of the tested analytes. Chromatographic data are shown in Table S2.† Blank samples showed no significant interference from the matrix at the retention times (peak positions) of the analytes. Calibration solutions were prepared by spiking studied analyte standards into negative urine (100 μL) and then extracting by using the described salting out protocol (see ESI S1,†). Linearity was verified over the concentration range of 0.5–20.0 μg mL^{-1}, 1.0–20.0 μg mL^{-1}, 1.5–20.0 μg mL^{-1} and 2.0–20.0 μg mL^{-1}, respectively, by injection of calibration solutions containing known concentrations of a mixture of THC, CBD, SCs and their metabolites (see Table S3,†). The six points calibration method was used; each point was measured three times. The calibration curves were obtained by plotting the peak areas as a function of analyte concentrations. The regression analysis was performed by calculating the coefficients of determination R^2, which ranged between 0.9980 and 0.9998. These values clearly confirm good linearity in the given calibration range (Table S3,†). 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. LOQ was taken as a concentration of the analyte where the signal-to-noise ratio was 10 : 1. The obtained LOD and LOQ values ranged between 0.15 and 0.52 μg mL^{-1} and 0.50 and 1.73 μg mL^{-1}, respectively.

The extraction recoveries were calculated by comparing the experimental results of two sets of solutions at two concentration levels. In the first set, eight blank urine samples were spiked with all analytes at 5.0 and 10.0 μg mL^{-1} final concentrations before the extraction step, while in the second set the spiked standard solutions (at the same concentrations) were made on the blank urine samples. Recovery data with the RSD values are reported in Table S4.† The percentage recovery for analytes at 5.0 μg mL^{-1} final concentrations was in the range of 74.2–124.3% and at 10.0 μg mL^{-1} final concentrations was in the range of 71.2–96.9%.

The one-way ANOVA statistical method was used for testing the robustness. Selected variable method parameters were: ACN content in the MP (7.0 ± 0.2 vol%), column temperature (39 °C, 40 °C and 41 °C) and BP (90 ± 5 bars). The robustness was determined for triplicate injections of 5.0 μg mL^{-1} of the

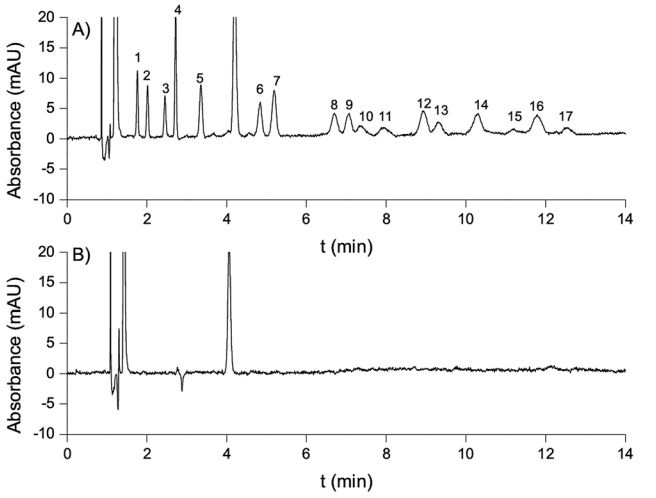

Fig. 1 Typical chromatograms of blank urine sample spiked with 5.0 μg mL^{-1} working solution of analytes (A) and blank urine (B) under optimized experimental conditions. MP composition: CO2/ACN 93/7 (v/v); flow rate 2.5 mL min^{-1}; column temperature 40 °C; 95 bars as BP; UV detection at 210 nm, injection volume 5 μL. Elution order: 1. THC, 2. CBD, 3. JWH-250, 4. JWH-073, 5. AM-2201, 6. JWH-019 5-hydroxyindole, 7. JWH-073 5-hydroxyindole, 8. JWH-018 6-hydroxyindole, 9. JWH-073 6-hydroxyindole, 10. JWH-210 N-(5-carboxypentyl), 11. JWH-018 N-pentanoic acid, 12. AM-2201 M1, 13. RCS-4 N-(4-hydroxypentyl), 14. JWH-018 N-(4-hydroxypentyl), 15. JWH-019 N-(6-hydroxyhexyl), 16. JWH-200, 17. JWH-200 4-hydroxyindole.

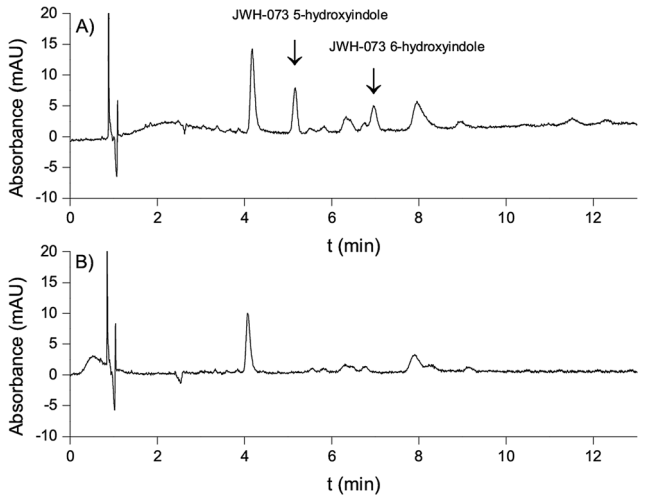

Fig. 2 Chromatograms of analysis of urine obtained from patient after JWH-073 abusing (A) and blank urine (B). See caption to Fig. 1 for details.

mixture of tested analytes. The effects of method parameters on peak areas and retention times were calculated. The hypothesis that errors resulted from a normal distribution was tested first. This hypothesis was accepted in all cases at a significant level ($\alpha = 0.05$). Consequently, the robustness of the method was examined using the one-way ANOVA. The null hypothesis (all medians are equal) was accepted in all cases (obtained *p*-values were higher than 0.05, data not shown), so the robustness of the selected parameters was verified.

The developed method for separation and detection of natural cannabinoids, SCs and their metabolites was used for screening these compounds in urine samples suspicious of JWH-073 abuse to demonstrate the potential of the method. The sample preparation and extraction was performed according to the procedure described in ESI S1.† Representative chromatograms of the urine sample analysis obtained from patients after JWH-073 administration and blank urine obtained from healthy volunteers are shown in Fig. 2. The identification of JWH-073 5-hydroxyindol and JWH-073 6-hydroxyindol metabolites in urine was carried out by the standard addition method and by the comparison of retention times and DAD spectra with standards. As it can be seen from Fig. 2, two hydroxy-metabolites were identified in urine. However, the absence of detailed information such as dosage, duration of administration and time delay between intoxication and sample collection makes it difficult to interpret the concentration ratio between identified metabolites. The concentrations of JWH-073 5-hydroxyindol and JWH-073 6-hydroxyindol were determined as 2.5 ± 0.2 μg mL^{-1} and 1.0 ± 0.2 μg mL^{-1}, respectively.

