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Studium kinetiky trypsinového štěpení peptidů a chirálních separací biologicky aktivních látek metodou HPLC

Study of peptide digestion kinetics by trypsin and chiral separations of biologically active compounds by HPLC

Dizertační práce

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Prohlášení:

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V Praze dne ……..

……..

Podpis

Poděkování

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Klíčová slova

Trypsin, kinetika štěpení, aminokyseliny, chirální separace, vysokoúčinná kapalinová chromatografie

Keywords

Trypsin, digestion kinetic, amino acids, chiral separations, high performance liquid chromatography

Abstrakt

Dizertační práce se skládá ze dvou tematických částí; první část se zabývá charakterizací trypsinu, enzymu využívaného v proteomickém výzkumu k identifikaci proteinových sekvencí, a rychlostí trypsinového štěpení peptidů. Druhá část je zaměřena na chirální separace biologicky aktivních látek.

V první části projektu byla studována kinetika štěpení syntetických peptidů trypsinem a vyvinuta metoda vysokoúčinné kapalinové chromatografie (HPLC) pro detekci těchto peptidů a jejich fragmentů. Za použití enzymové reakce v roztoku a metody HPLC byly stanoveny relativní rychlostní konstanty štěpné reakce problematických sekvenčních motivů. Bylo objasněno, které aminokyseliny v okolí štěpného místa zpomalují enzymovou reakci a v jaké poloze vůči štěpnému místu je jejich vliv největší. Dále bylo zjištěno, že trypsin je schopen nízké exopeptidázové aktivity, především při štěpení na konci peptidového řetězce. Dále byly zjištěny a porovnány účinnosti tří chromatografických kolon s imobilizovaným trypsinem. Imobilizace na pevný nosič se využívá pro zvýšení množství enzymu účastnícího se katalytické reakce a pro zajištění lepší opakovatelnosti a reprodukovatelnosti výsledků. Účinnost kolony syntetizované na University of North Carolina at Chapel Hill byla porovnána s účinností dvou komerčně dostupných kolon. Jako substrát byl použit N_{α}-benzoyl-L-arginin 4-nitroanilid hydrochlorid a testovací podmínky zahrnovaly použití separačních pufrů o různém pH, různých průtoků mobilních fází a teplot. Za některých podmínek, především při pH 9,0, vykazovala nově syntetizovaná kolona vyšší účinnost než kolony komerční.

Ve druhé části práce byly vypracovány a optimalizovány metody HPLC a kapilární elektroforézy (CE) pro separaci enantiomerů tryptofanu a jeho derivátů používaných pro různé účely např. ve farmaceutickém průmyslu. V případě CE byl jako chirální selektor použit cyklodextrin (CD) a jeho deriváty. Vývoj HPLC metody vyžadoval použití více různých chirálních stacionárních fází a separačních módů. Byly testovány stacionární fáze na bázi CD, cyklofruktanu (CF), teikoplaninu i polysacharidů. Bylo dosaženo separace všech analytů na základní linii. Na základě získaných výsledků byl diskutován separační mechanismus použitých kolon. Dizertační práce zahrnuje také rozsáhlé rešerše týkající se enantioseparací léčiv, aminokyselin a dalších chirálních látek metodami HPLC, superkritickou fluidní chromatografií (SFC), plynovou chromatografií (GC) a elektrokinetickou chromatografií.

Abstract

This dissertation thesis composes of two parts; the first part focus on the characterization of trypsin, enzyme frequently used in proteomic research for the investigation and identification of protein sequences, and its peptide digestion kinetics. The second part is aimed to the enantioseparations of biologically active compounds.

First part of this project focus on tryptic digestion of synthetic peptides and the development of HPLC method for the identification of synthetic peptides and their fragments. Using the in-solution digestion and HPLC method, relative kinetic constants were determined for problematic sequences. Amino acids responsible for the decrease in trypsin catalytic activity and their location towards the cleavage site were studied. Certain slight exopeptidase activity of trypsin was noted, especially at the end of peptide chain. Furthermore, three columns with immobilized trypsin used in HPLC were compared concerning their catalytic activity. The immobilization of enzymes on solid support is used to elevate the amount of enzyme present during digestion and to assure better repeatability and reproducibility of obtained results. Activity of a new trypsin column synthesized at the University of North Carolina at Chapel Hill was compared to two commercially available trypsin columns. N_{α}-benzoyl-L-arginine 4-nitroanilide hydrochloride was used as a substrate and the separation conditions tested included the use of various buffer pH, flow rates and temperatures. The results showed that the newly synthetized trypsin column showed much higher activity than the commercial ones, especially at pH 9.0 measurements.

The second part deals with HPLC and CE chiral separation methods for the determination of tryptophan and its unnatural derivatives. These analytes can be used for instance in pharmaceutical industry for various purposes. In the case of CE measurements, native cyclodextrin and its derivatives were used as chiral selectors. The optimization of HPLC method required application of various chiral stationery phases. Stationary phases based on derivatized cyclodextrins, cyclofructans, teicoplanin and polysaccharides were tested. All the analytes were baseline separated. Based on the obtained results separation mechanisms were discussed. This part also includes a vast research on the enantioseparation of pharmaceuticals, amino acids and other chiral compounds by HPLC, SFC, GC and EKC separation methods.

Obsah

Seznam použitých zkratek

ACN – acetonitril

BEH – ethylene bridged hybrid silica

CD - cyklodextrin

CE – kapilární elektroforéza

CF - cyklofruktan

CSP – chirální stacionární fáze

GC – plynová chromatografie

HILIC – hydrofilní interakční kapalinová chromatografie

HPLC – vysokoúčinná kapalinová chromatografie

MeOH – methanol

MP – mobilní fáze

RP – reverzní mód

SFC – superkritická fluidní chromatografie

UNC – University of North Carolina

UPLC – ultraúčinná kapalinová chromatografie

1. Úvod

Biologicky aktivní látky lze definovat jako sloučeniny, které jsou i v nízkých koncentracích schopny ovlivňovat životní pochody, jak pozitivním tak i negativním způsobem. Mezi biologicky aktivní látky řadíme např. makromolekuly typu proteinů, léčiva, vitamíny a aminokyseliny, látky přírodního i syntetického původu. Velké množství těchto látek je chirální - vyskytují se v odlišných prostorových uspořádáních, která navzdory stejnému sumárnímu vzorci mohou vykazovat různou biologickou aktivitu [1].

Základní složkou proteinů jsou L-enantiomery aminokyselin, ale vlivem vysokých teplot, stárnutím nebo činností mikroorganizmů postupně dochází k přeměně na jejich D-analogy. Ty však mohou zasahovat do metabolismu L-forem a způsobit toxickou reakci, v případě zabudování do proteinu mohou způsobit i ztrátu biologické funkce [2]. D-aminokyseliny lze nalézt např. v buněčných stěnách mnoha bakterií [3], odkud se mohou uvolňovat do okolí [4]. U vyspělejších biologických systémů, např. u člověka, může výskyt D-aminokyselin doprovázet rozvoj některých onemocnění, např. *Diabetes mellitus* [5] nebo Alzheimerovu chorobu [6]. V tělech vyspělejších organismů se přirozeně vytváří kyselina D-asparagová, především metabolickými pochody souvisejícími se stárnutím [2]. Z důvodu diagnostiky, prevence a boje proti různým chorobám je potřeba vývoje spolehlivých a efektivních metod pro detekci a identifikaci proteinů a jejich stavebních jednotek.

Identifikace proteinů a jejich aminokyselinového složení probíhá nejčastěji za využití specifických proteolytických enzymů [7]. Nejpoužívanějším enzymem ke štěpení proteinů za účelem jejich analýzy je pro vysokou specifitu trypsin [7,8], a to především k přípravě vzorků pro tvorbu peptidových map, stanovení primární struktury proteinů a post-translačních modifikací [9]. Ačkoli jsou optimální podmínky pro trypsinovou hydrolýzu detailně popsány [10], stanovení rychlosti štěpení tzv. problematických sekvencí bylo dosud opomíjeno. Trypsin štěpí aminokyselinové řetězce pouze na karboxylovém konci argininu a lysinu [11], ale aminokyseliny obklopující štěpné místo mohou tuto reakci významně zpomalovat. Nejčastěji jsou nekompletně naštěpené sekvence pozorovány v blízkosti kyselých aminokyselin (kyseliny asparagová a glutamová) nebo v případě, že je v sekvenci přítomno více štěpných míst jdoucích za sebou [12]. Znalost rychlostních konstant hydrolýzy těchto sekvencí by po zabudování do programů pro identifikaci peptidů mohla vést ke snížení množství falešně pozitivních výsledků a k podstatnému

urychlení vyhodnocování získaných dat. Enzymové štěpení lze provést dvěma způsoby. Konvenčně se používá reakce v roztoku, ale ta je zatížena mnoha nevýhodami, především autolýzou. Výrazné vylepšení postupu představuje imobilizace enzymu na pevný nosič, která umožňuje použití podstatně vyššího množství enzymu a eliminuje nevýhody reakce v roztoku [13,14]. Použití komerčních chromatografických kolon s imobilizovaným trypsinem by mělo vést k větší opakovatelnosti a spolehlivosti výsledků.

Chirální separace biologicky aktivních látek mají v dnešní době obrovský význam, protože mnoho léčiv, chemikálií a látek používaných v potravinářském průmyslu a zemědělství vykazuje chiralitu. Stále větší důraz je kladen na vypracování toxikologických a environmentálních analýz vlivu jednotlivých enantiomerů, se kterými je úzce spjata potřeba rychlých, spolehlivých a citlivých metod. Uplatnění v této problematice nalezla především kapalinová chromatografie, kterou lze použít pro různě polární analyty pocházející z různých typů matric [15]. Velké množství dostupných chirálních stacionárních fází ještě více upřednostňuje použití této techniky [16]. Příkladem biologicky aktivních látek mohou být neproteinogenní deriváty tryptofanu, používané např. ve farmacii k vývoji nových léčiv, jako biologické sondy nebo katalyzátory [17,18,19]. Dál lze zmínit nepřírodní deriváty prolinu, používané v peptidomimetice ke zlepšení biologické dostupnosti léčiv a odolnosti vůči enzymům [20], nebo β-aryl-substituované β-aminokyseliny, které působí jako inhibitory enzymů, mají neurologickou aktivitu a používají se při vývoji nových léčiv [21].

2. Cíle práce

Cílem dizertační práce bylo studium peptidů a aminokyselin vysokoúčinnou kapalinovou chromatografií.

Dílčí cíle lze rozdělit do následujících bodů:

- Studium rychlostních konstant štěpení problematických peptidových sekvencí trypsinem.
- Porovnání účinnosti komerčně dostupných kolon s imobilizovaným trypsinem vůči nově syntetizované koloně v laboratoři.
- Vývoj vhodných metod pro chirální separace tryptofanu a jeho neproteinogenních derivátů pro screeningové a purifikační účely.

3. Teoretický úvod do analýzy biologicky aktivních látek

3.1. Trypsinové štěpení aminokyselinové sekvence proteinů

Proteiny společně se sacharidy a lipidy patří mezi makromolekuly nezbytné k existenci života na Zemi. Základem proteinového řetězce jsou aminokyseliny, jejichž pořadí určuje nejen vyšší strukturu proteinů, ale také jejich funkci. V proteomickém výzkumu je protein izolován, denaturován a následně rozložen na krátké peptidové fragmenty, které jsou identifikovány nejčastěji hmotnostní detekcí. Štěpení je obvykle prováděno enzymatickou katalýzou. Aby mohlo dojít k hydrolytické reakci, musí být štěpné místo a okolní sekvence aminokyselin kompatibilní s aktivním centrem enzymu. Navázáním peptidu na enzym dochází ke změně konformace aktivního centra, kterou začíná samotná reakce. Některé aminokyseliny v bezprostředním okolí štěpného místa mohou zabránit navázání na enzym nebo mohou ovlivnit změny konformace na aktivním centru enzymu tak, že pro průběh reakce nebude výhodná a reakce bude probíhat pomalu [22]. Ve výsledné směsi pak pozorujeme peptidové fragmenty s vynechanými štěpnými místy (z angl. miscleavage), i když je reakční doba dostatečně dlouhá.

Trypsin je pro vysokou specifitu nejpoužívanějším enzymem pro štěpení proteinů [7,8]. Optimální podmínky pro trypsinové štěpení jsou udávány následovně: pH v rozmezí 7-9 jednotek (v závislosti na zdroji) [23,24] a teplota 37°C. Snížením pH na hodnoty 2-4 dojde k inaktivaci enzymu, aktivita se navrátí prakticky ihned po změně pH na optimální hodnotu, ale při zvýšení teploty nad 37°C enzym postupně denaturuje až do úplné ztráty aktivity. Specifita štěpení trypsinem spočívá ve schopnosti štěpit proteiny pouze na karboxylovém konci lysinu a argininu, pokud po nich nenásleduje prolin [9]. Mezi faktory, které negativně ovlivňují rychlost enzymové reakce, se řadí pH, teplota, přítomnost organických rozpouštědel, autolýza, vyšší struktury štěpeného proteinu a samotná sekvence aminokyselin. Kyselé aminokyseliny nacházející se v bezprostřední blízkosti štěpného místa snižují rychlost enzymové reakce. Jejich vzdálenost od štěpného místa je zásadní a má různý dopad na rychlost reakce [12,11]. Aminokyselinová sekvence obklopující štěpné místo se obvykle popisuje jako P4-P3-P2-P1-||-P1´-P2´-P3´-P4´, kde mezi aminokyselinami P1 a P1´ dochází ke štěpné reakci [25]. Při analýzách „miscleavage“ sekvencí se v okolí vynechaného štěpného místa nejčastěji objevují negativně nabité kyseliny asparagová a glutamová, zejména v pozicích P3, P4, P1’ a P2‘. Tyto dvě aminokyseliny vytváří solné můstky s bazickým argininem a lyzinem a zabraňují

tak jejich navázání na kyselinu asparagovou v aktivním centru trypsinu, čímž dochází k bránění interakce s enzymem [12]. „Miscleavage“ sekvence se objevují také v případech, kdy je v aminokyselinovém řetězci umístěno několik štěpných míst za sebou. Bylo popsáno, že trypsin není schopen pracovat jako exopeptidáza, tzn. nedokáže štěpit na úplném začátku nebo konci peptidového řetězce [26]. V sekvenci je obvykle hydrolyzováno pouze jedno štěpné místo, ostatní už enzymové reakci nepodléhají. Negativní vliv na štěpení peptidové vazby byl publikován také pro další aminokyseliny, histidin, tyrosin, tryptofan a fenylalanin [11], vyskytující se v blízkosti štěpného místa.

S výzkumem struktury a funkce proteinů je úzce spjata potřeba rychlých, nenáročných a efektivních separačních metod. V proteomickém výzkumu je nejčastěji využívanou technikou metoda HPLC v reverzním módu (RP) [7], dále se uplatňují také CE a gelová chromatografie. V případě HPLC a CE metod může štěpná reakce probíhat jak odděleně tak i přímo v separačním systému. Obrovskou výhodu on-line metod představuje posílení stability enzymu vůči autolýze, zvýšení odolnosti vůči teplotě, pH nebo přítomnosti organických rozpouštědel [13,14]. Pro použití v kapalinové chromatografii se enzym imobilizuje na membrány [14,27], polymerní nebo silikagelové nosiče [8], nanočástice [28] i magnetické částice [29]. Nevýhodou imobilizace enzymů je určitá ztráta účinnosti vlivem strukturních změn doprovázejících vazbu na pevný povrch [27], která je však kompenzována stechiometricky větším množstvím enzymu účastnícího se reakce. Použitím imobilizovaného enzymu tak lze výrazně zkrátit dobu štěpné reakce. Reakce v roztoku obvykle probíhá 12-24 hodin, oproti tomu reakce na imobilizovaných enzymových reaktorech může být ukončena po několika minutách, jak udávají výrobci komerčně dostupných trypsinových kolon [30,31].

3.2. Chirální separace

Chiralita je přirozenou vlastností přírodních látek. Chirální molekuly mají stejné složení, ale liší se prostorovým uspořádáním a v chirálním prostředí vykazují různé vlastnosti. Chirální látky se vyskytují ve formě izomerů, které si jsou navzájem zrcadlovými obrazy a nazýváme je enantiomery [1,32]. Většina léčiv, chemikálií a látek používaných v potravinářském průmyslu a zemědělství vykazuje chiralitu, a proto je nutné prozkoumat chování všech jejich izomerů nejen v lidském těle, ale také v tělech ostatních organizmů a v životním prostředí. Důvodem takto rozsáhlých toxikologických studií jsou právě rozdílné vlastnosti jednotlivých enantiomerů v chirálním prostředí, které mohou být důvodem rozdílných biologických účinků. Zatímco je jeden izomer aktivní, druhý může vykazovat nižší nebo žádnou farmakologickou účinnost, případně jiné až nežádoucí účinky [32,33]. Problematika chirálních léčiv vstoupila do širokého povědomí v šedesátých letech 20. století, kdy došlo k tragickému incidentu s thalidomidem a teratogenní účinek jednoho z enantiomerů byl zjištěn příliš pozdě [34]. Tato tragédie se stala mezníkem v regulaci léčiv. Dalším příkladem rozdílných účinků enantiomerů a problematiky správného výběru testovacích zvířat ve farmakologických studiích může být omeprazol, léčivo používané jako inhibitor protonových pump při poruchách trávení. Testy na potkanech ukázaly, že *R*-enantiomer má vyšší účinnost, zatímco při testech na psech nebyl zjištěn rozdíl v účinnosti enantiomerů a farmakologický výzkum u člověka ukázal o 90% vyšší účinnost *R*-enantiomeru [35].