Conclusions

A new, simple, and rapid SFC method for simultaneous separation of the natural cannabinoids, SCs and their metabolites in urine was developed and validated. It offers advantages such as short analysis time, high separation efficiency and low consumption of organic solvents. The method is precise, selective and robust with satisfactory linearity within the calibration range. The developed method for separation of SCs and their metabolites holds the potential for systematic toxicology analysis particularly in the case of hyphenation with sensitive detection, namely mass spectrometry.

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Notes and references

1 M. Johansson, D. Fransson, T. Rundlöf, N. H. Huynh and T. Arvidsson, *J. Pharm. Biomed. Anal.*, 2014, **100**, 215.
2 http://www.emcdda.europa.eu/topics/pods/synthetic-cannabinoids, downloaded 4th June 2015.
3 J. Lapoint, L. P. James, C. L. Moran, L. S. Nelson, R. S. Hoffman and J. H. Moran, *Clin. Toxicol.*, 2011, **49**, 760.
4 G. L. Buser, R. R. Gerona, B. Z. Horowitz, K. P. Vian, M. L. Troxell, R. G. Hendrickson, D. C. Houghton, D. Rozansky, S. W. Su and R. F. Leman, *Clin. Toxicol.*, 2014, **52**, 664.
5 A. Pourmand, P. Armstrong, M. Mazer-Amirshahi and H. Shokoohi, *Hum. Exp. Toxicol.*, 2014, **33**, 993.
6 J. Znaleziona, P. Ginterová, J. Petr, P. Ondra, I. Válka, J. Ševčík, J. Chastina and V. Maier, *Anal. Chim. Acta*, 2015, **874**, 11.
7 H. Koskela, U. Hakala, L. Loiske, P. Vanninen and I. Szilvay, *Anal. Methods*, 2011, **3**, 2307.
8 C. E. J. Emanuel, B. Ellion and C. E. Banks, *Anal. Methods*, 2010, **6**, 614.
9 M. Mazzarino, X. de la Torre and F. Botrè, *Anal. Chim. Acta*, 2014, **837**, 70.
10 D. Favretto, J. P. Pascali and F. Tagliaro, *J. Chromatogr. A*, 2013, **1287**, 84.
11 A. Grigoryev, S. Savchuk, A. Melnik, N. Moskaleva, J. Dzhurko, M. Ershov, A. Nosyrev, A. Vedenin, B. Izotov, I. Zabirova and V. Rozhanets, *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.*, 2011, **879**, 1126.
12 J. Kim, Y. Park, M. Park, E. Kim, W. Yang, S. Baeck, S. Lee and S. Han, *J. Pharm. Biomed. Anal.*, 2015, **102**, 162.
13 S. Beuck, I. Möller, A. Thomas, A. Klose, N. Schlörer, W. Schänzer and M. Thevis, *Anal. Bioanal. Chem.*, 2011, **401**, 493.
14 K. B. Schweidweiler and M. A. Huestis, *J. Chromatogr. A*, 2014, **1327**, 105.
15 C. Montesano, M. Sergi, S. Odoardi, M. C. Simeoni, D. Compagnone and R. Curini, *J. Pharm. Biomed. Anal.*, 2014, **91**, 169.

16 R. Gottardo, A. Bertaso, J. Pascali, D. Sorio, G. Musile, E. Trapani, C. Seri, G. Serpelloni and F. Tagliaro, *J. Chromatogr. A*, 2012, **1267**, 198.
17 G. Merola, Z. Aturki, G. D'Orazio, R. Gottardo, T. Machia, F. Tagliaro and S. Fanali, *J. Pharm. Biomed. Anal.*, 2012, **71**, 45.
18 C. Radcliffe, K. Maguire and B. Lockwood, *J. Biochem. Biophys. Methods*, 2000, **43**, 261.
19 K. Kalíková, T. Šlechtová, J. Vozka and E. Tesařová, *Anal. Chim. Acta*, 2014, **821**, 1.
20 E. G. Yanes and D. P. Lovett, *J. Chromatogr.B: Anal. Technol. Biomed. Life Sci.*, 2012, **909**, 42.
21 F. T. Peters, O. H. Drummer and F. Musshoff, *Forensic Sci. Int.*, 2007, **165**, 216.

Publikace VIII: Chirální separace a stanovení antidepresiva citalopramu a jeho prekursoru citadiolu

Citalopram (*RS*-CIT) je jedním z nejúčinnějších antidepresiv patřící do skupiny selektivních inhibitorů zpětného vychytávání serotoninu (*selective serotonin reuptake inhibitors*, SSRI). *RS*-CIT je tvořen ekvimolární směsí dvou enantiomerů, *R*-citalopramu (*R*-CIT) a *S*-citalopramu (*S*-CIT). Farmakologický efekt je přisuzován *S*-enantiomeru, zatímco *R*-enantiomer je neúčinný a dokonce inhibuje účinek *S*-isomeru [72,73]. V důsledku toho se na trhu s léčivy začal vyskytovat pouze enantiomerně čistý *S*-CIT. Citadiol (*RS*-CTD) je chirální prekursor a klíčový meziprodukt pro syntézu *RS*-CIT. V rámci předkládané **Publikace VIII** byla vyvinuta a validována HPLC metoda pro chirální separaci enantiomerů *RS*-CIT a *RS*-CTD. Dále byla testována i SFC metoda pro tyto účely. Navržená HPLC metoda je vhodná ke stanovení enantiomerní čistoty *RS*-CIT v lékových formách.

Při vývoji analytické metody bylo testováno široké spektrum CSF. CSF na bázi MA (Chirobiotic TAG, Chirobiotic V, Chirobiotic V2) a derivatizovaného CF (Larihc DMP-CF7) byly testovány v PO módu. Separace enantiomerů *RS*-CIT na základní linii ($R_{1/2}$ = 1,64) bylo dosaženo na vankomycinové koloně s větším pokrytím silikagelového nosiče (Chirobiotic V2) za použití MF: methanol/TEA/AcOH 100/0,05/0,05 (*v*/*v*/*v*). CSF na bázi derivatizované amylosy (Chiralpak AD-H) a celulosy (Chiralcel OD-H) byly studovány v NP módu a v RP HPLC systému (Chiralcel OD-RH).

Z porovnání chromatografických dat v jednotlivých módech HPLC jsme zjistili, že nejlepších výsledků bylo dosaženo při použití NP módu na koloně CHIRALCEL OD-H obsahující CS na bázi *tris*(3,5-dimethylfenylkarbamátu) celulosy. Nejvhodnější MF byla tvořena směsí *n*-hexan/propan-2-ol/TEA (*v*/*v*/*v*). Vliv přídavku TEA do MF s fixním poměrem *n*-hexan/propan-2-ol 96/4 (*v*/*v*) byl studován v rozmezí 0,00-0,20 objemových %. Vliv teploty kolony na enantioseparační proces byl sledován v systému s MF *n*-hexan/propan-2-ol/TEA 94/6/0,10 (*v*/*v*/*v*) v rozmezí teplot 5-25 °C a s MF *n*-hexan/propan-2-ol/TEA 96/4/0,10 (*v*/*v*/*v*) v rozmezí 20-35 °C. U obou analytů docházelo při zvýšení teploty ke snižování retence a enantioselektivity. Jako kompromis mezi retenčním časem analytů a rozlišením byl vybrán chromatografický systém tvořený kolonou CHIRALCEL OD-H, MF: *n*-hexan/propan-2-ol/TEA 96/4/0,10 (*v*/*v*/*v*), průtok 1 ml/min, teplota 25 °C, vlnová délka 250 nm. Chromatografické parametry enantiomerů *RS*-CIT a *RS*-CTD v optimalizovaném

chromatografickém systému byly následující: retenční faktor prvního eluujícího enantiomeru CIT k_1 = 3,45, faktor enantioselektivity, α = 1,23, rozlišení, $R_{1/2}$ = 2,16. Pro enantiomery CTD byl retenční faktor prvního eluujícího enantiomeru k_1 = 5,96; faktor enantioselektivity α = 1,18; rozlišení $R_{1/2}$ = 1,50.

Pro vývoj SFC metody byly testovány CSF na bázi derivatizovaných polysacharidů (CHIRALCEL OD-H, CHIRALPAK AD-H) a CF (Larihc DMP-CF7). Pouze částečná separace *RS*-CIT byla dosažena na celulosové koloně CHIRALCEL OD-H. Separace enantiomerů *RS*-CTD až na základní linii ($R_{1/2}$ = 1,58) byla docílena na amylosové SF za použití MF: CO_2/methanol/TEA 90/10/0,25 (*v*/*v*/*v*), průtok 4 ml/min, teplota kolony 40 °C a 120 barů jako zpětný tlak. Při použití kolony CHIRALPAK AD-RH bylo v systému SFC dosaženo rychlejší enantioseparace *RS*-CTD ve srovnání s HPLC metodou. Na druhou stranu nebyly pomocí SFC nalezeny vhodné podmínky pro chirální dělení *RS*-CIT, a proto validační parametry byly zkoumány pouze pro optimalizovanou HPLC metodu.

Za účelem validace metody pro stanovení enantiomerů CIT a CTD byly stanoveny tyto validační parametry: stabilita vzorků, přesnost, preciznost, linearita, mez detekce, mez stanovitelnosti a robustnost. Navržená HPLC metoda může být použita pro rutinní kontrolu enantiomerní čistoty léčiv obsahujících jak *RS*-CIT, tak i *S*-CIT.

Publikace VIII

Method for chiral separation and quantification of antidepressant citalopram and its precursor citadiol

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ORIGINAL

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

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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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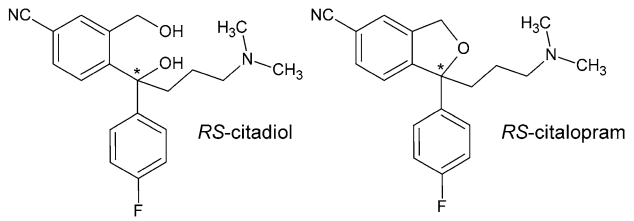

Fig. 1 Structures of the separated analytes

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.

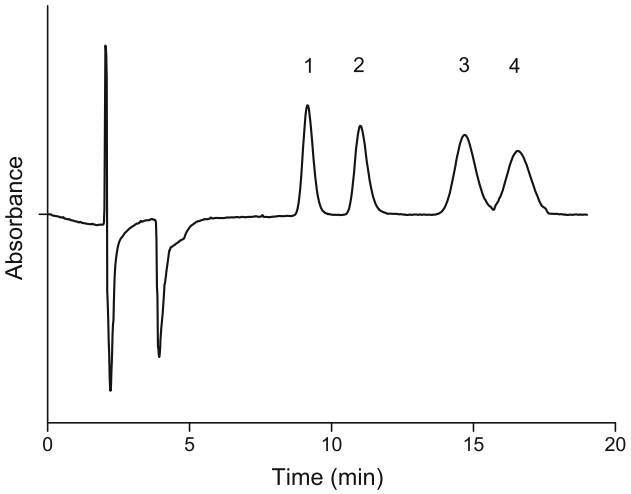

Fig. 2 HPLC separation of enantiomers of CIT and CTD under optimized conditions using Chiralcel OD-H column; mobile phase: hex/IPA/TEA 96/4/0.1 (*v*/*v*/*v*), flow rate 1.0 mL min^{-1}, UV detection at 250 nm; column temperature 25 °C, injection volume 10 µL. Resolution $R_{1/2} = 2.16$ (CIT) and 1.50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

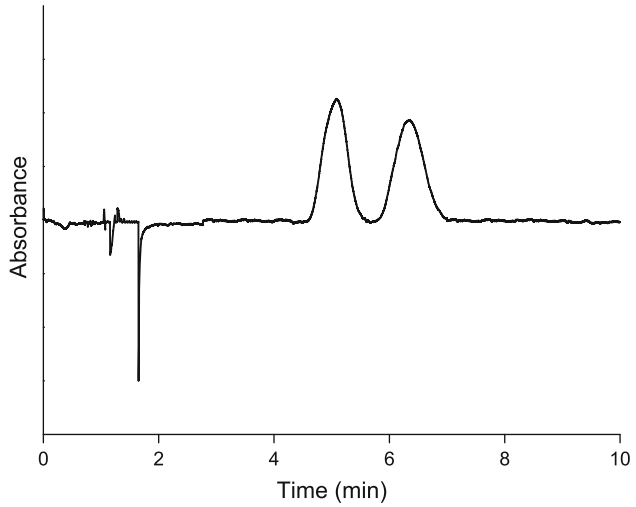

Fig. 3 SFC separation of CTD enantiomers using Chiralpak AD column; mobile phase: CO_2/methanol/TEA 90/10/0.25 (*v/v/v*), flow rate 4.0 mL min^{-1}, UV detection at 250 nm, injection volume 20 µL, column temperature 40 °C, 120 bars as back pressure