Účinnou metodou pro separace chirálních látek je především kapalinová chromatografie (LC). Mezi další významné metody patří superkritická fluidní chromatografie (SFC), CE a GC. [32]. Metoda HPLC je v současnosti nejpoužívanější technikou pro separaci chirálních látek vzhledem k velké robustnosti, opakovatelnosti a přenositelnosti analýz. Další výhodou této metody je možnost použití několika různých módů v závislosti na povaze separovaných látek. Podle polarity mobilní (MP) a stacionární fáze rozlišujeme tyto chromatografické módy: (i) normální mód – polární SP a nepolární MP; (ii) RP – nepolární SP a polární MP; (iii) polárně-organický mód – jako MP se používá směs acetonitrilu (ACN) s methanolem (MeOH), nebo samotný MeOH s malým přídavkem kyseliny octové nebo triethylaminu; (iv) hydrofilní-interakční kapalinová chromatografie (HILIC) - polární SP a MP složená z ACN a vodné fáze (pufru) do max. 30% objemu. [16]

Chirální separace lze v HPLC provést dvěma způsoby – přímým a nepřímým. Při přímých separacích jsou nejvíce používány systémy s chirální stacionární fází (CSP), která může být určena pro jeden nebo více módů. Využití CSP umožňuje snadnou identifikaci, popř. i izolaci enantiomerů. Dále lze použít chirální selektor jako aditivum v MP, ale tento postup není příliš využíván z důvodu nižší selektivity a účinnosti chromatografického systému a vysoké spotřebě aditiv. Při nepřímých separacích se využívá reakce enantiomerů s opticky čistým derivatizačním činidlem před vlastní analýzou. Vytvořené stabilní diastereoizomery jsou separovány v achirálním prostředí. Nevýhodu nepřímé metody představuje především nutnost vysoké optické čistoty derivatizačního činidla, možnost racemizace analytu během derivatizace a časová náročnost přípravy derivátu. Tato metoda není vhodná pro semipreparativní a preparativní účely. Hlavní nevýhodou přímých metod je vysoká cena CSP [16,32].

CSP používané v LC podléhají neustálému vývoji a zdokonalování, ať už v oblasti chirálních selektorů nebo částic, na které je selektor navázán. V současnosti jsou nejvíce využívány polysacharidové CSP na bázi derivatizované celulózy a amylózy. Dále je vhodné zmínit CSP na bázi makrocyklických antibiotik (teikoplanin, vankomycin), derivatizovaných i nativních CD a CF, syntetických polymerů nebo molekulárně vtištěných polymerů [16,36].

4. Výsledky a diskuze

Publikace I – Objasnění trypsinového štěpení: Určení a srovnání rychlostních konstant enzymatické hydrolýzy problematických sekvencí

Jak bylo zmíněno výše, jedním z negativních jevů provázejících trypsinové štěpení proteinů je produkce tzv. „miscleavage“ sekvencí. Přestože je tento enzym velice často používán v proteomickém výzkumu, nebyla dosud detailně prozkoumána kinetika štěpení problematických sekvencí. Objasnění afinity trypsinu k těmto sekvencím a určení rychlostních konstant štěpných reakcí je důležité nejen pro usnadnění a zpřesnění vyhledávání v databázích hmotnostních spekter, ale i pro vývoj nových enzymových modifikací. Zavedení rezistentních sekvencí do programů Peptide Mass Fingerprinting by významně urychlilo a zpřesnilo vyhodnocování výsledků a zmenšilo by množství falešně pozitivních identifikací.

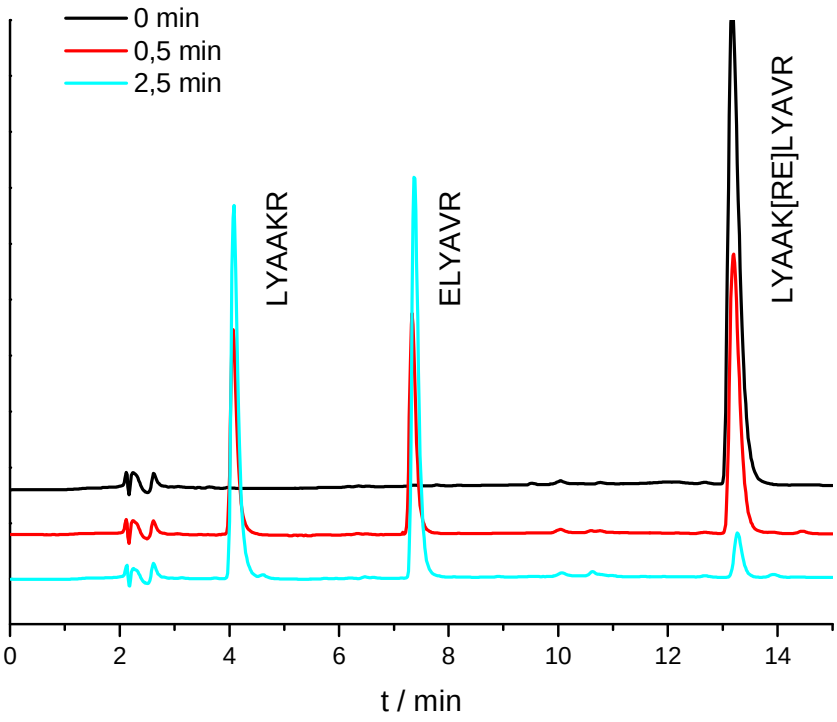
Publikace I se zabývá (i) vlivem kyselin glutamové a asparagové a (ii) několika po sobě jdoucích štěpných míst na rychlost trypsinového štěpení. Reakční podmínky byly zvoleny tak, aby bylo za pomalého průběhu reakce umožněno podrobné sledování změn v reakční směsi. Jako substrát byly použity syntetické peptidy s danou sekvencí aminokyselin bez sekundární struktury. Produkty enzymové reakce byly separovány vyvinutou RP-HPLC metodou (Obr. 1). Navržená metoda byla použita ke stanovení relativních rychlostních konstant štěpných reakcí různých sekvenčních motivů. Syntetické peptidy použité v této práci měly základní aminokyselinový řetězec LYAA[X]LYAVR[1]. Uprostřed sekvence se nacházely 1-3 aminokyseliny včetně štěpného místa. Dva tyrosiny (Y) v sekvenci umožnily detekci peptidů a jejich fragmentů při vlnové délce 280 nm, kdy je minimalizována interference nečistot a mobilní fáze.

Trypsin je vysoce specifický enzym, který štěpí pouze na karboxylovém konci aminokyselin argininu a lyzinu. Výsledky ukázaly, že trypsin preferuje štěpné místo obsahující arginin, pravděpodobně kvůli slabší vazbě lysinu do aktivního centra. Postranní řetězec argininu je delší, a proto může tvořit vodíkové vazby přímo s kyselinou asparagovou na jeho dně. Postranní řetězec lysinu vytváří vodíkové můstky se serinem a s kyselinou asparagovou je spojen nepřímo přes molekulu vody [37]. Z tohoto důvodu byly naměřené rychlostní konstanty peptidů obsahujících arginin vyšší.

[1] A – alanin, L – leucin, R - arginin, V – valin, Y – tyrozin

Dále byl sledován vliv pozice kyselých aminokyselin vůči štěpnému místu na rychlost enzymové reakce, a to na pozicích P3, P2, P2´ a P3´. Kyseliny asparagová a glutamová jsou známy svou schopností zpomalovat rychlost reakce, pokud se nacházejí v bezprostřední blízkosti štěpného místa (P1). Jejich vzdálenost vůči štěpnému místu je zásadní a má různý dopad na rychlost reakce. Bylo zjištěno, že umístění kyseliny glutamové nebo asparagové před štěpným místem zpomaluje rychlost reakce výrazněji. Sekvence obsahující tyto aminokyseliny na pozici P2 a P3 se štěpily velice obtížně, v průběhu několika hodin, zatímco obdobný peptid bez nich byl hydrolyzován během dvou minut. Podobný trend byl zaznamenán u obou štěpných míst. Jako silnější inhibitor reakce se projevila kyselina asparagová ve všech případech měření.

Sekvence obsahující několik po sobě jdoucích štěpných míst jsou označovány za nejčastěji se objevující „miscleavage“ motivy. V **Publikaci I** byly studovány sekvence se dvěma štěpnými místy, a to dvěma argininy, lysiny, nebo párem arginin-lysin a lysin-arginin. Tyto sekvence ochotně podléhají hydrolýze a trypsin si opět přednostně vybírá arginin jako štěpné místo. Hydrolyzováno je však pouze jedno ze dvou štěpných míst, což vede k produkci „miscleavage“ peptidů. B. Keil popsal, že trypsin není schopen pracovat jako exopeptidáza – neumí štěpit na úplném začátku nebo konci peptidového řetězce [26]. Toto tvrzení bylo částečně vyvráceno. Po 2 minutách enzymové reakce byl veškerý původní peptid naštěpen, ale stále docházelo ke změnám ploch píků vzniklých fragmentů. Bylo zjištěno, že trypsin je pravděpodobně schopen exopeptidázové aktivity, a to především pokud je štěpné místo na konci peptidového řetězce. Rychlost reakcí je však podstatně nižší než u původního peptidu.



Obr. 1 Ilustrativní chromatogramy separace štěpeného peptidu a jeho fragmentů v závislosti na době enzymové reakce. Peptid s aminokyselinovou sekvencí LYAA[RE]LYAVR, která obsahovala jako štěpné místo arginin (R) následované kyselinou glutamovou (E), byl trypsinem hydrolyzován na dva fragmenty. Separační podmínky: kolona Atlantis dC18; MP ACN/10 mM octan amonný, pH 3,0; lineární gradient MP 10-18% ACN (*v/v*) v čase 0-6 min, 6-20 min 18% ACN; 280 nm; teplota kolony 30°C; teplota autosampleru 20°C; dávkovaný objem 6 µl.

Publikace I

Insight into Trypsin Miscleavage: Comparison of Kinetic Constants of Problematic Peptide Sequences

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Insight into Trypsin Miscleavage: Comparison of Kinetic Constants of Problematic Peptide Sequences

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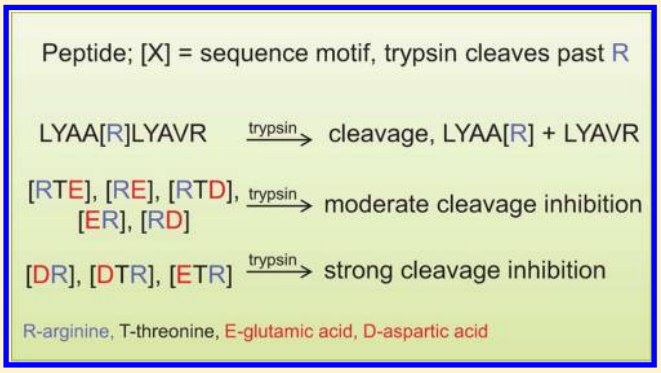
Supporting Information

ABSTRACT: Trypsin, a high fidelity protease, is the most widely used enzyme for protein digestion in proteomic research. Optimal digestion conditions are well-known and so are the expected cleavage products. However, missed cleavage sites are frequently observed when acidic amino acids, aspartic and glutamic acids, are present near the cleavage site. Also, the sequence motifs with successive lysine and/or arginine residues represent a source of missed cleaved sites. In spite of an adverse role of missed cleaved peptides on proteomic research, the digestion kinetics of these problematic sequences is not well-known. In this work, synthetic peptides with various sequence motifs were used as trypsin substrates. Cleavage products were analyzed with reversed-phase high performance liquid chromatography, and the kinetic constants for selected missed cleavage sites were calculated. Relative digestion speed for lysine and arginine sites is compared, including the digestion motifs flanked with aspartic and glutamic acid. Our findings show that DK and DTR motifs are cleaved by trypsin with 3 orders of magnitude lower speed than the arginine site. These motifs are likely to produce missed cleavage peptides in protein tryptic digests even at prolonged digestion times.



In the proteomic research, enzymatic digestion is one of the key steps in the identification of proteins and their posttranslational modifications. Proteins are usually identified using proteolytic enzymes, which are valued for their specificity.[1] In the forties of the last century, Kaj Linderstrøm-Lang[2] studied the interactions of proteolytic enzymes with proteins. He concluded that the peptide fragments should be formed until all cleavage sites are digested. The resulting peptide set is then referred to as "limit peptides". Such a reaction course would be ideal, but in practice, the enzymes often fail to cleave all scissile bonds, even though the reaction time is sufficiently long. This phenomenon may be in part caused by secondary and tertiary structure of proteins, when certain cleavable sites are sterically inaccessible for the enzyme. In addition, certain amino acid motifs are not cleavable or are cleaved with slow kinetics, despite the fact that they are freely accessible to the enzyme.

According to convention,[3] the amino acids surrounding the cleavage site are labeled as P4−P3−P2−P1−‖−P1′−P2′−P3′−P4′, where the position between the amino acids P1 and P1′ is the scissile bond. Cleavage site and the surrounding amino acid sequence must be compatible with the analogously arranged enzyme's active center to allow reaction to proceed. If any amino acid interferes with steric or electrostatic arrangement of the active center, the reaction will be significantly slower or will not proceed at all.[4]

Trypsin is the most widely used enzyme for protein digestion in proteomic research.[1] It produces relatively short peptide chains (10−12 amino acids long in average) bearing typically two positive charges, a useful feature for MS detection.[5] Trypsin is a highly specific protease; it cleaves peptide bonds at the C-terminal side of lysine (K) or arginine (R) residues, except when followed by proline (P).[6] Speed of proteolytic digestion is affected by well-known factors such as pH, temperature, presence of organic solvents, and autolysis[6,7] along with protein structure.[6−8]

An important, but often neglected, factor is the origin of trypsin. The authors of a recent study[9] compared bovine and porcine trypsins, which represent a majority of commercially available trypsin enzymes. They reported that bovine trypsin produced a higher number of peptides containing missed cleavages, whereas porcine trypsin produced more semitryptic peptides. In addition, cleavage sites showed variable digestion kinetics patterns, which could be related to differences in protein affinity into the substrate binding pockets.

The specificity of proteases including trypsin have been widely investigated.[10] Unfortunately, data available in the MEROPS database lack details about the effect of neighboring

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amino acids to the cleavage speed.[11] It is well-known that trypsin cleaves certain motifs with relatively slow kinetics; these incompletely cleaved peptides are termed missed cleavage peptides. Such digestion products are created when adjacent lysine and/or arginine are present in the sequence or in the vicinity of the scissile site up to the P4 residue.[6,7,12,13] The presence of glutamate or aspartate acidic residues (E or D) near the cleavage site also reduces the proteolysis speed.[6,13−15] The distance toward the cleavage site has an impact on the digestion rate; negative influence of glutamic and aspartic acids on digestion efficiency has been reported from position P4 to P3′.[6,13] These amino acids form salt bridges with basic arginine and lysine, competing with the complementary aspartic acid at the bottom of the trypsin active site.[6] Similar inhibition was reported for scissile sites flanked by phosphorylated serine and threonine residues, which also carry a negative charge.[16,17]

Yen and co-workers[7] suggested that the arginine digestion site is preferred over lysine. This can be caused by a different strength of lysine and arginine bonds with the enzyme. Binding of arginine is stronger due to its longer side chain, which is able to reach the bottom of the active center and form hydrogen bonds directly with aspartic acid. On the other hand, lysine forms hydrogen bonds with serine and it is indirectly attached to aspartic acid through a water molecule, which results in a less convenient position of lysine and its scissile bond.[18] According to this finding, different rules for digestion at lysine and arginine residues have been observed.[7] Therefore, it can be predicted that the kinetic parameters of enzymatic reactions will be affected differently by the presence of acidic amino acids. In-depth characterization of trypsin's affinity to problematic sequences and determination of their kinetic constants could lead to improved peptide detection protocols, signature peptide quantitation,[14] and development of new enzymatic modifications.

Recent studies investigated improved digestion strategies, such as trypsin immobilization on different supports.[19] Nevertheless, in-solution and in-gel digestion using a commercially available trypsin remains the primary method for sample processing in proteomics.[9,19]

The digestion speed of proteins strongly depends on their nature,[20] such as the presence of disulfide bridges, specific folds, solubility, glycosylation, and combinations of all the above features, which can make the cleavage motifs inaccessible to the enzyme. As far as we know, the only authors, who studied the digestion kinetic of trypsin in detail, were Brownridge and Beynon.[14] They compared the proteolysis of certain peptide sequences using standard and native proteins. They observed a decrease in kinetic constant values caused by acidic amino acid residues present in the vicinity of the cleavage site.