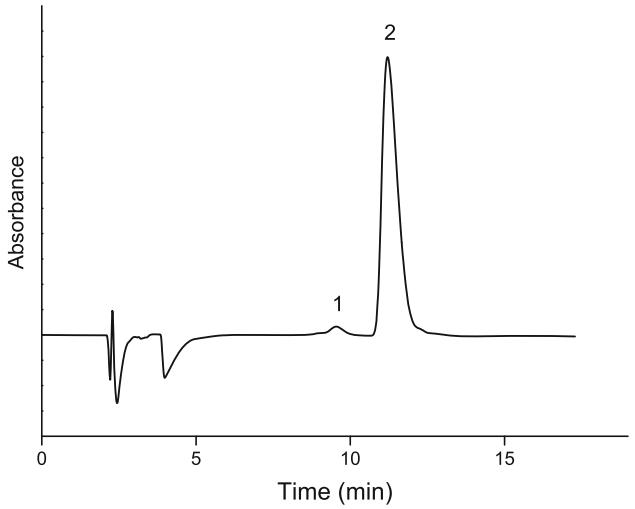

Fig. 4 HPLC chromatogram showing the separation of *S*-CIT (3.50 mg mL^{-1}) spiked with 1.0 % impurity of *R*-CIT (0.035 mg mL^{-1}) under optimized conditions—see caption to Fig. 2 for details. 1 *R*-CIT; 2 *S*-CIT

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.

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References

1. Hyttel J, Larsen JJ (1985) Acta Pharmacol Toxicol 56:146–153
2. Hyttel J, Bøgesø KP, Perregaard J, Sánchez C (1998) J Neural Transm Gen Sect 88:157–160
3. Baumann P (1996) Int Clin Psychofarmacol 11:5–11
4. Mørk A, Kreilgaard M, Sánchez C (2003) Neuropharmacology 45:167–173
5. Sanchez C, Bøgesø KP, Ebert B, Reines EH, Braestrup C (2004) Psychopharmacology 174:163–176
6. Wilson EA (2006) BioSocieties 1:125–131
7. Anderson IM (1999) Curr Anaesth Crit Care 10:32–39
8. Burke WJ, Kratochvil CJ (2002) Primary Care Companion J Clin Psychiatry 4:20–24
9. Vaswani M, Linda FK, Ramesh S (2003) Prog Neuropsychopharmacol Biol Psychiatry 27:85–102
10. Chen F, Larsen MB, Sanchez C, Wiborg O (2005) Eur Neuropsychofarmacol 15:193–198
11. Bøgesø KP, Perregaard J, US 4943590, 1990
12. Solares LF, Brieva R, Quirós M, Llorente I, Bayod M, Gotor V (2004) Tetrahedron: Asymmetry 15:341-345
13. Sungthong B, Jáč P, Scriba GKE (2008) J Pharm Biomed Anal 46:959–965
14. Chen D, Du Y (2010) J Chromatogr A 1217:1806–1812
15. Chen J, Du Y, Zhu F, Chen B (2010) J Chromatogr A 1217:7158–7163
16. Unceta N, Goicolea MA, Barrio RJ (2010) Biomed Chromatogr 25:238–257
17. Şentürk Z, Saka C, Tegin I (2011) Rev Anal Chem 30:87–122
18. Gupta VK, Ali I, Agarwal S (2011) Int J Electrochem Sci 6:5639–5648
19. Carlsson B, Norlander B (2001) Chromatographia 53:266–272
20. Mone KM, Chandrasekhar KB (2011) Chromatographia 73:985–992
21. Raman B, Sharma BA, Ghurage PD, Nandavadekar S, Singh D, Karmuse PK, Kumar A (2010) J Pharm Biomed Anal 53:895–901
22. Semreen MH, Aboul-Einen HY (2011) Acta Chromatographica 23:389–401
23. Rao RN, Raju AN, Nagaraju D (2006) J Pharm Biomed Anal 41:280–285
24. Rao RN, Raju AN (2007) J Pharm Biomed Anal 43:311–314
25. Lesellier E (2009) J Chromatogr A 1216:1881–1890
26. Taylor LT (2009) J Supercrit Fluids 47:566–573

7 Závěr

Předkládaná dizertační práce, tvořená komentovaným souborem osmi publikací, je zaměřena na výzkum a charakterizaci retenčních a enantiodiskriminačních mechanismů, které se uplatňují při separaci na chirálních stacionárních fázích na bázi derivatizovaných polysacharidů, cyklických oligosacharidů a makrocyklických antibiotik. Studované moderní stacionární fáze se v současnosti uplatňují zejména v systémech HPLC i SFC. Výsledky práce poslouží k lepšímu pochopení separačního mechanismu, a následně povedou ke snadnějšímu vývoji a optimalizaci metod pro separace širokého spektra analytů.

Enantioselektivní potenciál imobilizovaných chirálních stacionárních fází na bázi derivatizovaných polysacharidů lišících se povahou polymerního řetězce (amylosa *versus* celulosa) či typem derivatizační skupiny byl studován v podmínkách reversního i normálního módu HPLC. Amylosové chirální stacionární fáze vykazovaly v obou separačních módech vyšší hodnoty enantioselektivity a rozlišení především pro kyselé a bifunkční analyty. Chirální stacionární fáze na bázi derivatizované celulosy naopak ukázaly vyšší enantioselektivitu pro chirální báze. Imobilizované polysacharidové chirální stacionární fáze prokazují vzájemné komplementární vlastnosti a jejich kombinace umožní chirální separace širokého spektra strukturně odlišných sloučenin.

Dále byl zkoumán a porovnáván separační potenciál dvou polysacharidových stacionárních fází obsahujících totožný chirální selektor: *tris*(3,5-dimethylfenylkarbamát) amylosy, avšak lišících se navázáním chirálního polymeru na silikagelový nosič (pokrytá *versus* imobilizovaná chirální stacionární fáze). Srovnání chromatografických dat ukázalo, že obě chirální stacionární fáze vykazovaly srovnatelný separační potenciál pro kyselé analyty, zatímco pro enantioseparaci bazických látek je vhodnější pokrytá amylosová chirální stacionární fáze.

Následně byla v rámci práce testována enantioselektivita amylosové chirální stacionární fáze v systému SFC pro chirální separaci biologicky aktivních bazických látek. Podrobně byly studovány různé faktory ovlivňující separaci a na základě získaných výsledků byly nalezeny chromatografické podmínky, které umožnily separaci na základní linii všech 27 testovaných chirálních bází.