Two types of studies of tryptic substrate specificity have been described in the literature. The first approach utilizes combinatorial synthesis of libraries of labeled peptide substrates. This library is subjected to enzymatic reaction, the extent of which is monitored in parallel with plate readers employing colorimetric or fluorometric assay.[15,21,22] Various strategies for synthesis of combinatorial substrates have been recently reviewed by Poreba and Drag.[23] The second popular approach utilizes LC/MS proteomic methods; the enzyme specificity is inferred from the peptides generated by global proteome digest. These products of digestion are sequenced and identified by mass spectrometry.[5,6,13,14,24] The hybrid approach of synthetic peptide libraries cleaved by an enzyme and investigated by MS methods has also been utilized.[22,23]

The advantage of such strategies is that the complete, or nearly complete, matrix of possible neighboring amino acids can be investigated. However, for investigation of eight amino acid long motifs consisting of XXXB−||−XXXX, where X is any of the 20 amino acids and B is R or K, a cleavage site would require $2 \times 20^7 = 2560$ million experiments. In reality, less complex studies are typically carried out.[23]

Although the labeled peptide libraries permit quantitative measurement of the nearest neighbor effect on the digestion speed, the combinatorial studies have traditionally been aimed toward a simpler goal: elucidation of the enzyme primary specificity. Little quantitative data have been collected on relative tryptic digestion speed of various amino acid motifs.[23] The studies utilizing the LC/MS proteomic approach and global digest suffer from a different drawback: only qualitative or relative estimates of tryptic digestion speed can be inferred from the existing data.[6,7,24]

The trypsin digestion fidelity is of major importance in LC/MS proteomic research. Highly complex mixtures of peptides generated in global proteome digest present a challenge even for the high resolution LC/MS instruments.[25−27] Because the protein/peptide identification is performed via a theoretical database search, a complication of an already daunting problem by including missed cleaved, partially tryptic, and nontryptic peptides is undesirable.[9,28,29] Relaxing the search criteria to accommodate such peptides may result in a greater number of identifications, but the probability of false positive identification steeply increases.[30−32]

The negative effect of the aspartate and glutamate residues in proximity of scissile site on digestion speed was discussed in several articles.[6,7,13,14,16,33] The relative digestion speed of such sequence motifs is not known, but they are a potential source of missed cleavage peptides. Using longer digestion time or greater concentration of trypsin will drive the reaction to completion; however, it may also produce undesirable semi-tryptic peptides due to potential chymotryptic activity present with the trypsin.[5,9,29]

The proteomic LC/MS experiment could benefit from better understanding of tryptic cleavage kinetics. Search and scoring algorithms can be adjusted such that the most probable missed cleavage motifs are not penalized.[24] The knowledge of poorly cleavable primary sequence motifs may enable more rational design of proteomic experiments.

The main motivation behind our work was to gain a better understanding of tryptic digestion kinetics. For this purpose, porcine trypsin and short synthetic peptides free of the secondary structure were used. We selected a limited set of peptide substrates with primary sequence motifs expected to produce missed cleavages.[6,13,14,16] The sequence motifs contained acidic amino acid residues in the vicinity of the cleavage site or two successive lysine and/or arginine residues.

EXPERIMENTAL SECTION

Chemicals and Reagents. Porcine trypsin T0303, N_α-benzoyl-L-arginine 4-nitroanilide hydrochloride (L-Bapna, purity ≥99%), acetonitrile (ACN, gradient grade), formic acid (reagent grade ≥95%), acetic acid (purity ≥99.8%), trifluoroacetic acid (TFA, purity 99%), and ammonium acetate (purity ≥99%) were supplied by Sigma-Aldrich (St. Louis, MO, USA). Sodium bicarbonate (purity p.a. ≥ 99.7%) was supplied by Fluka (Steinheim, Germany). The synthetic peptide samples (purity ≥80%) of general sequence LYAA[X]LYAVR, where the [X] represents 1−3 amino acids including the digestion site,

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were manufactured by GenScript USA Inc. (Piscataway, NJ, USA). The deionized water was purified with a Rowapur and Ultrapur system from Watrex (Prague, Czech Republic). The 10 mM ammonium acetate solution was prepared by dissolving an appropriate amount of ammonium acetate in deionized water and adjusted with acetic acid to reach pH 3.0.

Instrumentation and Methods. For the peptide purification procedure, the Agilent 1220 Infinity Gradient HPLC System with a G4290C series autosampler, column oven, and integrated variable wavelength detector, all controlled by OpenLAB CDS ChemStation software from Agilent Technologies (Santa Clara, CA, USA), was used. The analytical column was Atlantis dC18, 150 × 3.0 mm, 5 μm (Waters, Milford, MA, USA).

The HPLC method used for kinetic constants determination utilized Waters Alliance System with Waters 2690 Separation Module, Waters 2487 UV−vis 2-channel detector, an 717 Plus autosampler, and Waters Alliance Series column heater. The instrument control and data collection were performed with Empower software. Peptide analysis was carried out using Atlantis dC18 column (Waters, Milford, MA, USA), 150 × 3.0 mm, 3 μm, equipped with a 0.45 μm precolumn filter.

Peptide Purification. Mobile phase consisted of ACN and 0.02% TFA. The linear gradient rose from 10% to 18% ACN in 6 min and was held for 15 min at 18% ACN. The column was re-equilibrated for 30 min. The flow rate was 0.4 mL/min, column oven temperature was 30 °C, injection volume was 100 μL, and the detection wavelength was 280 nm. Peptide samples (15−19 mg) were diluted in 0.4 mL of 50% TFA and 0.1 mL of ACN. After scouting runs, 100 μL aliquots were injected on the HPLC system. Main peak hearth-cutting and fraction collection were performed manually. Collected solutions were dried using a water bath at 25 °C.

Repeatability Test. To evaluate the repeatability of the LC method and kinetic constant measurement, L-Bapna was used as a substrate. L-Bapna and trypsin stock solutions were diluted in 50 mM $NaHCO_3$ to a 1 mg/mL concentration. Trypsin solution was prepared fresh for every reaction and stored for 3 min on ice. L-Bapna was thermostated at 22 °C before trypsin addition. Trypsin to substrate mass ratio was 1:100. Reaction was stopped by addition of 35 μL of concentrated formic acid to 35 μL of digestion solution. Repeatability was determined on two substrate concentration levels, 0.5 and 1 mg/mL. Kinetic constants were determined 10 times for both concentration levels. For every measurement, 25 fractions were taken and analyzed three times by HPLC. The standard deviance was 5% for both concentration levels. The LC method for monitoring the L-Bapna digestion was performed using Atlantis dC18 column, 150 × 3.0 mm, 3 μm. Mobile phase consisted of 40/60 (v/v) deionized water and ACN; flow rate was 0.4 mL/min, column temperature was 30 °C, injection volume was 4 μL, and the detection wavelength was 254 nm.

Kinetic Constants Determination. Peptide samples (0.5 mg) were dissolved in 50 μL of 0.1% TFA and sonicated for 15 min, and then, 950 μL of 50 mM $NaHCO_3$ was added. Trypsin (1 mg/mL) was dissolved in 50 mM $NaHCO_3$; the solution was prepared fresh for each reaction and rested for 3 min on ice. Peptide solutions were thermostated for 15 min at 22 °C prior to addition of trypsin. On the basis of preliminary testing, trypsin to substrate ratio 1:100 was chosen to provide a suitable reaction time for comparison of digestion kinetics of different sequence motifs. Reaction was stopped by addition of 35 μL of concentrated formic acid to 35 μL of digestion solution. The kinetic constants for each peptide were measured five times. For each kinetic measurement, 25 time points were used for reaction progress monitoring. Aliquots were taken at selected time intervals and analyzed three times by gradient HPLC. Peptide analysis was performed using an Atlantis dC18, 150 × 3.0 mm, 3 μm column. Mobile phase consisted of 10 mM ammonium acetate solution, pH 3.0, and acetonitrile. Linear gradient rose from 10% to 18% ACN in 6 min, followed by a hold for 10 min at 18% ACN. Column re-equilibration was performed for 25 min. The flow rate was 0.4 mL/min, column oven temperature was 30 °C, and injection volume was 6 μL. Detection wavelength was 280 nm, because peptides contained tyrosine (Y) residues. This detection wavelength produced a chromatogram with flat baseline; few impurities were detected.

Data Analysis. The digestion speed of peptide substrates was calculated from the decrease of original peptide peak area (%) with time. Figure 1 shows that LYAA[DK]LYAVR peptide

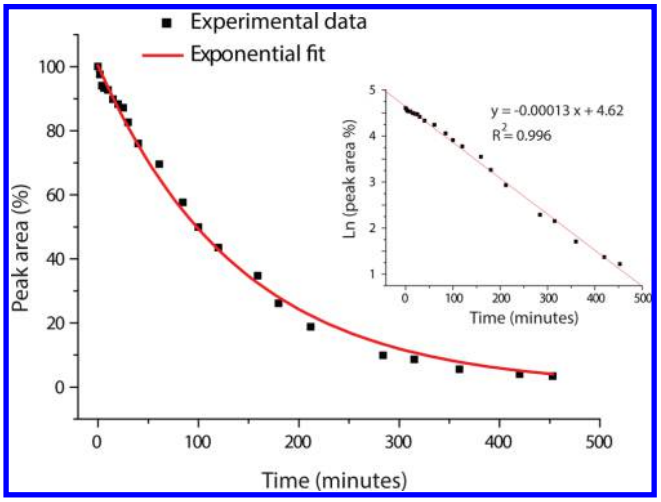

Figure 1. Data analysis of tryptic digestion of peptide LYAA[DK]-LYAVR. Raw data followed an exponential decay function; the first order kinetic constant was obtained from the linear fit of natural logarithm of peak area versus the digestion time.

area (%) decreases exponentially during digestion, suggesting the first order kinetics. The dependency of the natural logarithm of peak area (%) on time was fitted linearly; the absolute value of the slope represents the kinetic constant value. This approach was used for evaluation of kinetic constants of all studied sequence motifs. For long time in-solution digestions, it is possible that trypsin is partially inactivated due to autolysis. However, no trypsin inactivation was apparent in our experiments up to 8 h.

RESULTS AND DISCUSSION

Kinetic Constants Repeatability Measurement. To evaluate the repeatability of the whole procedure of kinetic constant measurement (pipetting error, LC repeatability, curve fitting), L-Bapna was used as a substrate. Repeatability for ten experiments (n = 10) determined on two concentration levels (0.5 and 1 mg/mL) was less than 5% relative standard deviation, which is sufficient for our purposes. The repeatability of peptide digestion kinetic constants measurement, shown in Table 1, based on three-to-five repetitions (n = 3−5) is usually less than 10% with a few exceptions exceeding 13%. We suspect that this added variability is caused by weighting error when preparing working solutions of peptides and trypsin and

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Table 1. Sequence Motifs and Related Kinetic Constants Obtained for Studied Peptides[a]

digestion motif	kinetic constant [$\times 10^{-3} s^{-1}$]	RSD[b] [%]	sequence motif	kinetic constant [$\times 10^{-3} s^{-1}$]	RSD[b] [%]
[R]	41.49	3.6	[K]	13.40	5.3
[RTE]	18.73	1.7	[KTE]	8.54	5.9
[RE]	18.48	2.8	[KE]	8.89	6.2
[RTD]	18.41	5.1	[KTD]	12.53	8.6
[ER]	15.29	8.0	[EK]	3.20	7.8
[RD]	13.87	9.6	[KD]	2.24	6.1
[ETR]	1.87	1.3	[ETK]	4.65	13.8
[DR]	1.27	13.0	[DK]	0.13	3.0
[DTR]	0.24	7.0	[DTK]	3.24	3.7
[RR]	30.27	4.9	[KK]	10.84	5.8
[KR]	20.15	11.5	[RK]	21.67	1.4
[RR*][d]	15.83	4.9	[KK*][d]	3.43	5.8
[R*R][d]	14.44	4.9	[K*K][d]	7.41	5.8
[KR*][d]	14.70	11.5	[K*R][d]	5.45	1.4
[R*K][d]	17.67	1.4	[RK*][d]	3.99	11.5
LYAAR*R[d]	2 41	–[c]	LYAAK*K[d]	0.94	–[c]
R*LYAVR[d]	0.015	–[c]	K*LYAVR[d]	0.002	–[c]

[a]Synthetic peptides have general sequence LYAA[X]LYAVR where [X] represents a sequence motif consisting of 1−3 amino acids including the lysine/arginine (K/R) cleavage site. Glutamate/aspartate acidic amino acids (E/D) were located before/after the cleavage site directly or separated by threonine (T). Sequences with two successive lysine and/or arginine residues were also studied. The kinetic constant was calculated as a slope of linear relationship of peptide ln(peak area %) versus time. [b]Relative standard deviation, $n = 3–5$. [c]Not estimated. [d]* = Cleavage site position.

pipetting error when sampling the aliquots of reaction mixture in selected time intervals. Measured kinetic constants are valid for the model peptide substrate and digestion procedure using 1:100 substrate to enzyme ratio. Change in the conditions will affect the absolute values of kinetic constants; however, the relative trends should not change.

Comparison of Peptide Digestion Kinetic. Studied sequence motifs are comprised of (i) simple LYAA[X]LYAVR peptides where [X] is either arginine or lysine cleavage site and (ii) motifs where arginine and lysine are flanked with acidic amino acids, aspartic and glutamic acids. Those amino acid residues followed the cleavage site, preceded it, or were separated by one threonine residue. (iii) In addition, motifs with successive lysine and/or arginine residues such as [KK], [RR], [KR], or [RK] were studied. Cleavage site sequences and results are summarized in Table 1.

Peptides with Single Digestion Site. First, the peptides with simple [K] and [R] digestion sites (not surrounded by the "problematic" amino acids) were compared. As suggested in the literature,[7] arginine bond cleavage is preferred over the lysine. Peptide bond at N terminus of arginine was cleaved approximately 3 times faster than the one of lysine (Table 1). This is most probably related to lysine's weaker binding in the trypsin's active site pocket.[18]

Figure 2 compares the digestion speed of peptides containing [R], [RD], [DR], [RTD], and [DTR] digestion motifs. It is apparent that the cleavage speed is affected by proximity of acidic amino acid. The acidic amino acids (aspartic acid in this case) preceding the location of cleavage in either the P2 or P3 position lower the digestion speed more significantly than the same acidic amino acids following the cleavage site (P1′ or P2′ positions). This cleavage inhibition is more significant when the acidic amino acids are separated by another amino acid residue, in our case by threonine. Similar trends are observed for both aspartic and glutamic acids flanking both arginine or lysine cleavage sites (Table 1).

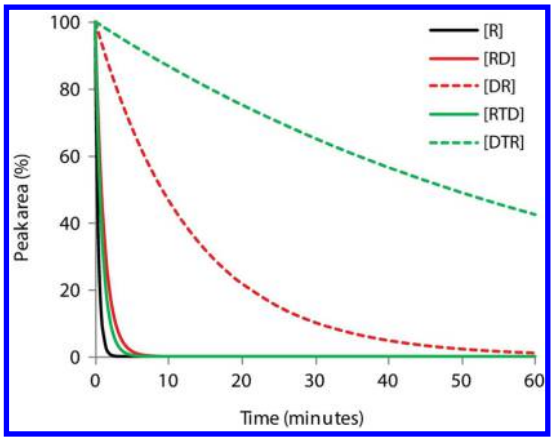

Figure 2. Dependencies of digestion progress of selected peptides on time. General peptide sequence is LYAA[X]LYAVR, where [X] represents R, RD, DR, RTD, and DTR, sequence motifs. The decay rate of selected peptides was calculated as follows: Peak area % = 100×10^{-k}. For kinetic constants k, see Table 1.

Figure 3 plots a relative digestion speed of peptides used in this study normalized to digestion speed of LYAA[R]LYAVR (100%). Comparison of reaction speed suggests that in general the aspartic acid is a more potent digestion inhibitor than glutamic acid. This effect could be caused by the higher acidity of the aspartic acid side chain and the fact that it is naturally present in the active site pocket of trypsin. The pK_a values of side chains of aspartic and glutamic acids are 3.9 and 4.3, respectively.[34]

The relative digestion speed of arginine sequence motifs can be sorted in descending order as [R] > [RTE] ~ [RE] ~ [RTD] > [ER] > [RD] > [ETR] ~ [DR] > [DTR]. The digestion of [DTR] motif was about 170-fold slower than digestion of [R]; it takes only 2 min to digest [R] motif, whereas [DTR] requires 5.3 h to completion (99% of substrate consumed).

Kinetic constants obtained for the lysine digestion site have a slightly different order: [K] ~ [KTD] > [KE] ~ [KTE] > [ETK] > [DTK] ~ [EK] ~ [KD] > [DK] (Figure 3 and Table 1). In agreement with previously reported trypsin's preference for arginine,[7] the kinetic constants measured for the lysine digestion site are generally lower compared to their counterparts with arginine, except the DTK and ETK motifs. The lysine site exhibited similar trends in digestion kinetics as arginine; proximity of aspartic acid lowered the digestion speed more significantly than glutamic acid in all cases (Figure 3 and Table 1).

Data in Figure 3 confirm the trends illustrated in Figure 2. Peptides with a cleavage site followed by an acidic amino acid (D, E) are digested more readily than peptides with a cleavage site preceded by D or E. Figure 3 (Table 1) shows that similar trends are observed for both arginine and lysine digestion sites flanked with aspartic and glutamic acid. Both the nature (D vs E) and the position of acidic amino acid in the vicinity of the cleavage site affect the speed of digestion. According to our findings, [DK] and [DTR] are the most likely motifs to produce missed cleavage peptides in protein tryptic digests.

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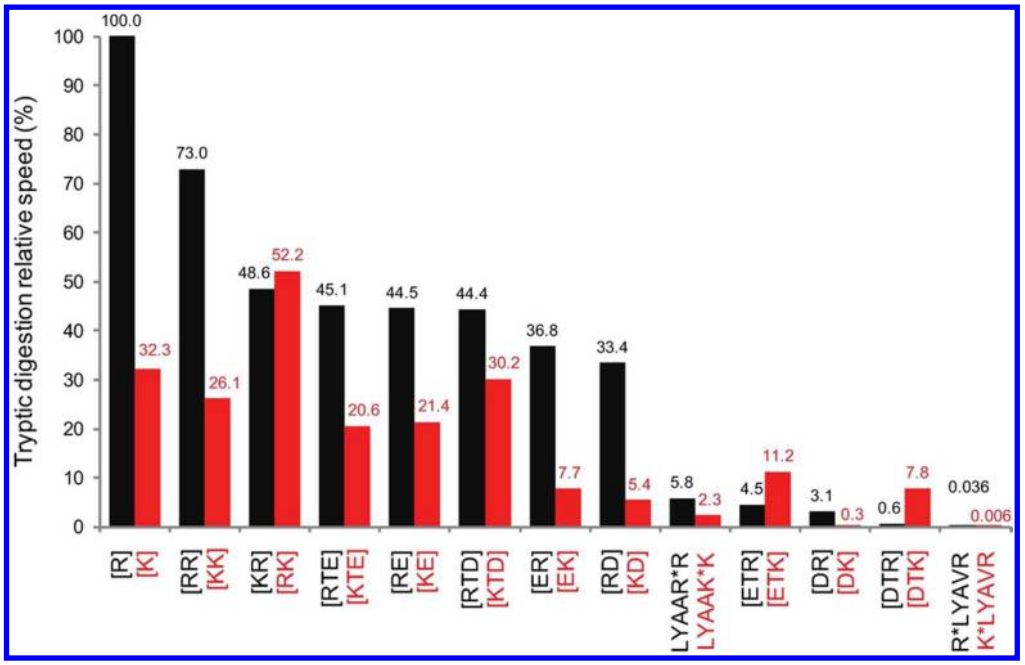

Figure 3. Relative digestion speed of peptides investigated in this study. Digestion speed is normalized to peptide with [R] sequence motif, representing 100% digestion rate.

Successive Lysine and Arginine Motifs. According to the "Keil rules",[35] the sequences with successive lysine and/or arginine are frequently thought of as missed cleavage patterns.[6,12,33] Our experiments show that such peptides are digested relatively rapidly (Table 1). However, the products of [RR], [KK], [KR], and [RK] peptide digest are missed cleavage peptides, because they cannot be further cleaved into limit peptides. For example, the peptide LYAA[RR]LYAVR with two arginine sites is cleaved into LYAAR and RLYAVR products, or in an alternative reaction, to LYAARR and LYAVR peptides. LYAARR and RLYAVR cannot be readily cleaved into final products due to lack of trypsin exopeptidase activity (inability to cleave at peptide termini).[35] Russell and co-workers[36] suggested that missed cleavage of these sequences can also be caused by the presence of a high concentration of organic solvents in the digestion solution. The results summarized in Table 1, and reported by Yen and co-workers,[7] suggest that missed cleavage of peptides with successive lysines/arginines is not caused by their competition for bonding with trypsin's active site pocket[6] but mainly by the lack of trypsin exopeptidase activity.[7,13] In other words, the KLYAVR, RLYAVR, or in general peptides with K/R at N-terminus (at P1 position) are not good substrates for trypsin, because they are missing the P4−P3−P2 amino acids involved in enzyme pocket binding. The peptide with missed cleaved motifs at C-terminus, such as LYAARR and LYAAKK, have full binding motif P4−P3−P2−P1−‖−P1′. One expects that P1′ terminal arginine or lysine will be readily cleaved. The relatively slow observed digestion speed may be due to the proximity of the acidic moiety (C-terminus carboxyl), which has presumably a similar inhibitory effect as the proximity of aspartate or glutamate amino acids to a scissile bond.[16]

Chromatograms in Figure 4 show the digestion of LYAA-[RR]LYAVR (panel A) and LYAA[KK]LYAVR (panel B). In contrast to peptides with a single scissile bond generating two products, the tryptic digestion of peptides with two cleavage sites produces four peptides. The products were identified according to their retention. A complete digestion of parent peptides in Figure 4 is finished in approximately 2 min (Figure 4). However, the product peak areas continue to change, although with a slower speed. Most noticeably, the LYAARR (panel A) and LYAAKK (panel B) cleavage products are subsequently digested into LYAAR and LYAAK limit peptides. The liberated arginine or lysine do not absorb UV light at 280 nm; therefore, they are not detected in the chromatograms. Detailed insets in Figure 4 reveal that KLYAVR and RLYAVR peptides are also gradually cleaved; their area decreases very slowly, while the area of product peptide LYAVR increases in time. This reaction is either the result of minor exoprotease activity of trypsin or an outcome of trypsin catalyzed reversed reaction, peptide elongation, followed by cleavage to limit peptides. Both mechanisms can explain the observed results.

Table 1 lists the kinetic constants of peptides with [RR], [KK], [KR], and [RK] motifs calculated from the disappearance of the parent peptides. The kinetic constants decrease in descending order as [RR] > [RK] ~ [KR] > [KK]. This is consistent with findings of Yen et al.[7] and our results, suggesting that trypsin preferably cleaves at R over K positions. The observed digestion speed of [RR] peptide is approximately 3-fold greater than that of [KK] peptide; a similar reaction speed ratio to that observed for [R] and [K] peptides (Table 1). The relative digestion speeds for peptides with single or double digestion sites were [R] > [RR] and [K] > [KK]. This finding is counterintuitive, because the two parallel reactions for double cleavage sites should result in a faster digestion speed of parent peptide. It suggests that the presence of the second basic amino acid inhibits the reaction speed at both cleavage positions. Competition for the bond in the active site pocket of trypsin and less favorable ionic interactions may be responsible for this observation.

As mentioned above, we calculated the kinetic constants for [RR], [KK], [KR], and [RK] peptides. However, since we monitor specific products of the peptides cleavage, we can also calculate the kinetic constants for each specific scissile site. To

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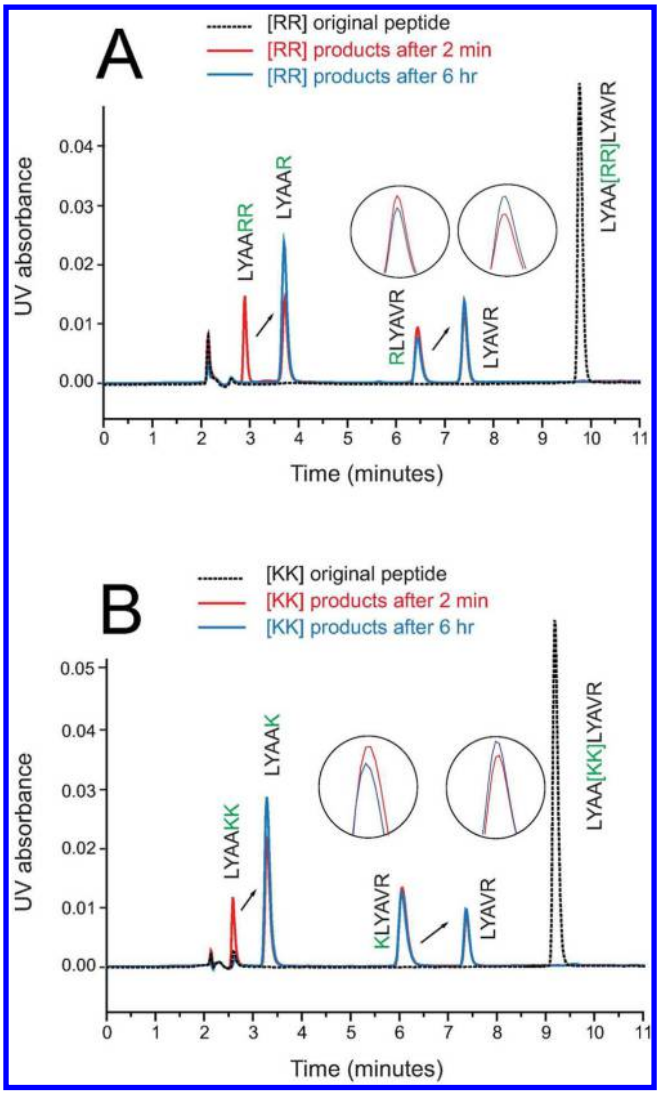

Figure 4. Chromatograms of digestion reaction of peptides LYAA-[RR]LYAVR (panel A) and LYAA[KK]LYAVR (panel B). Parent peptides and four cleavage products are well resolved in chromatograms. For LC conditions, see the Experimental Section.

clarify the location of the site, it is marked with an asterisk in Table 1 and in the following discussion when appropriate.

The digestion speed of peptide [RR] is a sum of parallel [R*R] and [RR*] reactions. Consequently, the kinetic constant $k_{[RR]}$ can be calculated as sum of kinetic constants $k_{[R*R]}$ and $k_{[RR*]}$ (eq 1).

$$k_{[RR]} = k_{[R*R]} + k_{[RR*]} \quad (1)$$

The digestion kinetics for each specific cleavage position can be calculated from the ratio of peak areas of specific digestion products. For LYAA[RR]LYAVR, the reaction generates LYAARR and LYAVR peptides for the [RR*] reaction (cleavage after the second arginine) and LYAAR and RLYAVR peptides for the [R*R] reaction (cleavage after the first arginine (Figure 4A)). The situation is complicated by the fact that LYAARR peptide is cleaved in a subsequent reaction into LYAAR and R; as discussed previously, the area of the LYAARR peak in Figure 4A decreases in time, while the area of the LYAAR peptide visibly increases. For this reason, we chose to calculate the kinetics of [R*R] and [RR*] parallel reactions using the relative area of RLYAVR and LYAVR peptides. Although RLYAVR is being slowly cleaved into LYAVR, the speed of this subsequent reaction is several orders of magnitude slower; hence, it does not affect the specific peak areas within the time scale of our experiment.

Assuming that RLYAVR and LYAVR peptides have the same molar absorption at UV 280 nm (both contain a single aromatic tyrosine in the same LYA sequence motif) and that kinetic constants for two parallel reactions are additive as given in eq 1, the kinetic constants $k_{[RK*]}$ and $k_{[R*K]}$ can be calculated from areas of observed peaks at any point during the reaction using the eqs 2a and 2b. The site specific kinetics of [KK], [KR], and [RK] peptides can be calculated in the same fashion.

$$k_{[R*R]} = k_{[RR]} \times \frac{A_{RLYAVR}}{A_{RLYAVR} + A_{LYAVR}} \quad (2a)$$

$$k_{[RR*]} = k_{[RR]} \times \frac{A_{LYAVR}}{A_{RLYAVR} + A_{LYAVR}} \quad (2b)$$

The values of site-specific kinetic constants (Table 1) show that sequence motifs KR and RK are preferably cleaved at the arginine position. This is consistent with the report from Yen et al.[7] and with the other results listed in Table 1. The values of site specific digestion constants suggest that the presence of the second arginine/lysine residue adjacent to the cleavable arginine/lysine position slows down the digestion speed approximately 2- to 3-fold.

As pointed out earlier, the parent peptides [RR], [KK], [KR], and [RK] are cleaved relatively rapidly (within 2 min) to produce missed cleavage peptides. As shown in Figure 4 for [RR] and [KK] examples, the missed cleavage products are further digested by trypsin at a slower reaction speed. Digestion speed of selected peptides was measured by extending the reaction time up to 360 min. We found that digestion speed of LYAAR*R and LYAAK*K peptides is approximately 15-fold reduced compared to [R] and [K] motif peptides, respectively (Table 1). The cleavage of terminal arginine in the R*R motif was about 3-fold faster than K*K motif digestion. The measurement of K*LYAVR and R*LYAVR peptides digestion kinetics was more difficult, because the reactions progressed at extremely low speed. These kinetic constants listed in Table 1 should be treated as approximate estimates.

The knowledge of digestion kinetics of partially trypsin resistant motifs may be applicable in biopharmaceutical research.[37−40] Mass spectrometry compatible surfactants used for denaturation of proteins for enzymatic digestion[41−43] reduce constraints of protein solubility and higher order structure (accessibility of scissile bonds). However, poorly cleavable motifs identified in this work remain a hindrance for protein digestion speed.[14] Ren and colleagues[37] illustrated that the typical alkaline buffer pH used in tryptic digestion induces artificial modifications such as asparagine deamidation and N-terminal glutamine cyclization. The amount of these modifications and production semitryptic peptides[5,9,29] is directly proportional to the incubation time. The knowledge of poorly cleavable motifs could lead to alternative peptide mapping strategies. For example, the monoclonal therapeutic antibody Trastuzamab sequence contains three poorly cleavable motifs DK and one DR (sequence with highlighted sites is provided as Figure S1, Supporting Information). By accepting that cleavage of such motifs proceeds in orders of magnitude slower speed than the cleavage of the remaining sites, one may choose to accept the selected missed cleaved peptides and

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reduce the digestion time significantly[37] without compromising the peptide mapping repeatability or sequence coverage.

CONCLUSIONS

In this work, we measured relative tryptic digestion speed for synthetic peptides with cleavage sites consisting of arginine or lysine flanked with neutral, acidic, or basic amino acids. The estimated kinetic constants are valid for short peptides without a secondary/tertiary structure and used experimental conditions. The digestion motif relative speed rules should be extended to proteins with caution; the impact of protein higher order structure cannot be neglected.[8] The conclusions from our experiments are as follows: (i) The arginine peptide bond is hydrolyzed approximately 3-fold faster than the lysine bond. (ii) Proximity of acidic amino acid (aspartate or glutamate residue) significantly reduces the digestion speed at both arginine and lysine positions. (iii) Aspartic acid is a more potent inhibitor than glutamic acid in most cases. (iv) Acidic amino acids preceding the arginine/lysine scissile bond reduce the digestion speed more significantly than the acidic amino acid following the cleavage position. (v) Peptides with [DR], [DK], [ETR], and [DTR] motifs were cleaved approximately 2 orders of magnitude slower compared to peptides with simple arginine/lysine site. (vi) The presence of the second lysine/arginine adjacent to the lysine/arginine digestion site slows down the digestion speed in both cleavage positions. Digestion speed is decreased approximately 2−3-fold. The produced missed cleavage peptides (LYAARR or LYAAKK) were further digested by trypsin to limit peptides at about a 15-fold slower reaction speed compared to LYAA[R]LYAVR and LYAA[K]-LYAVR peptides, respectively. K*LYAVR and R*LYAVR missed cleavage peptides were also further digested. However, the reaction speed was 3000−6000-fold slower compared to digestion of LYAA[R]LYAVR and LYAA[K]LYAVR peptides, respectively. (vii) The a priori knowledge of poorly cleavable motifs could be used to adjust the proteomic LC/MS search and scoring algorithms. (viii) The knowledge of relative digestion kinetics of protein sequence motifs could be used for rational development of the digestion protocols. When accepting a limited number of missed cleaved peptides with poorly cleavable motifs, the digestion times can be shortened, resulting in a minimization of undesirable products (deamidated or semitryptic peptides).