Do práce jsou také zařazeny dvě ukázky praktického využití získaných poznatků pro vývoj a validaci chromatografických metod.

Seznam literatury

[1] Ribeiro, A.R.; Maia, A.S.; Cass, Q.B.; Tiritan, M.E., Enantioseparation of chiral pharmaceuticals in biomedical and environmental analyses by liquid chromatography: An overview, *Journal of Chromatography B* **2014**, 968, 8-21.

[2] Izake, E.L., Chiral discrimination and enantioselective analysis of drugs: An overview, *Journal of Pharmaceutical Sciences* **2007**, 96, 1659-1676.

[3] Cavazzini, A.; Marchetti, N.; Guzzinati, R.; Pierini, M.; Ciogli, A.; Kotoni, D.; D´Acquarica, I.; Villani, C., Enantioseparation by ultra-high-performance liquid chromatography, *Trends in Analytical Chemistry* **2014**, 63, 95-103.

[4] De Klerck, K.; Mangelings, D.; Heyden, Y.V., Supercritical fluid chromatography for the enantioseparation of pharmaceuticals, *Journal of Pharmaceutical and Biomedical Analysis* **2012**, 69, 77-92.

[5] Ali, I.; Al Othman, Z.A.; Al-Warthan, A.; Asnin, L.; Chudinov, A., Advances in chiral separations of small peptides by capillary electrophoresis and chromatography, *Journal of Separation Science* **2014**, 37, 2447-2466.

[6] Ward, K.D.; Ward, T.J., Recent progress in chiral stationary phase development and current chiral applications, *LC GC North America* **2014**, 32, 20-23.

[7] Červinka, O., Chiralita a pojmy s ní související, *Chemické listy* **1999**, 93, 294-305.

[8] McMurry, J.E., *Organic Chemistry* **2004**, Sixth Edition.

[9] Loukotková, L.; Rambousková, M.; Bosáková, Z.; Tesařová, E., Cellulose *tris*(3,5-dimethylphenylcarbamate)-based chiral stationary phases as effective tools for enantioselective HPLC separation of structurally different disubstituted binaphthyls, *Chirality* **2008**, 20, 900-909.

[10] Neff, R.K.; Frantz, D.E., Recent applications of chiral allenes in axial-to-central chirality transfer reactions, *Tetrahedron* **2015**, 71, 7-18.

[11] Nubbemeyer, U., Planar chirality - synthesis and transformations of 8- to 10-membered heterocycles bearing (*E*)-olefins, *European Journal of Organic Chemistry* **2001**, 10, 1801-1816.

[12] Starý, I.; Stará, I.G., Helikálně chirální aromáty, *Chemické listy* **2014**, 108, 293-300.

[13] Hordern, B.K., Pharmacologically active compounds in the environment and their chirality, *Chemical Society Reviews* **2010**, 39, 4466-4503.

[14] Sekhon, B.S., Exploiting the power of stereochemistry in drugs: An overview of racemic and enantiopure drugs, *Journal of Modern Medicinal Chemistry* **2013**, 1, 10-36.

[15] Agranat, I.; Caner, H.; Caldwell, J., Putting chirality to work: The strategy of chiral switches, *Nature Reviews* **2002**, 1, 753-768.

[16] Zhang, Y.; Wu, D.R.; Wang-Iverson D,B.; Tymiak, A.A., Enantioselective chromatography in drug discovery, *Drug Discovery Today* **2005**, 10, 571-577.

[17] Jovanović, M.; Rakić, T.; Jančić-Stojanović, B., Theoretical and empirical models in hydrophilic interaction liquid chromatography, *Instrumentation Science and Technology* **2014**, 42, 230-266.

[18] Nováková, L.; Grand-Guillaume Perrenoud, A.; Nicoli, R.; Saugy, M.; Veuthey, J.L.; Guillarme, D., Ultra high performance supercritical fluid chromatography coupled with tandem mass spectrometry for screening of doping agents. I: Investigation of mobile phase and MS conditions, *Analytica Chimica Acta* **2015**, 853, 637-646.

[19] Nováková, L.; Rentsch, M.; Grand-Guillaume Perrenoud, A.; Nicoli, R.; Saugy, M.; Veuthey, J.L.; Guillarme, D., Ultra high performance supercritical fluid chromatography coupled with tandem mass spectrometry for screening of doping agents. II: Analysis of biological samples, *Analytica Chimica Acta* **2015**, 853, 647-659.

[20] Plotka, J.M.; Biziuk, M.; Morisson, C.; Namiesnik, J., Pharmaceutical and forensic drug applications of chiral supercritical fluid chromatography, *Trends in Analytical Chemistry* **2014**, 56, 74-89.

[21] Nováková, L.; Grand-Guillaume Perrenoud, A.; Francois, I.; West, C.; Lesellier, E; Guillarme, D., Modern analytical supercritical fluid chromatography using columns packed with sub-2 µm particles: A tutorial, *Analytica. Chimica. Acta* **2014**, 824, 18-35.

[22] Tarafder, A.; Kaczmarski, K.; Ranger, M.; Poe, D.P.; Guiochon, G., Use of the isopycnic plots in designing operations of supercritical fluid chromatography: IV. Pressure and density drops along columns, *Journal of Chromatography A* **2012**, 1238, 132-145.

[23] Deye, J.F.; Berger, T.A.; Anderson, A.G., Nile red as a solvatochromic dye for measuring solvent strength in normal liquids and mixtures of normal liquids with supercritical and near critical fluids, *Analytical Chemistry* **1990**, 62, 615-622.

[24] Khater, S.; West, C., Insights into chiral recognition mechanisms in supercritical fluid chromatography V. Effect of the nature and proportion of alcohol mobile phase modifier with amylose and cellulose *tris*-(3,5-dimethylphenylcarbamate) stationary phases, *Journal of Chromatography A* **2014**, 1373, 197-210.

[25] Wang, T.; Wenslow Jr., R.M., Effects of alcohol mobile-phase modifiers on the structure and chiral selectivity of amylose *tris*(3,5-dimethylphenylcarbamate) chiral stationary phase, *Journal of Chromatography A* **2003**, 1015, 99-110.

[26] Ye, Y.K.; Lynam, K.G.; Stringham, R.W., Effect of amine mobile phase additives on chiral subcritical fluid chromatography using polysaccharide stationary phases, *Journal of Chromatography A* **2004**, 1041, 211-217.

[27] Stringham, R.W., Chiral separation of amines in subcritical fluid chromatography using polysaccharide stationary phases and acidic additives, *Journal of Chromatography A* **2005**, 1070, 163-170.