To our knowledge, these results are the first direct measurement of proteolytic digestion kinetics of selected missed cleavage motifs. This study should enhance the understanding of relative tryptic digestion speed, in order to obtain reproducible, efficient, and predictable digestions of protein samples. We believe that these results could considerably impact the proteomic research and aid the development of new enzymatic modifications.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in the text. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b00866.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) He, Y.; Zhong, W.; Yeung, E. S. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2002**, *782*, 331−341.
(2) Linderstrøm-Lang, K. *Proteins and Enzymes*; Stanford University Press: Stanford, 1952.
(3) Schechter, I.; Berger, A. *Biochem. Biophys. Res. Commun.* **1967**, *27*, 157−162.
(4) Perona, J. J.; Craik, C. S. *J. Biol. Chem.* **1997**, *272*, 29987−29990.
(5) Burkhart, J. M.; Schumbrutzki, C.; Wortelkamp, S.; Sickmann, A.; Zahedi, R. P. *J. Proteomics* **2012**, *75*, 1454−1462.
(6) Siepen, J. A.; Keevil, E. J.; Knight, D.; Hubbard, S. J. *J. Proteome Res.* **2007**, *6*, 399−408.
(7) Yen, C. Y.; Russell, S.; Mendoza, A. M.; Meyer-Arendt, K.; Sun, S.; Cios, K. J.; Ahn, N. G.; Resing, K. A. *Anal. Chem.* **2006**, *78*, 1071−1084.
(8) Wenschuh, H.; Halada, P.; Lamer, S.; Jungblut, P.; Krause, E. *Rapid Commun. Mass Spectrom.* **1998**, *12*, 115−119.
(9) Walmsley, S. J.; Rudnick, P. A.; Liang, Y.; Dong, Q.; Stein, S. E.; Nesvizhskii, A. I. *J. Proteome Res.* **2013**, *12*, 5666−5680.
(10) Rawlings, N. D.; Waller, M.; Barrett, A. J.; Bateman, A. *Nucleic Acids Res.* **2014**, *42*, D503−509.
(11) Rawlings, N. D.; Barrett, A. J. *MEROPS, the Peptidase Database*; 2014, http://merops.sanger.ac.uk/.
(12) Monigatti, F.; Berndt, P. *J. Am. Soc. Mass Spectrom.* **2005**, *16*, 13−21.
(13) Lawless, C.; Hubbard, S. *J. OMICS* **2012**, *16*, 449−456.
(14) Brownridge, P.; Beynon, R. J. *Methods (Amsterdam, Neth.)* **2011**, *54*, 351−360.
(15) Zabłotna, E.; Dysasz, H.; Lesner, A.; Jaskiewicz, A.; Kazmierczak, K.; Miecznikowska, H.; Rolka, K. *Biochem. Biophys. Res. Commun.* **2004**, *319*, 185−188.
(16) Gershon, P. D. *J. Proteome Res.* **2014**, *13*, 702−709.
(17) Schlosser, A.; Pipkorn, R.; Bossemeyer, D.; Lehmann, W. D. *Anal. Chem.* **2001**, *73*, 170−176.
(18) Craik, C. S.; Largman, C.; Fletcher, T.; Roczniak, S.; Barr, P. J.; Fletterick, R.; Rutter, W. J. *Science (Washington, DC, U. S.)* **1985**, *228*, 291−297.
(19) Safdar, M.; Spross, J.; Janis, J. *J. Chromatogr. A* **2014**, *1324*, 1−10.
(20) Proc, J. L.; Kuzyk, M. A.; Hardie, D. B.; Yang, J.; Smith, D. S.; Jackson, A. M.; Parker, C. E.; Borchers, C. H. *J. Proteome Res.* **2010**, *9*, 5422−5437.
(21) Petrassi, H. M.; Williams, J. A.; Li, J.; Tumanut, C.; Ek, J.; Nakai, T.; Masick, B.; Backes, B. J.; Harris, J. L. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 3162−3166.
(22) Furlong, S. T.; Mauger, R. C.; Strimpler, A. M.; Liu, Y. P.; Morris, F. X.; Edwards, P. D. *Bioorg. Med. Chem.* **2002**, *10*, 3637−3647.
(23) Poreba, M.; Drag, M. *Curr. Med. Chem.* **2010**, *17*, 3968−3995.
(24) Rodriguez, J.; Gupta, N.; Smith, R. D.; Pevzner, P. A. *J. Proteome Res.* **2008**, *7*, 300−305.

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Anal. Chem. 2015, 87, 7636−7643

(25) Li, G. Z.; Vissers, J. P.; Silva, J. C.; Golick, D.; Gorenstein, M. V.; Geromanos, S. *J. Proteomics* **2009**, *9*, 1696−1719.
(26) Elias, J. E.; Gygi, S. P. *Nat. Methods* **2007**, *4*, 207−214.
(27) Abbatiello, S. E.; Schilling, B.; Mani, D. R.; Zimmerman, L. J.; Hall, S. C.; MacLean, B.; Albertolle, M.; Allen, S.; Burgess, M.; Cusack, M. P.; Ghosh, M.; Hedrick, V.; Held, J. M.; Inerowicz, H. D.; Jackson, A.; Keshishian, H.; Kinsinger, C. R.; Lyssand, J.; Makowski, L.; Mesri, M.; Rodriguez, H.; Rudnick, P.; Sadowski, P.; Sedransk, N.; Shaddox, K.; Skates, S. J.; Kuhn, E.; Smith, D.; Whiteaker, J. R.; Whitwell, C.; Zhang, S.; Borchers, C. H.; Fisher, S. J.; Gibson, B. W.; Liebler, D. C.; MacCoss, M. J.; Neubert, T. A.; Paulovich, A. G.; Regnier, F. E.; Tempst, P.; Carr, S. A. *Mol. Cell. Proteomics* **2015**, DOI: 10.1074/mcp.M114.047050.
(28) Nesvizhskii, A. I. *J. Proteomics* **2010**, *73*, 2092−2123.
(29) Loziuk, P. L.; Wang, J.; Li, Q.; Sederoff, R. R.; Chiang, V. L.; Muddiman, D. C. *J. Proteome Res.* **2013**, *12*, 5820−5829.
(30) Gilar, M.; Olivova, P.; Chakraborty, A. B.; Jaworski, A.; Geromanos, S. J.; Gebler, J. C. *Electrophoresis* **2009**, *30*, 1157−1167.
(31) Geromanos, S. J.; Vissers, J. P.; Silva, J. C.; Dorschel, C. A.; Li, G. Z.; Gorenstein, M. V.; Bateman, R. H.; Langridge, J. I. *Proteomics* **2009**, *9*, 1683−1695.
(32) Geromanos, S. J.; Hughes, C.; Golick, D.; Ciavarini, S.; Gorenstein, M. V.; Richardson, K.; Hoyes, J. B.; Vissers, J. P.; Langridge, J. I. *Proteomics* **2011**, *11*, 1189−1211.
(33) Thiede, B.; Lamer, S.; Mattow, J.; Siejak, F.; Dimmler, C.; Rudel, T.; Jungblut, P. R. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 496−502.
(34) Frey, P. A.; Ables, R. H. *Enzymatic Reaction Mechanism*; Oxford University Press: New York, 2007.
(35) Keil, B. *Specificity of Proteolysis*; Springer-Verlag: New York, 1992.
(36) Russell, W. K.; Park, Z. Y.; Russell, D. H. *Anal. Chem.* **2001**, *73*, 2682−2685.
(37) Ren, D.; Pipes, G. D.; Liu, D.; Shih, L. Y.; Nichols, A. C.; Treuheit, M. J.; Brems, D. N.; Bondarenko, P. V. *Anal. Biochem.* **2009**, *392*, 12−21.
(38) Lesur, A.; Varesio, E.; Hopfgartner, G. *J. Chromatogr. A* **2010**, *1217*, 57−64.
(39) Xie, H.; Chakraborty, A.; Ahn, J.; Yu, Y. Q.; Dakshinamoorthy, D. P.; Gilar, M.; Chen, W.; Skilton, S. J.; Mazzeo, J. R. *mAbs* **2010**, *2*, 379−394.
(40) Gilar, M.; Yu, Y. Q.; Ahn, J.; Xie, H.; Han, H.; Ying, W.; Qian, X. *Anal. Biochem.* **2011**, *417*, 80−88.
(41) Ji, Y.; Liu, M.; Bachschmid, M. M.; Costello, C. E.; Lin, C. *Anal. Chem.* **2015**, *87*, 5500−5504.
(42) Yu, Y. Q.; Gilar, M.; Lee, P. J.; Bouvier, E. S.; Gebler, J. C. *Anal. Chem.* **2003**, *75*, 6023−6028.
(43) Yu, Y. Q.; Gilar, M.; Gebler, J. C. *Rapid Commun. Mass Spectrom.* **2004**, *18*, 711−715.

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Publikace II – Porovnání katalytické účinnosti tří trypsinových kolon používaných v kapalinové chromatografii

Imobilizace enzymů na pevný nosič je vhodným prostředkem jak zabránit nechtěným jevům, které doprovází enzymové štěpení v roztoku. V případě trypsinu je těchto nevýhod několik. Nejvýznamnější je časová náročnost enzymové reakce, která obvykle trvá 12-24 hodin. Trypsin se do reakční směsi přidává v malém množství, obvykle v poměru 1:50, aby nedocházelo k autolýze. Molekuly trypsinu jsou schopny se navzájem hydrolyzovat, a tak dochází ke kontaminaci analyzovaného vzorku nechtěnými peptidovými fragmenty. Další nevýhody představuje malá odolnost vůči organickým rozpouštědlům a vysokým teplotám, jejichž použití by mohlo podstatně zvýšit efektivitu štěpné reakce [13,14]. Všem těmto nevýhodám se lze vyhnout imobilizací enzymu, která zabraňuje autolýze, umožňuje tisícinásobné zvýšení množství enzymu účastnícího se štěpné reakce, použití vyšších teplot a organických rozpouštědel. Velkou výhodu představuje také přímé zapojení do HPLC aparatury, čímž lze omezit manipulaci se vzorkem a vnášení chyb do analýz. Produkci „miscleavage“ sekvencí, která byla diskutována v předešlé publikaci, se tímto způsobem nelze vyhnout, ale vzhledem k velkému zrychlení enzymové reakce by se jejich množství mělo významně snížit.

Publikace II obsahuje určení a srovnání účinnosti tří trypsinových kolon, z nichž dvě, Perfinity a Poroszyme, jsou komerčně dostupné. Kolony Poroszyme a Perfinity jsou již nějakou dobu dostupné a používané, jejich výrobci slibují vysokou účinnost, rychlé reakční časy v rámci několika minut, odolnost vůči vysokým tlakům, teplotám a opakovatelné výsledky pro nástřik více než 250 vzorků. Třetí kolona je prototypem vytvořeným ve skupině prof. J. Jorgensona na University of North Carolina at Chapel Hill (UNC), USA a stále podléhá vývoji. Jako jediná by měla být kompatibilní i s UPLC přístroji. U zmíněných trypsinových kolon byla určena a porovnána katalytická účinnost vyjádřená jako úbytek plochy píku substrátu štěpné reakce po aplikaci různých separačních podmínek. Byl studován vliv pH separačního pufru, průtoku mobilní fáze a teploty. Zvolené testovací podmínky v této práci jsou: pH 7,0; 8,0; 9,0; teploty 20, 25, 30 a 37°C; průtok mobilní fáze 0,1, 0,2 a 0,3 ml/min. Změna rychlosti průtoku mobilní fáze souvisí s dobou setrvání analytu v koloně. Vzhledem k rozměrům UNC prototypu byla doba setrvání analytu v koloně 44, 22 and 15 s při průtocích 0,1, 0,2 a 0,3 ml/min. Práce se zaměřuje také na opakovatelnost analýz a celého procesu. Jako substrát pro enzymovou

reakci byl zvolen N_α-benzoyl-L-arginin 4-nitroanilid hydrochlorid (Obr. 2), který je obvykle využíván k ověření účinnosti trypsinu [38,39].

Vybrané kolony se liší nosičem, na kterém je imobilizován trypsin, velikostí pórů a původem použitého enzymu (vepřový/hovězí). Rozdíly mezi kolonami shrnuje Tabulka 1. V případě UNC kolony byl trypsin imobilizován na hybridní silikagelové částice (BEH, 300 Å) modifikovaným postupem popsaným v literatuře [40]:

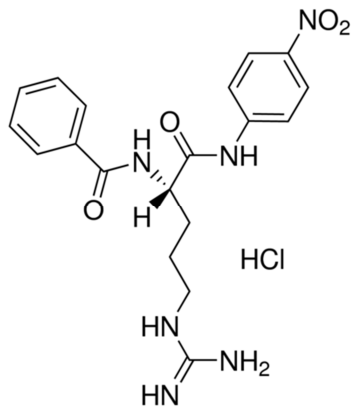
1) 0,6 g BEH částic bylo přes noc zahříváno na 110 °C.
2) Po ochlazení bylo přidáno 220 µl triethoxysilyl butyraldehydu ve 4 ml ethanolu a směs byla míchána 2 h při pokojové teplotě.
3) Do reakční směsi bylo přidáno 10 ml 50 mM $(NH_4)_2CO_3$ a částice byly třikrát propláchnuty 20-30 ml stejného roztoku. Po propláchnutí byly k částicím přidány 4 ml $(NH_4)_2CO_3$.
4) 80 mg trypsinu bylo rozpuštěno ve 4 ml $(NH_4)_2CO_3$ a 2 ml 1 M $NaCNBH_3$. Směs byla přidána k BEH částicím a 2 h ponechána při pokojové teplotě. Následně byly přidány 2 ml 1 M ethanolaminu a směs reagovala dalších 30 min a částice byly třikrát propláchnuty 20-30 ml $(NH_4)_2CO_3$.
5) Nakonec byly částice s imobilizovaným trypsinem rozmíchány ve vodě s kyselinou trifuoroctovou, pH 4, a vpraveny do kovové kolony o rozměrech 2,1 × 30 mm.

Množství imobilizovaného trypsinu bylo stanoveno spektrofotometricky při vlnové délce 562 nm reakcí s hovězím sérovým albuminem. Na 1 mg BEH sorbentu bylo imobilizováno 84 mg trypsinu. Účinnost imobilizovaného trypsinu byla stanovena spektrofotometricky za použití substrátu N_α-benzoyl-L-arginin ethyl ester hydrochloridu jako změna absorbance při vlnové délce 253 nm. Byla porovnána směrnice závislosti absorbance na době reakce v roztoku vůči reakci stejného množství trypsinu imobilizovaného na BEH částicích rozptýlených v reakčním pufru. Množství trypsinu imobilizovaného v koloně bylo 5,7 mg a enzym si zachoval 35-40 % účinnosti vůči čerstvě připravenému roztoku trypsinu. I s takovou ztrátou účinnosti se reakce účastní o tři řády více trypsinu než při reakci v roztoku, kde se obvykle používá množství kolem 1 µg.

Při porovnání účinnosti výše zmíněných trypsinových kolon bylo zaznamenáno několik trendů. (i) Reakční rychlost roste s rostoucím pH pufru. Nejlépe pozorovatelný byl tento trend na UNC prototypu, který vykazovat podstatně vyšší účinnost při pH 9,0 než při

pH 7,0 a 8,0. (ii) Rychlost štěpení roste s rostoucí teplotou. (iii) Se snižujícím se průtokem mobilní fáze se zvyšuje množství naštěpeného substrátu. Výjimka v tomto chování byla zaznamenána u kolony Poroszyme při měření v pufrech o pH 7,0 a 8,0.

Optimální rozsah pH pro trypsinové štěpení udávají výrobci Sigma Aldrich a Worthington Biochemical Corporation jako pH 7-9 [23,24]. Experimentálně byl však zjištěn významný pokles účinnosti studovaných kolon při pH 7,0 a nejvyšší účinost byla zaznamenána při použití reakčního pufru o pH 9,0.



Obr. 2 Struktura substrátu, N_{α}-benzoyl-L-arginin 4-nitroanilid hydrochloridu, použitého pro porovnání účinnosti trypsinových kolon.

Tab. 1 Obecné parametry testovaných kolon s imobilizovaným trypsinem.

	Poroszyme	Perfinity	UNC prototyp
Rozměry	2,1 × 30 mm, 20 µm	2,1 × 33 mm, 20 µm	2,1 × 30 mm, 5 µm
Velikost pórů	500-10000 Å	>>1000 Å	300 Å
Maximální tlak	2500 psi	2500 psi	15000 psi
Pracovní teplota	25-67 °C	25-67 °C	20-60 °C
Sorbent	PDVB	PDVB	BEH
Původ trypsinu	Hovězí	Vepřový	Hovězí

PDVB – polystyrendivinylbenzen, BEH – ethylen bridged hybrid silica

Publikace II

Comparison of catalytic activity of three trypsin columns used in liquid chromatography

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Comparison of catalytic activity of three trypsin columns used in liquid chromatography

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Abstract

Trypsin is the most widely used enzyme in proteomic research due to its high specificity. Although the in-solution digestion is predominantly used, it has several drawbacks, such as long digestion times, autolysis, and intolerance to high temperatures or organic solvents. To overcome these shortcomings trypsin was covalently immobilized on solid support and tested for its proteolytic activity. Trypsin was immobilized on bridge-ethyl hybrid silica sorbent with 300 Å pores, packed in 2.1 × 30 mm column and compared with Perfinity and Poroszyme trypsin columns. Catalytic activity of enzymatic reactors was tested using N_{α}-Benzoyl-L-arginine 4-nitroanilide hydrochloride as a substrate. The impact of buffer pH, mobile phase flow rate, and temperature on enzymatic activity was investigated. Digestion speed generally increased with the temperature from 20 to 37 °C. Activity also increased with pH from 7.0 to 9.0; the activity of prototype enzyme reactor was highest at pH 9.0, when its activity exceeded both commercial reactors.

Introduction

Enzymatic proteolysis is a common method for liquid chromatography - mass spectrometry (LC-MS) identification, and characterization of proteins. The choice of enzyme is typically trypsin, a highly specific protease, which cleaves peptide bonds only after arginine and lysine residues [1]. The use of trypsin is associated with a number of difficulties. In-solution digestion can be time consuming (up to 24 hours); speeding up reaction by adding more trypsin leads to enzyme autolysis and production of undesired peptides. Other drawbacks are loss of trypsin activity at elevated temperatures (partly resulting from increased autolysis) [2] or in the presence of organic solvents [3].

Some of these limitations can be overcome by enzyme immobilization on solid support. Immobilized enzymes can be used in either batch mode digestion or as flow through reactors. Both approaches have been investigated over the past two decades and several commercial products have been introduced. However, adoption of immobilized enzyme reactors (IMERs) for protein analysis has been slow due to their price, stability, and in many cases an incorrect application. For example, although IMERs can be highly active, capable of digesting the proteins in seconds, the speed of proteolysis can be reduced by secondary/tertiary protein sequence (inaccessible digestion sites). Presence of chaotropic agents used to denature proteins may inactivate immobilized enzyme, while the presence of primary sequence motifs with slow digestion kinetics may result in generation of missed cleavage peptides. The dependence of tryptic digestion kinetics on primary protein sequence was recently studied [4-6].

Despite these shortcomings, IMERs hold the promise of protein digestion within minutes, if not seconds, depending on immobilized enzyme activity. Since the intermolecular collisions of trypsin are minimal in immobilized reactors, high enzyme concentration can be used, promoting speed of digestion, while minimizing enzyme autolysis. Packed IMERs can be easily used with LC instrumentation in flow through mode, reducing the sample manipulations and possible contamination. Trypsin can be immobilized using a wide variety of carriers; monoliths, silica particles, nano particles, membranes etc. [7-9]. The nature of the carrier is the most important factor in determining activity stability, and capacity of enzymatic reactors; stationary phase can either stabilize or denature the enzyme [7]. As suggested in a recent review [8], the development of carriers with superior characteristics, such as high mechanical strength, large surface area, low back pressure, high enzyme loading capacity, and good biocompatibility is the main goals of current IMER research.

The commercially available IMERs are reportedly stable in organic solvents, high flow rates, elevated temperatures, and compatible with LC working pressures. The most frequently used is Poroszyme® Immobilized Trypsin Cartridge from Applied Biosystems® based on polystyrene divinylbenzene (PDVB) particles. The manufacturer states, that this column provides enhanced biomolecule access *via* large pores, fast 1-5 min digestion, high sample recovery, and chemical stability [10]. Poroszyme columns have been available since 1995, applied mainly to peptide mapping [11-30]. A similar product StyrosZyme™ TPCK-Trypsin column manufactured by OraChrom was applied to on-line BSA digestion [31] and compared to other trypsin columns [32]. Perfinity Biosciences offer two trypsin columns based on a similar carrier. The company suggests that digestion does not require a reduction

and alkylation of proteins prior on-line proteolysis. Perfinity Biosciences used Shimadzu LC multi-column workstation for automated protein digestion, peptide separation and MS analysis. This approach was used for identification of transferrin peptides [33], characterizing the post-translational modifications of human serum albumin [34] and for analysis of therapeutic proteins [35]. Although the immobilized enzymatic reactors have gained considerable attention in past years, few articles compared activity of research IMER prototypes to the commercial ones [29,32].

In this work we investigated the activity of a prototype immobilized trypsin reactor based on 5 μm bridged ethyl hybrid (BEH) silica particles with 300 Å pores. The prototype IMER was compared to Poroszyme and Perfinity IMERs available in similar column hardware dimensions (Table 1). We investigated catalytic activity and digestion repeatability of three IMERs at various temperatures, flow rates (residence time) and buffer pH. N_{α}-Benzoyl-L-arginine 4-nitroanilide hydrochloride, a common digestion substrate, was utilized to evaluate the trypsin activity in this study.

Experimental section

Chemicals and reagents

N_{α}-Benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPNA, ≥99%), N_{α}-Benzoyl-L-arginine ethyl ester hydrochloride (BAEE), acetonitrile (ACN, gradient grade), chloroacetic acid (ACS reagent, 37%), calcium chloride (Dri^{TM} ≥97%), Trizma® base (Tris, ≥99.9%), ethanolamine (≥98%) and trypsin (T1426, TPCK Treated, essentially salt-free, lyophilized powder, ≥10,000 BAEE units/mg protein) were supplied by Sigma Aldrich (St. Louis, MO, USA). Micro BCA Protein Assay Reagent Kit and trifluoroacetic acid (≥99.5%) were supplied by Thermo Fisher Scientific (Waltham, MA, USA). ALD Coupling Solution which contained 1 M $NaCNBH_3$ was obtained from Sterogene (Carlsbad, CA, USA), triethoxysilyl butyraldehyde (90%) from Gelest Inc. (Morrisville, PA, USA), ammonium bicarbonate (≥99.5%) from Fluka (Buchs, Switzerland), anhydrous ethanol (≥99.5%) from Acros Organics (Belgium, NJ, USA). Non-bonded bridged ethyl hybrid (BEH) silica particles (5 μm, 300 Å) were supplied by Waters Corporation (Milford, MA, USA). Deionized water was purified with a Rowapur and Ultrapur system from Watrex (Prague, Czech Republic). 50 mM ammonium bicarbonate buffer was made by dissolving the appropriate amount of ammonium bicarbonate in deionized water and adjusted to pH 9.0 with 1 M NaOH. Tris digestion buffer composed of 0.05 M Tris, 0.01 M calcium chloride titrated to desired pH by 2 M chloroacetic acid.

Preparation of IMER prototype

The trypsin immobilization was carried out at the University of North Carolina at Chapel Hill (UNC) using protocol adapted from Ahn *et al*. [36]. Approximately 0.6 g of BEH silica particles supplied by Waters Corporation were added to a 25 mL round bottom flask and placed in an oven (110 °C) overnight prior to immobilization. When cooled, the flask and its contents were kept under a constant flow of nitrogen. Then 220 µL of triethoxysilyl butyraldehyde in 4 mL of anhydrous ethanol was added to the flask. The mixture underwent magnetic stirring for 2 hr at room temperature. Approximately 10 mL of 50 mM ammonium bicarbonate buffer (pH 9.0) was added to the reaction and the mixture was transferred into a 50 mL centrifuge tube. The particles were washed three times *via* centrifugation with 20-30 mL of 50 mM ammonium bicarbonate buffer to remove unreacted silane. After the final decant, particles were slurried in 4 mL of buffer in a round bottom flask with magnetic stirrer. 80 mg of trypsin dissolved in 4 mL of 50 mM ammonium bicarbonate buffer, followed by the addition of 2 mL of ALD Coupling Solution (containing 1 M $NaCNBH_3$). Reaction was performed for 2 hr at room temperature. Afterwards, 2 mL of 1 M ethanolamine was added to quench the reaction for another 30 min at room temperature. Particles were washed three times with 50 mM ammonium bicarbonate buffer to remove excess of trypsin and ethanolamine using the above described protocol. BEH particles with immobilized trypsin were slurried in water with trifluoroacetic acid (pH 4.0) and packed into the 2.1 × 30 mm column hardware.

The amount of immobilized trypsin was estimated using Micro BCA Protein Assay Reagent Kit. Presence of protein was measured as a color reaction at 562 nm using UV-VIS µQuant microplate spectrophotometer (BioTek, Winooski, VT, USA). BSA was used to construct the calibration plot of absorbance versus mg of protein. The amount of immobilized trypsin per 1 g of BEH sorbent was approximately 84 mg.

To determine the activity of immobilized trypsin, BAEE was used as substrate. BAEE digestion was monitored as change in UV absorbance at 253 nm using Labda35 UV/VIS spectrometer (Perkin Elmer, Waltham , MA, USA). Slope of the UV absorbance change versus time obtained for trypsin in-solution digestion was compared to equal amount of trypsin immobilized on BEH particles dispersed in the reaction buffer. The retained activity of immobilized trypsin was ~35-40 % compared to freshly prepared trypsin solution.

Instrumentation and Methods

The activity of immobilized trypsin (IMERs) was determined by Waters CapLC 2487 spectrophotometer with the absorbance measured at 253 nm and the amount of immobilized enzyme was determined at 562 nm.

HPLC analyses were carried out using a Waters Alliance System composed of Waters 2690 Separation Module, Waters 2487 UV-VIS 2-channel detector, 717 Plus autosampler and Waters Alliance Series column heater and cooler (Waters Corp., Milford, MA, USA). Instrument control and data collection were performed by Empower 2 software. For enzymatic digestion, three different trypsin columns were used, *i.e.* Poroszyme® (Applied Biosystems, Foster City, CA, USA), Perfinity (Perfinity Biosciences Inc., West Lafayette, IN, USA) and prototype IMER synthesized at University of North Carolina at Chapel Hill using a modified method described previously [36]. Separation of digestion products was carried out using an XTerra® RP18 column (Waters, Milford, MA, USA), 150 × 3.0 mm, 3.5 μm. In Table 1 we list IMER data provided by manufacturers. Although the operating temperature range is up to 67 °C, digestion temperatures above 40 °C may result in reduction of IMER lifetime, especially in combination with organic solvents.

Table 1. General parameters of tested trypsin IMERs.

	Poroszyme	Perfinity	UNC Prototype
Dimension	2.1 × 30 mm, 20 μm	2.1 × 33 mm, 20 μm	2.1 × 30 mm, 5μm
Pore size	500-10000 Å	>>1000 Å	300 Å
Maximum pressure	2500 psi	2500 psi	15000 psi
Operating temperature	25-67 °C	25-67 °C	20-60 °C
Sorbent	PDVB	PDVB	BEH
Trypsin origin	Bovine	Porcine	Bovine

Digestion experiments were carried out using BAPNA as a substrate. BAPNA was prepared at concentration 6 mg/mL in mixture of 25% ACN, 25% water and 50% digestion buffer (*v/v/v*). 100 μL of BAPNA substrate was injected on IMER connected in series to separation column (mobile phase gradient passed through both IMER and separation column). Substrate BAPNA and its yellow digestion product *p*-nitroaniline were identified and quantified using UV-VIS detector set at 254 and 410 nm detection wavelengths. Mobile phase consisted of Tris buffer (pH 7.0, 8.0 or 9.0) and ACN. Three different gradients and flow rates were used. The rationale for changing flow rate was to alter the residence time of substrate on the IMER column. The completeness of digestion is expected to decrease at

elevated flow rates. Mobile phase gradient started at 95/5 Tris/ACN volume ratio held for 1 min; it was raised to 50/50 in 110, 70 and 50 min for flow rates 0.1, 0.2, and 0.3 mL/min, respectively. Given the physical dimension of IMERs and volume fraction of reactor containing mobile phase ($\varepsilon_t \sim 0.7$), the estimated residence times of substrate on the IMERs were 44, 22 and 15 seconds for flow rates 0.1, 0.2, and 0.3 mL/min, respectively. Separation and digestion temperature was 20, 25, 30 and 37 °C. The autosampler temperature was set to 25 °C.

The IMER experiments started at pH 8.0 (recommended pH for tryptic digestion) followed by pH 7.0 and pH 9.0. The order of temperature experiments for each IMER at given pH was 20, 25, 30, and 37 °C; each digestion condition was tested at 0.3, 0.2 and 0.1 mL/min flow rate, respectively. Initial mobile phase composition, *i.e.* 5/95 (*v/v*) ACN/Tris buffer, is recommended by column manufacturers to enhance digestion. Since the presence of organic modifier in the buffers affects the activity of hydronium ions [37], we report pH of aqueous buffer before the addition of acetonitrile. On-line digestion measurements were performed in eight repetitions for each separation conditions; the calculated relative standard deviation (RSD) values are reported. Enzyme in all columns was treated by *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) by manufacturer to minimize the undesired chymotrypsin activity.

Results and discussion

With the advancements of methods in proteomic research, fast, repeatable and more reliable protein digestions are needed. The application of IMER technology has the potential to accomplish that and simplify the digestion procedure significantly. When the IMER hardware and packing are compatible with high-pressure, the IMERs can be utilized in flow-through mode serially connected to an HPLC column. In such case, protein sample can be introduced by HPLC injector, transported by the stream of mobile phase to the IMER, digested, and the resulting peptides trapped onto an HPLC column, followed by gradient elution of digestion products. The manufacturers of commercial IMERs indicate that the proteins can be repeatedly digested within several minutes. This is significantly faster reaction speed than typically achieved with in-solution digestions. The degree of protein digestion can be controlled by adjusting the mobile phase flow rate, and IMERs can be used for hundreds of analyses with reproducible results.

In our setup the mobile phase gradient was passed *via* IMERs; enzyme was repeatedly exposed to high concentration of acetonitrile (up to 50%) and a slight decrease in trypsin catalytic activity was noted. To insure meaningful comparison we performed the pH, flow rates and temperature experiments in the same order for all three tested IMERs.

In this article we compared activity of two commercially available and one in-house made prototype trypsin IMERs. To evaluate the LC-UV-VIS analysis repeatability we first performed analysis of BAPNA substrate without the IMER in the flow path and compared it with the setup where the IMER was placed between the injector and a RP18 HPLC column. Obtained RSD values of eight repetitive analyses are summarized in Table 2. The LC analysis repeatability (no IMER) was 1.4%; the variability with IMER was several time higher (see Table 2). The increased variability is in part due to the digestion process, and in part caused by integration error of substantially smaller peak (BAPNA substrate is partially or nearly completely converted by the IMER to digestion product). Average RSD obtained for trypsin digestion in tandem with separation on RP18 column was 5.4%.

Table 2. RSD values of injections obtained with XTerra RP18 column only and trypsin reactors connected in series with XTerra RP18 column for various IMER's and pH conditions. RSDs were calculated from substrate peak area. All experiments were performed in eight replicates (n=8).

	Perfinity RSD [%]	Poroszyme RSD [%]	UNC Prototype RSD [%]	RP18 RSD [%]
pH 7.0	2.6	3.7	4.8	1.3
pH 8.0	6.1	6.7	3.1	1.4
pH 9.0	5.4	9.2	6.7	1.3

IMER activity data (percent of BAPNA substrate remaining) are summarized in Table 3. Several common trends were observed for all three IMERs: (i) Digestion speed increases with increasing pH. This is most obvious for the UNC prototype, which had significantly higher activity at pH 9.0 than pH 8.0 or 7.0. (ii) With exception of a few experiments at pH 7.0 (suboptimal pH) the IMERs activity increases with temperature. The most complete digestion was observed at 37 °C. (iii) Digestion improves at lower flow rate (longer residence time of substrate on IMER). Notable exceptions from this trend are the Poroszyme IMER at pH 8.0 and experiments at the suboptimal pH 7.0.

Table 3. Activity of Perfinity, Poroszyme and UNC prototype IMERs measured as percent of undigested peak area of BAPNA substrate at indicated pH, temperatures, and flow rates. All experiments were performed in eight replicates (n=8); RSDs were comparable with values shown in Table 2. Complete RSD data are provided in supplementary Table S1.

Perfinity pH 7.0			
	0.3 mL/min	0.2 mL/min	0.1 mL/min
20 °C	58.2 %	62.8 %	59.9 %
25 °C	59.2 %	61.0 %	62.0 %
30 °C	59.4 %	58.0 %	58.0 %
37 °C	58.7 %	63.1 %	54.2 %

Poroszyme pH 7.0			
	0.3 mL/min	0.2 mL/min	0.1 mL/min
20 °C	63.7 %	56.0 %	58.0 %
25 °C	61.1 %	53.6 %	55.4 %
30 °C	60.6 %	53.2 %	52.1 %
37 °C	59.5 %	58.5 %	56.5 %

UNC prototype pH 7.0			
	0.3 mL/min	0.2 mL/min	0.1 mL/min
20 °C	62.5 %	66.9 %	53.1 %
25 °C	61.5 %	55.7 %	50.1 %
30 °C	58.7 %	55.7 %	47.2 %
37 °C	54.0 %	53.6 %	52.4 %

Perfinity pH 8.0			
	0.3 mL/min	0.2 mL/min	0.1 mL/min
20 °C	35.1 %	29.2 %	17.6 %
25 °C	26.0 %	20.3 %	11.1 %
30 °C	18.2 %	11.9 %	8.4 %
37 °C	11.0 %	6.0 %	6.7 %

Poroszyme pH 8.0			
	0.3 mL/min	0.2 mL/min	0.1 mL/min
20 °C	40.7 %	21.4 %	23.5 %
25 °C	32.4 %	13.8 %	16.8 %
30 °C	22.4 %	10.7 %	12.1 %
37 °C	16.1 %	7.7 %	12.1 %

UNC prototype pH 8.0			
	0.3 mL/min	0.2 mL/min	0.1 mL/min
20 °C	40.4 %	17.3 %	10.3 %
25 °C	33.9 %	13.7 %	7.8 %
30 °C	27.3 %	8.2 %	5.2 %
37 °C	21.7 %	5.7 %	3.0 %

Perfinity pH 9.0			
	0.3 mL/min	0.2 mL/min	0.1 mL/min
20 °C	32.4 %	25.2 %	19.1 %
25 °C	25.6 %	18.4 %	13.9 %
30 °C	20.1 %	11.0 %	5.2 %
37 °C	11.8 %	3.2 %	0.4 %

Poroszyme pH 9.0			
	0.3 mL/min	0.2 mL/min	0.1 mL/min
20 °C	41.0 %	34.0 %	16.6 %
25 °C	32.8 %	25.5 %	9.3 %
30 °C	25.8 %	16.0 %	4.7 %
37 °C	16.3 %	5.3 %	0.5 %

UNC prototype pH 9.0			
	0.3 mL/min	0.2 mL/min	0.1 mL/min
20 °C	7.8 %	1.3 %	0.0 %
25 °C	6.5 %	1.5 %	0.0 %
30 °C	6.0 %	2.1 %	0.0 %
37 °C	4.0 %	0.3 %	0.0 %

The optimal pH range for tryptic digestion is 7-9 according to Sigma Aldrich [38] or 7.8-8.7 according to Worthington Biochemical Corporation [39]. Our results indicate great loss of trypsin activity at pH 7.0 for all IMERs used in the study. Interestingly, the UNC prototype (prepared using TPCK treated bovine trypsin from Sigma Aldrich) exhibited significantly higher activity at pH 9.0; at flow rate 0.1 ml/min no undigested BAPNA substrate remained.