[28] Blackwell, J.A.; Stringham, R.W.; Weckwerth, J.D., Effect of mobile phase additives in packed-column subcritical and supercritical fluid chromatography, *Analytical Chemistry* **1997**, 69, 409-415.

[29] Wang, C.; Zhang, Y., Effects of column back pressure on supercritical fluid chromatography separations of enantiomers using binary mobile phases on 10 chiral stationary phases, *Journal of Chromatography A* **2013**, 1281, 127-134.

[30] Lou, X.; Janssen, H.G.; Cramers, C.A., Temperature and pressure effects on solubility in supercritical carbon dioxide and retention in supercritical fluid chromatography, *Journal of Chromatography A* **1997**, 785, 57-64.

[31] West, C., Enantioselective separations with supercritical fluids - Review, *Current Analytical Chemistry* **2014**, 10, 99-120.

[32] Kalíková, K.; Šlechtová, T.; Vozka, J.; Tesařová E., Supercritical fluid chromatography as a tool for enantioselective separation; A review, *Analytica Chimica Acta* **2014,** 821, 1-33.

[33] Lesellier, E., Retention mechanisms in super/subcritical fluid chromatography on packed columns, *Journal of Chromatography A* **2009**, 1216, 1881-1890.

[34] Saito, M., History of supercritical fluid chromatography: Instrumental development, *Journal of Bioscience and Bioengineering* **2013**, 115, 590-599.

[35] Galushko, S.V., The calculation of retention and selectivity in reversed-phase liquid chromatography II. Methanol-water eluents, *Chromatographia* **1993**, 36, 39-42.

[36] Kimata, K.; Iwaguchi, K.; Onishi, S.; Jinno, K.; Eksteen, R.; Hosoya, K.; Araki, M.; Tanaka, N., Chromatographic characterization of silica C_{18} packing materials. Correlation between a preparation method and retention behavior of stationary phase, *Journal of Chromatography Science* **1989**, 27, 721-728.

[37] Engelhardt, H.; Jungheim, M., Comparison and characterization of reversed phases, *Chromatographia* **1990**, 29, 59-68.

[38] Walters, M.J., Classification of octadecyl-bonded liquid chromatography columns, *Journal - Association of Official Analytical Chemists* **1987**, 70, 465-469.

[39] Vitha, M.; Carr, P.W., The chemical interpretation and practice of linear solvation energy relationships in chromatography, *Journal of Chromatography A* **2006**, 1126, 143-194.

[40] Abraham, M.H.; Ibrahim. A.; Zissimos, A.M., Determination of sets of solute descriptors from chromatographic measurements, *Journal of Chromatography A* **2004**, 1037, 29-47.

[41] Vonk, E.C.; Lewandowka, K.; Claessens, H.A.; Kaliszan, R.; Cramers, C.A., Quantitative structure-retention relationships in reversed-phase liquid chromatography using several stationary and mobile phases, *Journal of Separation Science* **2013**, 23, 777-792.

[42] West, C.; Guenegou, G; Zhang, Y,; Morin-Allory, L., Insights into chiral recognition mechanisms in supercritical fluid chromatography. II. Factors contributing to enantiomer separation on *tris*-(3,5-dimethylphenylcarbamate) of amylose and cellulose stationary phases, *Journal of Chromatography A* **2011**, 1218, 2033-2057.

[43] Khater, S.; Zhang, Y.; West, C., Insights into chiral recognition mechanism in supercritical fluid chromatography III. Non-halogenated polysaccharide stationary phases, *Journal of Chromatography A* **2014**, 1363, 278-293.

[44] Franco, P.; Zhang, T., Common screening approaches for efficient analytical method development in LC and SFC on columns packed with immobilized polysaccharide-derived chiral stationary phases, *Methods in Molecular Biolology* **2013**, 970, 113-126.

[45] Hesse, G.; Hagel, R., A complete separation of a racemic mixture by elution chromatography on cellulose triacetate, *Chromatographia* **1973**, 6, 277-280.

[46] Yashima, E., Polysaccharide-based chiral stationary phases for high-performance liquid chromatographic enantioseparation, *Journal of Chromatography A* **2001**, 906 105-125.

[47] Ikai, T.; Okamoto, Y., Structure control of polysaccharide derivatives for efficient separation of enantiomers by chromatography, *Chemical Reviews* **2009**, 109, 6077-6101.

[48] Chankvetadze, B., Recent developments on polysaccharide-based chiral stationary phases for liquid-phase separation of enantiomers, *Journal of Chromatography A* **2012**, 1269, 26-51.

[49] Younes, A.A.; Ates, H.; Mangelings, D.; Heyden, Y.V., A separation strategy combining three HPLC modes and polysaccharide-based chiral stationary phases, *Journal of Pharmaceutical and Biomedical Analysis* **2013**, 75, 74-85.

[50] Khater, S.; Zhang, Y.; West, C., Insights into chiral recognition mechanism in supercritical fluid chromatography IV. Chlorinated polysaccharide stationary phases, *Journal of Chromatography A* **2014**, 1363, 294-310.

[51] Pelluso, P.; Cossu, S., Comparative HPLC enantioseparation of thirty-six aromatic compounds on four columns of the Lux ® series: Impact of substituents, shapes and electronic properties, *Chirality* **2013**, 25, 709-718.

[52] Younes, A.A.; Mangelings, D.; Heyden, Y.V., Chiral separations in normal phase liquid chromatography: Enantioselectivity of recently commercialized polysaccharide-based selectors. Part I: Enantioselectivity under generic screening conditions, *Journal of Pharmaceutical and Biomedical Analysis* **2011**, 55, 414-423.

[53] Younes, A.A.; Mangelings, D.; Heyden, Y.V., Chiral separations in reversed-phase liquid chromatography: Evaluation of several polysaccharide-based chiral stationary phases for a separation strategy update, *Journal of Chromatography A* **2012**, 1269, 154-167.

[54] Peng, L.; Jayapalan, S.; Chankvetadze, B.; Farkas, T., Reversed-phase chiral HPLC and LC/MS analysis with *tris*(chloromethylphenylcarbamate) derivatives of cellulose and amylose as chiral stationary phases, *Journal of Chromatography A* **2010**, 1217, 6942-6955.

[55] Zhang, T.; Kientzy, C.; Franco, P.; Ohnishi, A.; Kagamihara, Y.; Kurosawa, H., Solvent versatility of immobilized 3,5-dimethylphenylcarbamate of amylose in enantiomeric separations by HPLC, *Journal of Chromatography A* **2005**, 1075, 65-75.

[56] Zhang, T.; Nguyen, D.; Franco, P., Reversed-phase screening strategies for liquid chromatography on polysaccharide-derived chiral stationary phases, *Journal of Chromatography A* **2010**, 1217, 1048-1055.