Given that 2.1 × 30 mm IMER hardware has ~0.1 mL volume, packing density of BEH is 0.7 g/mL, and amount of trypsin immobilized on UNC prototype is ~78 mg/mL, the amount of trypsin in the prototype IMER is approximately 5.7 mg. Even if only 35-40% remains active, the amount of trypsin available for digestion is three orders of magnitude higher than the amount typically used for in-solution protein digestion (microgram amounts of trypsin). This explains why IMER's has a potential to perform faster digestions compared to in-solution proteolysis typically practiced today. Out of the three IMERs tested in this study, the UNC prototype outperformed the Poroszyme and Perfinity at pH 9.0 and

performed comparably well at pH 7.0 and 8.0. It should be noted that BEH sorbent is stable in such pH range.

Conclusion

IMERs based on pressure resistant particles are suitable for direct on-line digestions in flow through mode. High amount of trypsin immobilized on sorbent facilitates faster digestion times, compared to traditional in-solution digestion methods. Trypsin activity was not significantly affected at elevated pressure used in this study even with mobile phases containing high acetonitrile concentrations. We found that the activity of all the IMERs were suboptimal at pH 7.0; several fold faster digestion was observed at pH 8.0 and 9.0. The highest activity of the UNC prototype was measured at pH 9.0 for all temperatures and flow rates. At this pH, the UNC prototype IMER activity surpassed the commercial enzymatic reactors used in this study. Temperature had moderate effect on digestion speed; the IMERs activity improved 2-3 fold when increasing the digestion temperature from 20 to 37 °C.

All three IMERs selected for this study proved to stable at elevated working pressure (2500 psi). The UNC prototype IMER is compatible with high operational pressure up to 15000 psi. Immobilizing trypsin onto a high pressure compatible media opens a possibility of future studies that require high flow rate and high pressure operation with IMER serially connected to long columns or columns packed with sub-two micron particles.

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References

[1] Keil, B. *Specificity of Proteolysis*; Springer-Verlag: New York, 1992.

[2] Stefansson, B.; Helgadóttir, L.; Olafsdottir, S.; Gudmundsdottir, Á.; Bjarnason, J.B. *Comp. Biochem. Phys B* **2010**, *155*, 186–194.

[3] Slysz, G.W.; Schriemer, D.C. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 1044–1050.

[4] Šlechtová, T.; Gilar, M.; Kalíková, K.; Tesařová, E. *Anal. Chem.* **2015**, *87*, 7636−7643.

[5] Yen, C. Y.; Russell, S.; Mendoza, A. M.; Meyer-Arendt, K.; Sun, S.; Cios, K. J.; Ahn, N. G.; Resing, K. A. *Anal. Chem.* **2006**, *78*, 1071−1084.

[6] Monigatti, F.; Berndt, P. *J. Am. Soc. Mass Spectrom.* **2005**, *16*, 13−21.

[7] Safdar, M.; Spross, J.; Jänis, J. *J. Chromatogr. A* 2014, *1324*, 1–10.

[8] Ma, J.; Zhang, L.; Liang, Z.; Zhang, W.; Zhang, Y. *Anal. Chim. Acta* **2009**, *632*, 1–8.

[9] Qiao, J.; Kim, J.Y.; Wang, Y.Y.; Qi, L.; Wang, F.Y.; Moon, M.H. *Anal. Chim. Acta* **2016**, *906*, 156-164.

[10] General product information, available 30.6.2016 from https://www.thermofisher.com/order/catalog/product/2312800

[11] Lombardo, C.R.; Consler, T.G.; Kassel, D.B. *Biochemistry* **1995**, *34*, 16456−16466.

[12] Boerner, R.J.; Kassel, D.B.; Barker, S.C.; Ellis, B.; DeLacy, P.; Knight, W.B. *Biochemistry* **1996**, *35*, 9519−9525.

[13] Hsieh, Y.L.F.; Wang, H.Q.; Elicone, C.; Mark, J.; Martin, S.A.; Regnier, F. *Anal. Chem.* **1996**, *68*, 455−462.

[14] Nadler, T.; Blackburn, C.; Mark, J.; Gordon, N.; Regnier, F.E.; Vella, G. *J. Chromatogr. A* **1996**, *743*, 91−98.

[15] van der Donk, W.A.; Zeng, C.; Biemann, K.; Stubbe, J.A. *Biochemistry* **1996**, *35*, 10058−10067.

[16] Lebrun, B.; Romi-Lebrun, R.; Martin-Eauclaire, M.-F.; Yasuda, A.; Ishiguro, M.; Oyama, Y.; Pongs, O.; Nakajima, T. *Biochem. J.* **1997**, *328*, 321−327.

[17] Blackburn, R.K.; Anderegg, R.J. *J. Am. Sot. Mass Spectrom.* **1997**, *8*, 483−494.

[18] Richards, G.P.; Liang, Y.M.; Chao, J.; Chao, L. *Comp. Biochem. Physiol.* **1997**, *118C*, 39–48.

[19] Fukuda, H.; Irie, K.; Nakahara, A.; Ohigashi, H.; Wender, P.A. *Bioorg. Med. Chem.* **1999**, *7*, 1213–1221.

[20] Rocklin, R.D.; Ramsey, R.S.; Ramsey, J.M. *Anal. Chem.* **2000**, *72*, 5244–5249.

[21] Hara, S.; Katta, V.; Lu, H.S. *J. Chromatogr. A* **2000**, *867*, 151–160.

[22] Riggs, L.; Sioma, C.; Regnier, F.E. *J. Chromatogr. A* **2001**, *924*, 359–368.

[23] Vecchione1, G.; Casetta, B.; Santacroce1, R.; Margaglione, M. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 1383–1390.

[24] Canarelli, S.; Fisch, I.; Freitag, R. *J. Chromatogr. B* **2002**, *775*, 27–35.

[25] Samskog, J.; Bylund, D.; Jacobsson, S.P.; Markides, K.E. *J. Chromatogr. A* **2003**, *998*, 83–91.

[26] Carol, J.; Gorseling, M.C.J.K.; de Jong, C.F.; Lingeman, H.; Kientz, C.E.; van Baar, B.L.M.; Irth, H. *Anal. Biochem.* **2005**, *346*, 150–157.

[27] Nicoli, R.; Gauda, N.; Stella, C.; Rudaz, S.; Veuthey, J.-L. *J. Pharm. Biomed. Anal.* **2008**, *48*, 398–407.

[28] Hoehenwarter, W.; van Dongen, J.T.; Wienkoop, S.; Steinfath, M.; Hummel, J.; Erban, A.; Sulpice, R.; Regierer, B.; Kopka, J.; Geigenberger, P.; Weckwerth, W. *Proteomics* **2008**, *8*, 4214–4225.

[29] Nicoli, R.; Rudaz, S.; Stella, C.; Veuthey, J.-L. *J. Chromatogr. A* **2009**, *1216*, 2695–2699.

[30] Schneiderheinze, J.; Walden, Y.; Dufield, R.; Demarest, C. *J. Chromatogr. B* **2009**, *877*, 4065–4070.

[31] Hoos, J.S.; Sudergat, H.; Hoelck, J.-P.; Stahl, M.; de Vlieger, J.S.B.; Niessen, W.M.A.; Lingeman, H.; Irth, H. *J. Chromatogr. B* **2006**, *830*, 262–269.

[32] Cingöz, A.; Hugon-Chapuis, F.; Pichon, V. *J. Chromatogr A* **2008**, *1209*, 95–103.

[33] Rivera-Burgos, D.; Regnier, F.E. *J. Sep. Sci.* **2013**, *36*, 454–460.

[34] Jelinek, C.A. *Characterizing the post-translational modifications of human serum albumin as they correlate to cardiac ischemia, Chapter IV: Sample Preparation Strategies for use in Clinical MS Assays*, dissertation thesis, Johns Hopkins University 2012, Baltimore, Maryland, available 30.6.2016 from http://gradworks.umi.com/35/28/3528552.html

[35] Lewis, E.; Meyer, K.; Hao, Z.; Herold, N.; Bennett, P. *Rapid Peptide Mapping via Automated Integration of On-line Digestion, Separation and Mass Spectrometry for the Analysis of Therapeutic Proteins*, Thermo Scientific Poster Note, 06/13S, available 30.6.2016 from http://apps.thermoscientific.com/media/cmd/ASMS-TNG-Roadshow/TNG/resouces/805_PN_ASMS13_WP24_ELewis.pdf

[36] Ahn, J.; Jung, M.C.; Wyndham, K.; Yu, Y.Q.; Engen, J.R. *Anal. Chem.* **2012**, *84*, 7256–7262.

[37] Subirats, X.; Rosés, M.; Bosch, E. *Sep. Purif. Rev.* **2007**, *36*, 231–255.

[38] Trypsin, general informations, available 30.6.2016 from http://www.sigmaaldrich.com/life-science/metabolomics/enzyme-explorer/analytical-enzymes/trypsin.html

[39] *Introduction to enzymes*, Worthington Biochemical Corporation, 2015, available 30.6.2016 from http://www.worthington-biochem.com/introbiochem/Enzymes.pdf

Suplementary Table S1: RSD values (%) for Perfinity, Poroszyme and UNC prototype IMERs digestion using BAPNA as a substrate. All experiments at indicated pH, temperatures, and flow rates were performed in eight replicates (n=8). Large RSD values were observed for experiments where BAPNA substrate is nearly consumed (peak integration becomes difficult). Values n.a. represent measurements where BAPNA substrate was digested completely, no RSD's can be calculated.

Perfinity pH 7.0			
	0.3 mL/min	0.2 mL/min	0.1 mL/min
20 °C	0.3 %	6.2 %	4.1 %
25 °C	1.4 %	2.0 %	3.3 %
30 °C	1.6 %	5.6 %	1.8 %
37 °C	0.3 %	8.6 %	3.6 %

Poroszyme pH 7.0			
	0.3 mL/min	0.2 mL/min	0.1 mL/min
20 °C	2.2 %	6.2 %	4.9 %
25 °C	1.7 %	6.6 %	2.7 %
30 °C	1.9 %	3.7 %	6.0 %
37 °C	2.4 %	2.9 %	4.13 %

UNC prototype pH 7.0			
	0.3 mL/min	0.2 mL/min	0.1 mL/min
20 °C	2.2 %	3.1 %	10.1 %
25 °C	1.6 %	5.9 %	6.9 %
30 °C	2.7 %	1.3 %	5.3 %
37 °C	1.6 %	2.3 %	5.7 %

Perfinity pH 8.0			
	0.3 mL/min	0.2 mL/min	0.1 mL/min
20 °C	2.9 %	1.2 %	2.5 %
25 °C	0.1 %	3.7 %	3.0 %
30 °C	4.5 %	9.8 %	9.9 %
37 °C	4.4 %	4.2 %	10.2 %

Poroszyme pH 8.0			
	0.3 mL/min	0.2 mL/min	0.1 mL/min
20 °C	2.3 %	4.3 %	3.1 %
25 °C	4.9 %	7.3 %	4.5 %
30 °C	4.3 %	5.1 %	9.1 %
37 °C	10.0 %	8.3 %	2.3 %

UNC prototype pH 8.0			
	0.3 mL/min	0.2 mL/min	0.1 mL/min
20 °C	3.1 %	7.1 %	8.1 %
25 °C	3.5 %	6.9 %	2.4 %
30 °C	3.0 %	18.3 %	9.2 %
37 °C	2.1 %	8.5 %	5.5 %

Perfinity pH 9.0			
	0.3 mL/min	0.2 mL/min	0.1 mL/min
20 °C	2.7 %	0.9 %	2.7 %
25 °C	2.3 %	2.6 %	1.9 %
30 °C	5.3 %	0.4 %	30.4 %
37 °C	20.5 %	8.5 %	83.0 %

Poroszyme pH 9.0			
	0.3 mL/min	0.2 mL/min	0.1 mL/min
20 °C	3.1 %	0.5 %	4.7 %
25 °C	5.1 %	2.6 %	7.6 %
30 °C	6.0 %	12.6 %	3.3 %
37 °C	6.8 %	12.2 %	46.8 %

UNC prototype pH 9.0			
	0.3 mL/min	0.2 mL/min	0.1 mL/min
20 °C	4.6 %	15.5 %	n.a.
25 °C	7.1 %	22.4 %	n.a.
30 °C	44.9 %	12.9 %	n.a.
37 °C	50.4 %	55.3 %	n.a.

Publikace III – Přímé CE a HPLC metody k separaci tryptofanu a jeho neproteinogenních derivátů

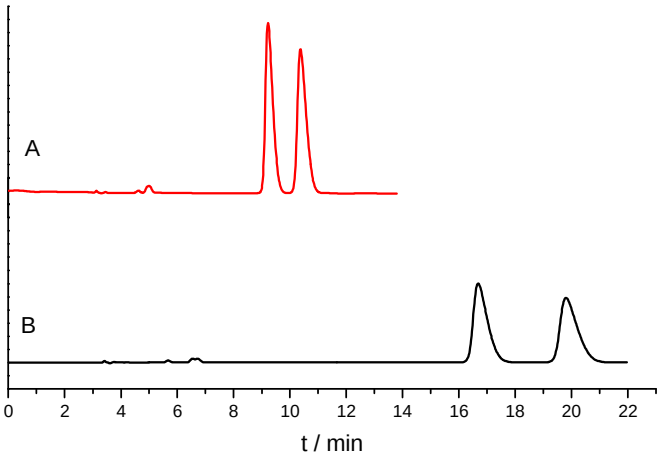
Tryptofan a jeho neproteinogenní deriváty jsou často používány k vývoji a syntéze léčiv nebo jako chirální katalyzátory [19]. Dále se tyto aminokyseliny zavádí do řetězců bioaktivních peptidů, např. pro zvýšení jejich odolnosti vůči enzymům [41].

Publikace III se zabývá vývojem a optimalizací separace enantiomerů tryptofanu a jeho derivátů metodami HPLC a CE. Analyty bylo možné rozdělit do tří skupin: (i) bazické – tryptofan methyl ester, tryptofan butyl ester, tryptofan oktyl ester, tryptofan benzyl ester, tryptofanol; (ii) kyselé – N_α-BOC-tryptofan; (iii) amfoterní – tryptofan, 5-fluoro tryptofan, 5-hydroxy tryptofan.

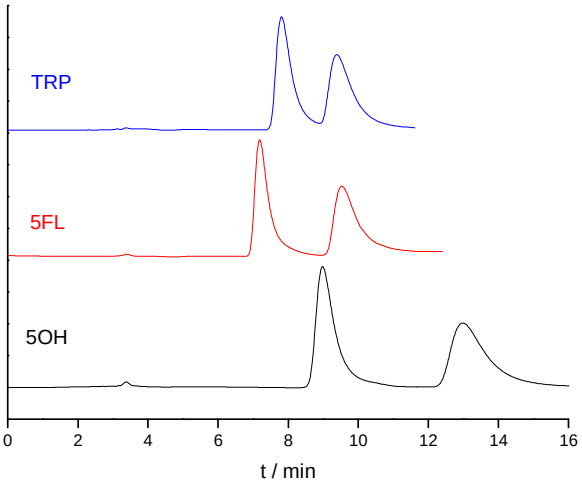
Metody CE byly vyvinuty za použití CD a jeho derivátů jako chirálních selektorů a jsou vhodné především pro kontrolu čistoty enantiomerů. Nejlepších výsledků bylo pro bazické deriváty tryptofanu dosaženo za použití sulfatovaného γ-CD a acetátového pufru (10 mM LiOH/20 mM kyselina octová, pH 4,74). Enantiomery tryptofan oktyl esteru byly separovány v duálním systému β-CD + sulfatovaný γ-CD v pufru o stejném složení. Enantiomery tryptofanu a jeho amfoterních derivátů byly separovány za použití sulfatovaného γ-CD a fosfátového pufru, pH 2,09. Nejnáročnější bylo nalezení vhodných podmínek pro separaci enantiomerů kyselého N_α-BOC-tryptofanu. Při použití heptakis(6-amino-6-deoxy)-β-CD a acetátového pufru byly i tyto enantiomery separovány na základní linii. Metodou CE byly separovány enantiomery všech analytů na základní linii a čas separace nepřekročil 8 min.

Při vývoji vhodných HPLC metod bylo nutné použít různé typy chirálních stacionárních fází a separačních módů vzhledem k velkým strukturním rozdílům mezi analyty. Byly testovány chirální stacionární fáze na bázi teikoplaninu, CD, CF a polysacharidů. Vyvinuté HPLC metody jsou vhodné pro kontrolu čistoty enantiomerů i pro semipreparativní účely. Nejlepší enantioseparace N_α-BOC-tryptofanu (R = 1,79) bylo dosaženo v normálním módu za použití kolony Chiralpak IB, obsahující tris(3,5-dimethylfenylkarbamát) celulózy jako chirální selektor a MP složené z hexan/izopropanol/kyselina trifluoroctová v objemovém poměru 80/20/0,1. Enantiomery bazických derivátů tryptofanu byly separovány v normálním módu na stejné koloně, MP obsahovala stejné organické složky a jako aditivum ethanolamin nebo kyselinu trifluoroctovou. Vliv složení MP na separaci enantiomerů butyl esteru tryptofanu v RP

módu ilustruje Obr. 3. Tryptofan a jeho amfoterní deriváty byly na teikoplaninových kolonách Chirobiotic T a Chirobiotic T2 separovány na základní linii v HILIC i polárně organickém módu. Obr. 4 zobrazuje separaci enantiomerů těchto analytů v HILIC módu na koloně T2. Nejjednodušší MP pro separaci tryptofanu a jeho amfoterních derivátů se skládala ze 100 % MeOH. Na kolonách obsahujících teikoplanin byl testován vliv množství MeOH na retenci enantiomerů tryptofanu a jeho amfoterních derivátů. Bylo potvrzeno, že kolony na bázi teikoplaninu vykazují tzv. „mixed mode“ chování – v závislosti na množství organické fáze vykazují chování charakteristické pro více chromatografických módů. Při nízkém obsahu MeOH v MP se systém chová jako reverzní, zatímco při vysokém obsahu MeOH přechází do HILIC módu. U obou chromatografických systémů se sice používají obdobné složky MP, ale systém retence je opačný. V RP módu se na retenci analytů nejvíce podílí hydrofobní interakce, takže látky s vyšší polaritou eluují nejdříve a s rostoucím množstvím organické složky MP retence klesá. V HILIC systému, podobně jako při separacích na normální fázi, se retence zvyšuje s polaritou analytu a snižuje se zvýšením polarity MP – s rostoucím množstvím organické složky v MP roste retence. Proto je HILIC systém vhodnější pro separace polárních látek, např. pro peptidy nebo polární léčiva, které jsou v RP systému eluovány v blízkosti mrtvého času kolony.