[57] Shen, J.; Ikai, T.; Okamoto, Y., Synthesis and application of immobilized polysaccharide-based chiral stationary phases for enantioseparation by high-performance liquid chromatography, *Journal of Chromatography A* **2014**, 1363, 51-61.

[58] Zhang, T.; Franco, P.; Nguyen, D.; Hamasaki, R.; Miyamoto, S.; Ohnishi, A.; Murakami, T., Complementary enantiorecognition patterns and specific method optimization aspects on immobilized polysaccharide-derived chiral stationary phases, *Journal of Chromatography A* **2012**, 1269, 178-188.

[59] De Klerck, K.; Heyden, Y.V.; Mangelings, D., Pharmaceutical-enantiomers resolution using immobilized polysaccharide-based chiral stationary phases in supercritical fluid chromatography, *Journal of Chromatography A* **2014**, 1328, 85-97.

[60] Xiao, Y.; Ng, S.; Tan, T.T.Y.; Wang, Y., Recent development of cyclodextrin chiral stationary phases and their applications in chromatography, *Journal of Chromatography A* **2012**, 1269, 52-68.

[61] Lin, Y.; Zhou, J.; Tang, J.; Tang, W., Cyclodextrin clicked chiral stationary phases with functionalities-tuned enantioseparations in high performance liquid chromatography, *Journal of Chromatography A* **2015**, 1406, 342-346.

[62] Schneiderman, E.; Stalcup, A.M., Cyclodextrins: a versatile tool in separation science, *Journal of Chromatography B* **2010**, 745, 83-102.

[63] Sun, P.; Wang, C.; Breitbach, Z.S.; Zhang, Y.; Armstrong, D.W., Development of new HPLC chiral stationary phases based on native and derivatized cyclofructans, *Analytical Chemistry* **2009**, 81, 10215-10226.

[64] Aranyi, A.; Bagi, A.; Ilisz, I.; Pataj, Z.; Fülöp, F.; Armstrong, D.W.; Antal, P., High-performance liquid

chromatographic enantioseparation of amino compounds on newly developed cyclofructan-based chiral stationary phases, *Journal of Separation Science* **2012**, 35, 617-624.

[65] Woods, R.M.; Patel, D.C.; Lim, Y.; Breitbach, Z.S.; Gao, H.; Keene, C.; Li, G.; Kürti, L.; Armstrong, D.W., Enantiomeric separation of biaryl atropisomers using cyclofructan based chiral stationary phases, *Journal of Chromatography A* **2014**, 1357, 172-181.

[66] Meier, V.; Kalíková, K.; Přibylka, A.; Vozka, J.; Smuts, J.; Švidrnoch, M.; Ševčík, J.; Armstrong, D.W.; Tesařová, E., Isopropyl derivative of cyclofructan 6 as chiral selector in liquid chromatography and capillary electrophoresis, *Journal of Chromatography A* **2014**, 1338, 197-200.

[67] Sun, P.; Wang, C.; Padivitage, N.L.T.; Nanayakkara Y.S.; Perera, S.; Qiu, H.; Zhang, Y.; Armstrong, D.W., Evaluation of aromatic-derivatized cyclofructans 6 and 7 as HPLC chiral selectors, *Analyst* **2011**, 136, 787-800.

[68] Breitbach, A.S.; Lim, Y.; Xu, Q.L.; Kürti, L.; Armstrong, D.W.; Breitbach, Z.S., Enantiomeric separations of α-aryl ketones with cyclofructan chiral stationary phases *via* high performance liquid chromatography and supercritical fluid chromatography, *Journal of Chromatography A* **2016**, 1427, 45-54.

[69] Ilisz, I.; Pataj, Z.; Aranyi, A.; Péter, A., Macrocyclic antibiotic selectors in direct HPLC enantioseparations, *Separation and Purification Reviews* **2012**, 41, 207-249.

[70] Scriba, G.E., Chiral recognition mechanisms in analytical separation sciences, *Chromatographia* **2012**, 75, 815-838.

[71] Ilisz, I.; Aranyi, A.; Péter, A., Chiral derivatizations applied for the separation of unusual amino acid enantiomers by liquid chromatography and related techniques, *Journal of Chromatography A* **2013**, 1296, 119-139.

[72] Sanchez, C.; Bogeso, K.P.; Ebert, B.; Reines, E.H.; Braestrup, C. Escitalopram *versus* citalopram: the surprising role of the *R*-enantiomer, *Psychofarmacology* **2004**, 174, 163-176.

[73] Mørk, A.; Kreilgaard, M.; Sanchez, C., The *R*-enantiomer of citalopram counteracts escitalopram-induced increase in extracellular 5-HT in the frontal cortex of freely moving rats, *Neuropharmacology* **2003**, 45, 167-173.

Přílohy

A. **Seznam publikací**

Publikace I: Kučerová, G.; Vozka, J.; Kalíková, K.; **Geryk, R.;** Plecitá, D.; Pajpanová, T.; Tesařová, E., Enantioselective separation of unusual amino acids by high performance liquid chromatography, *Separation and Purification Technology* **2013**, 119, 123-128 (IF: 3,091).

Publikace II: Riesová, M.; **Geryk, R.;** Kalíková, K.; Šlechtová, T.; Voborná, M.; Martínková, M.; Bydžovská, A.; Tesařová, E., Direct CE and HPLC methods for enantioseparation of tryptophan and its unnatural derivatives, *Separation and Purification Technology* **2016**, 158, 24-30 (IF: 3,091).

Publikace III: Geryk, R.; Kalíková, K.; Vozka, J.; Plecitá, D.; Schmid, M.G.; Tesařová, E., Enantioselective potential of chiral stationary phases based on immobilized polysaccharides in reversed phase mode, *Journal of Chromatography A*, **2014**, 1363, 155-161 (IF: 4,612).

Publikace IV: Geryk, R.; Kalíková, K.; Vozka, J.; Tesařová, E., Immobilized polysaccharide-based stationary phases for enantioseparation in normal *versus* reversed phase HPLC, *Chromatographia* **2015**, 78, 909-915 (IF: 1,457).

Publikace V: Kalíková, K.; **Geryk, R.;** Vozka, J.; Tesařová, E., Evaluation of differences between CHIRALPAK IA and CHIRALPAK AD-RH amylose-based chiral stationary phases in reversed-phase high-performance liquid chromatography, *Journal of Separation Science*, **2015**, 38, 711-719 (IF: 2,594).

Publikace VI: Geryk, R.; Kalíková, K.; Schmid, M.G.; Tesařová, E., Enantioselective separation of biologically active basic compounds in ultra-performance supercritical fluid chromatography, *Analytica Chimica Acta* **2016**, 932, 98-105 (IF: 4,513).

Publikace VII: Geryk, R.; Švidrnoch, M.; Přibylka, A.; Kalíková, K.; Maier, V.; Tesařová, E., A supercritical fluid chromatography method for the systematic

toxicology analysis of cannabinoids and their metabolites, *Analytical Methods* **2015**, 7, 6056-6059 (IF: 1,821).

Publikace VIII: Geryk, R.; Vozka, J.; Kalíková, K.; Tesařová, E. HPLC Method for chiral separation and quantification of antidepressant citalopram and its precursor citadiol, *Chromatographia* **2013**, 76, 483-489 (IF: 1,457).

B. Seznam konferenčních příspěvků

Přednášky:

Comparison of enantioseparation potential of chiral stationary phases based on immobilized polysaccharides in reversed phase mode

Radim Geryk, Denisa Plecitá, Květa Kalíková, Eva Tesařová

9th International Students Conference "Modern Analytical Chemistry", Praha, Česká Republika, 2013

Comparison of chiral stationary phases based on immobilized polysaccharides in reversed phase mode

Radim Geryk, Denisa Plecitá, Květa Kalíková, Eva Tesařová

CECE 2013, Brno, Česká Republika, 2013

Experience with saccharide-based chiral stationary phases for separation of enantiomers

Květa Kalíková, **Radim Geryk**, Jiří Vozka, Gabriela Kučerová, Eva Tesařová

20th International Symposium on Separation Sciences, Praha, Česká republika, 2014

How to influence (enantio)separation in HPLC? Stationary phase *versus* mobile phase

Radim Geryk, Gabriela Kučerová, Květa Kalíková, Eva Tesařová

Česká chromatografická škola, Rožnov pod Radhoštěm, Česká Republika, 2015

HPLC *versus* CE competition: Chiral separation of tryptophan derivatives

Radim Geryk, Martina Riesová, Květa Kalíková, Markéta Voborná, Monika Martínková, Adéla Bydžovská, Eva Tesařová

15th International Symposium and Summer School on Bioanalysis, Targu Mures, Rumunsko, 2015

Plakátová sdělení:

Chiral stationary phases based on derivatized cyclofructan for chiral HPLC separation

Květa Kalíková, Lucie Janečková, **Radim Geryk**, Daniel W. Armstrong, Eva Tesařová

37th International Symposium on HPLC Separations & Related Techniques,, Budapešť, Maďarsko, 2011

HPLC method for chiral separation of enantiomers of citalopram and its precursor citadiol

Radim Geryk, Jiří Vozka, Květa Kalíková, Eva Tesařová

12th International Symposium and Summer School on Bioanalysis, Cluj-Napoca, Rumunsko, 2012

Optimization and validation of HPLC method for chiral separation of citalopram and its precursor citadiol

Radim Geryk, Jiří Vozka, Květa Kalíková, Eva Tesařová

29th International Symposium on Chromatography, Toruň, Polsko, 2012

Enantioseparation of profens on immobilized polysaccharide-based columns in reverse phase separation systems

Radim Geryk, Denisa Plecitá, Květa Kalíková, Eva Tesařová

13th International Symposium and Summer School on Bioanalysis, Debrecen, Maďarsko, 2013

Enantioselective separation of profens using immobilized polysaccharide-based columns in reverse phase separation systems

Radim Geryk, Denisa Plecitá, Květa Kalíková, Eva Tesařová

39th International Symposium on HPLC Separations & Related Techniques, Amsterdam, Nizozemsko, 2013

Comparison of enantioselective separation of profens on immobilized polysaccharide-based columns in reverse phase separation systems

Radim Geryk, Denisa Plecitá, Květa Kalíková, Eva Tesařová

Česká chromatografická škola 2013, Seč, Česká Republika, 2013

Supercritical fluid chromatography: A powerful tool for the analysis of synthetic cannabinoids and their metabolites
Radim Geryk, Martin Švidrnoch, Adam Přibylka, Ivo Válka, Peter Ondra, Vítězslav Maier
52nd Annual Meeting of The International Association of Forensic Toxicologists, Buenos Aires, Argentina, 2014

Comparison of enantioselective potential of chiral stationary phases based on immobilized polysaccharides in reversed phase mode
Radim Geryk, Denisa Plecitá, Jiří Vozka, Květa Kalíková, Eva Tesařová
Chiranal 2014, Olomouc, Česká Republika, 2014

Comparison of enantioselective potential of amylose-based chiral stationary phases in reversed-phase mode
Květa Kalíková, Jiří Vozka, **Radim Geryk**, Eva Tesařová
41st International Symposium on HPLC Separations & Related Techniques, New Orleans, USA, 2014

The comparison of normal and reversed phase modes for enantioseparation on immobilized polysaccharides-based columns in HPLC
Radim Geryk, Květa Kalíková, Jiří Vozka, Eva Tesařová
30th International Symposium on Chromatography, Salzburg, Rakousko, 2014

Normal *versus* reversed phase modes for enantioseparation on immobilized polysaccharides-based columns in HPLC
Radim Geryk, Květa Kalíková, Jiří Vozka, Eva Tesařová
20th International Symposium on Separation Sciences, Praha, Česká republika, 2014

Comparison of enantioselective potential of polysaccharide-based chiral stationary phases in normal and reversed phase modes
Radim Geryk, Květa Kalíková, Jiří Vozka, Eva Tesařová
14th International Symposium and Summer School on Bioanalysis, Smolenice,

Slovensko, 2014

Supercritical fluid chromatography method for the screening analysis of synthetic cannabinoids and their metabolites

Radim Geryk, Martin Švidrnoch, Adam Přibylka, Květa Kalíková, Vítězslav Maier, Eva Tesařová

42th International Symposium on HPLC Separations & Related Techniques, Ženeva, Švýcarsko, 2015

SFC method for the analysis of synthetic cannabinoids and their metabolites

Radim Geryk, Martin Švidrnoch, Adam Přibylka, Květa Kalíková, Vítězslav Maier, Eva Tesařová

CECE 2015, Brno, Česká Republika, 2015

Enantioseparation of basic biological active compounds in supercritical fluid chromatography

Radim Geryk, Květa Kalíková, Martin G. Schmid, Eva Tesařová

43th International Symposium on HPLC Separations & Related Techniques, San Francisko, USA, 2016

Supercritical fluid chromatography as a tool for enantioseparation of biologically active compounds

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28th International Symposium on Chiral Discrmination, Heidelberg, Německo, 2016