Obr. 3 Vliv složení MP na enantioseparaci bazického butyl esteru tryptofanu v RP módu na koloně s derivatizovaným β-CD (Astec Cyclobond^{TM} I 2000 HP-RSP). (A) MeOH/10 mM octan amonný, pH 4,0 40/60; (B) MeOH/10 mM octan amonný, pH 4,0 30/70 (*v/v*).



Obr. 4 Separace enantiomerů tryptofanu, 5-fluoro tryptofanu a 5-hydroxy tryptofanu na koloně Chirobiotic T2 v MP MeOH/10 mM octan amonný 95/5 (*v/v*), pH 4,0.

Publikace III

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.

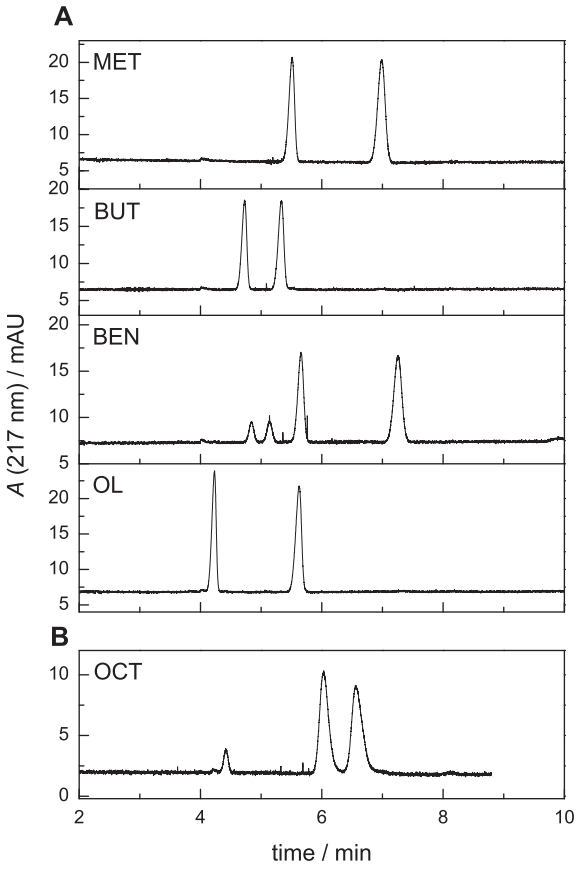

Fig. 1. Enantioseparation of basic Trp derivatives (A) MET, BUT, OCT, BEN with 5.0 mM S-γ-CD and (B) OL with mixed CSs: 1.0 mM β-CD + 4.0 mM S-γ-CD; acetate buffer (10 mM LiOH/20 mM acetic acid, pH 4.74), separation voltage −15 kV, additional pressure +30 mbar.

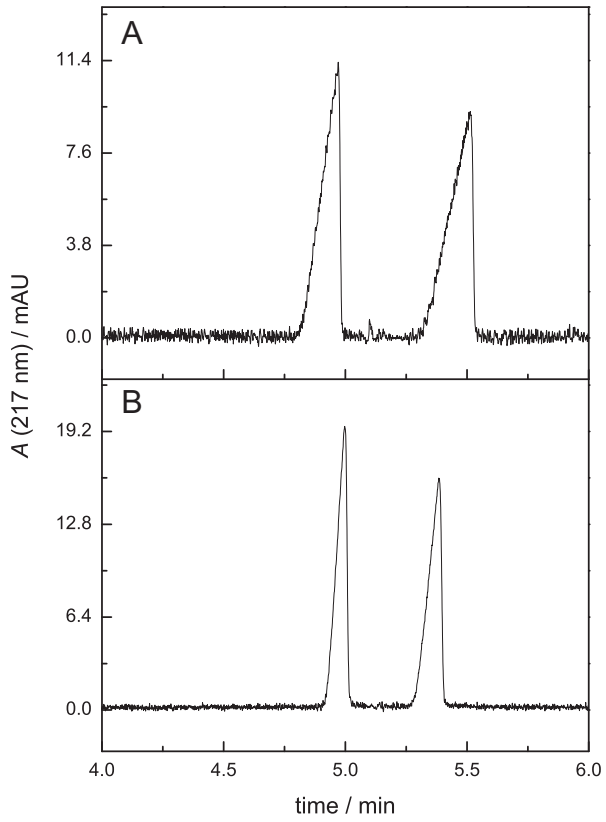

Fig. 2. Enantioseparation of BTrp in acetate buffer (10 mM LiOH/20 mM acetic acid, pH 4.74); separation voltage +25 kV, no additional pressure; (A) 1.0 mM A-β-CD; (B) 2.5 mM A-β-CD.

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)

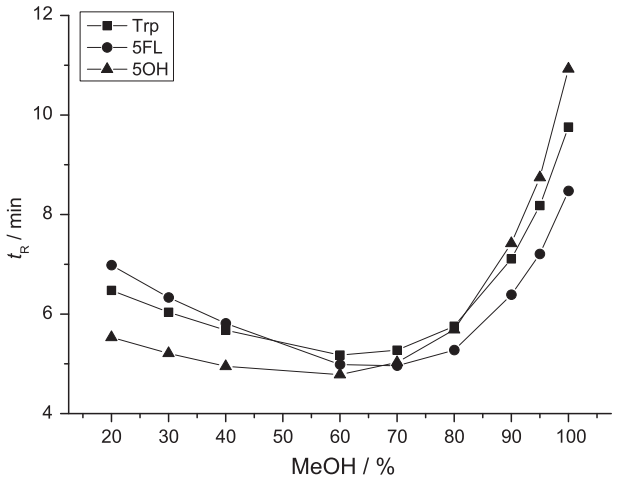

Fig. 3. The effect of MeOH content in mobile phase on retention time of the first eluted enantiomer of Trp, 5FL and 5OH.

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 IV – Chirální stacionární fáze na bázi cyklických oligosacharidů používaných pro kontrolu čistoty léčiv

Na enantiomerní čistotu léčiv jsou v dnešní době kladeny vysoké nároky, především od nechvalně známého incidentu s thalidomidem v šedesátých letech minulého století [34]. Uvedení léčiva na trh dnes nutně předchází rozsáhlé studie na toxicitu a farmakologické účinky jednotlivých enantiomerů [32,33]. Se zajištěním enantiomerní čistoty léčiv je spojen vývoj efektivních a citlivých separačních metod, které budou schopny detekovat i stopová množství nežádoucích příměsí. Nejčastěji se používá metoda HPLC s CSP, dále v této oblasti nalezly uplatnění GC, SFC, CE, micelární elektrokinetická chromatografie a kapilární elektrochromatografie.

Publikace IV shrnuje metody vhodné pro separaci léčiv za použití CSP na bázi cyklických oligosacharidů – CD, CF a jejich derivátů – v rozmezí let 2000 a 2015. Základním rozdílem mezi těmito chirálními selektory jsou sacharidové jednotky, ze kterých se skládají, a velikost jejich kavity. Vzhledem k těmto rozdílům vykazují rozdílné separační vlastnosti. CD jsou tvořeny 6 - 8 glukopyranosovými jednotkami, které vytváří hydrofobní kavitu [42]. CF jsou tvořeny obdobným počtem fruktofuranosových jednotek vytvářejících kavitu hydrofilní, která je menší než u CD [43]. Oba chirální selektory mají v nativním stavu nižší enantioselektivní účinnost než jejich deriváty. Vzhledem k rozdílným fyzikálně-chemickým vlastnostem nalezly v HPLC CD CSP uplatnění především v RP módu a CF CSP v normálním a polárně-organickém módu. CSP na bázi CF byly použity také v HILIC pro achirální separace léčiv a jejich nečistot. Lze očekávat, že v budoucnosti budou s rostoucí oblibou ultrarychlých separací upřednostňovány metody SFC a UPLC, ačkoli je vývoj stacionárních fází kompatibilních s těmito technikami poměrně náročný.

Publikace IV

Cyclic Oligosaccharide-Based Chiral Stationary Phases Applicable to Drug Purity Control; A Review

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Cyclic Oligosaccharide-Based Chiral Stationary Phases Applicable to Drug Purity Control; A Review

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Abstract: Oligosaccharide-based chiral stationary phases are frequently used for enantioselective separations by different chromatographic techniques, namely gas chromatography, high performance liquid chromatography, supercritical fluid chromatography or capillary electrochromatography. Their multimodal application potential (they are compatible with both polar and/or non-polar mobile phases) makes them suitable chiral selector candidates for separation of a wide variety of structurally diverse compounds. In this paper, separation systems utilizing cyclodextrin- or cyclofructan-based chiral stationary phases in analyses of pharmacologically active compounds are summarized. The review covers the period from 2000 to 2015. This review article can be helpful to analysts searching for an appropriate method for the separation/determination of pharmaceuticals of their interest.

Keywords: Cyclodextrin, cyclofructan, pharmaceuticals, drugs, enantioseparation, enantioselectivity, chromatography

1. INTRODUCTION

It is a well-known fact that different enantiomers of drugs can exhibit distinct biological effects in the chiral environment of living organisms. The unequal behavior of drug enantiomers was alerted by the case of thalidomide in the sixties of the 20th century, when the teratogenic effect of one enantiomer was recognized too late. The thalidomide tragedy was a landmark in drug regulation [1,2], but the proposal that the tragedy could have been avoided if only single enantiomer had been used is misleading. Later, it was proved that both enantiomers are teratogenic [3] and the drug racemizes in the body [4]. Efficient separation methods are needed for the separation, determination and isolation of even traces of unwanted enantiomers present in pharmaceuticals, since the differences in efficiency, toxicity and pharmacological effects must be considered before a new drug appears on the market and can be administered. Among the methods used, high performance liquid chromatography (HPLC) employing chiral stationary phases (CSPs) is a technique of choice for its reliability [5-7]. Nevertheless, other chromatographic methods such as gas chromatography (GC), supercritical fluid chromatography (SFC) or capillary electrochromatography (CEC) are also applied, mainly for the analytical purposes. The utilization of enantioselective techniques for separation of pharmaceuticals is reviewed in many publications [for example 8-13], but none of them focused directly on the use of cyclic oligosaccharide-based CSPs, namely cyclodextrin (CD), cyclofructan (CF) and their derivatives. This review displays these CSPs used for analyses of drugs by various chromatographic techniques, namely GC, HPLC, SFC and CEC for the period between years 2000 and 2015. Reviewed articles deal either with (i) development, description and validation of the enantioselective methods for drug analyses, or (ii) preparation of new CSPs and evaluation of their separation potential by a set of analytes including drug representatives.

1.1. Cyclodextrins *versus* cyclofructans

CDs and CFs are both cyclic oligosaccharides composed of several monosaccharide units. CDs consist of glucopyranoses, while CFs are formed by fructofuranoses. Despite certain structural similarity, CD- and CF-based CSPs behave differently. CDs (α, β and γ) compose of 6, 7 or 8 D-glucopyranose units and possess a hydrophobic cavity. Its size is proportional to the number of saccharide units - 5.2 Å for α-CD, 6.4 Å for β-CD and 8.3 Å for γ-CD. Precise fit of analyte or its hydrophobic part into rigid cavity of CD is the main/leading "interaction" of the retention mechanism in reversed phase (RP) mode [14,15]. CFs consist preferentially of 6, 7 or 8 D-fructofuranose units (abbreviations: CF6, CF7, CF8, respectively) connected by β-(2,1) linkages creating a crown-ether skeleton. CFs have much smaller cavity with inner diameters 2.3 Å for CF6, 4.1 Å for CF7 and 4.7 Å for CF8. CF core is hydrophilic due to the presence of crown oxygens [16,17]. Compared to their derivatives, both chiral selectors (CSs) possess lower enantioselective abilities in the native state. The derivatization procedure offers new or modified interaction properties compared to the native CSs.

2. SEPARATION/ANALYSIS IN GC

The right choice of CSP is essential for successful direct chiral separation in GC. There is no other possibility for modifying the enantioselective environment in GC than altering the CSP. Although several reviews concerning the use of CD-based stationary phases in GC were published [15,18,19], none of them directly focus on the analysis of pharmaceutical compounds in the year period of 2000-2015.

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GC methods on CD-based CSPs suitable for pharmaceutical analyses of different drug enantiomers are summarized in Table 1 [20-25]. Derivatized CD-based CSPs were used in all cases and in the majority of publications derivatization of the analytes was required before separation.

Analytes of great interest were methylamphetamine and its precursors and intermediates [23-25]. Various capillary Astec Chiraldex columns were used to simultaneously separate enantiomers of all these compounds in one run.

Table 1.

3. SEPARATION/ANALYSIS IN HPLC

In HPLC, enantioselective separation can be substantially affected not only by the choice of CSP but also by mobile phase (MP) composition. Separation mode determines the interaction mechanism which is responsible for the enantioselective recognition. CSPs based on CDs or CFs and their derivatives can be used in all separation modes, *i.e.* normal phase (NP), RP, polar organic (PO) mode or hydrophilic interaction liquid chromatography (HILIC). In NP mode, polarity of MP is lower than polarity of the stationary phase. Usual MP components are *n*-hexane (HEX) or *n*-heptane, some alcohol and often a small amount of acidic or basic additives. In RP mode, polarity of MP is higher than that of stationary phase. Water or buffer phase is combined with organic modifier, usually acetonitrile (ACN) or methanol (MeOH). Typical MPs for PO mode compose of MeOH or ACN as the main component and acidic or basic additives. In HILIC, typical MP contains ACN (min 60-70 volume %) and aqueous part (water or buffer).

3.1. Native cyclodextrin-based CSPs

3.1.1. Chiral separations

Despite the fact that derivatized CD-based CSPs usually provide better enantioseparation results, some authors also successfully applied native CD-based CSPs. Native β-CD CSP (commercial name Cyclobond I (β) column) was successfully used for simultaneous separation of enantiomers of PNU-83894 and its metabolite PNU-83892 in plasma [26]. PNU-83894 and PNU-83892 are active circulating metabolites of an anticonvulsant drug PNU-54494A [27]. Optimized RP separation conditions were: ACN/water containing 0.2% triethylamine (TEA) adjusted with acetic acid (HAc) to pH 6.0, 20/80 (*v/v*) as a MP, temperature 21-23 °C. Optimized method was subsequently validated. β-CD-based CSP composed of β-CD polymer coated silica support was prepared and successfully used for the enantioseparation of warfarin [28]. Separation conditions were: MeOH/0.1M sodium acetate buffer, pH 4.0, 20/80 (*v/v*) as a MP, temperature 25 °C. Good enantioselectivity, $\alpha = 1.3$, was obtained under above mentioned conditions. Simple LC-MS method for simultaneous determination of enantiomers of anticholinergic drugs produced as racemates, *i.e.* trihexyphenidyl, biperiden and procyclidine in human plasma was developed [29]. PO mode chromatographic conditions were: ACN/MeOH/HAc/TEA 95/5/0.5/0.3 (*v/v/v/v*) and native β-CD CSP (Cyclobond I 2000 column). Quantitation was performed by electrospray ionization MS using diphenidol as an internal standard. HPLC-tandem MS method for the analysis of warfarin enantiomers in human plasma was developed and validated [30]. Baseline resolution of warfarin and internal standard enantiomers (*p*-chlorowarfarin) was achieved on a β-CD column with MP composed of ACN/HAc/TEA 100/0.3/0.25 (*v/v/v*) at a room temperature. Analysis time did not exceed 8 min. Monolithic silica columns with chemically bonded β-CD were prepared and successfully used for the enantioseparation of some chiral drugs [31]. Enantiomers of oxazepam, cromakalim, norgestrel, methadone and prominal were baseline separated in RP mode under various chromatographic conditions depending on the drug structure. A big advantage of the use of monolithic columns is the possibility to operate at high flow rates to fasten the separation while maintaining high efficiency. Cyclobond I 2000 column was used for the enantioseparation of anti-allergic drug azelastine and its three metabolites [32]. Under optimized conditions, ACN/MeOH/45 mM ammonium acetate, pH 4.7, 9/21/70 (*v/v/v*), temperature 5 °C, enantiomers of azelastine and its major metabolite demethylazelastine were separated simultaneously. Weaker performance was observed for two other chiral metabolites, 6-hydroxy-azelastine and 7-hydroxyazelastine. Nevertheless, chiral impurity of more than 0.5% was detected in both azelastine metabolites. Afterwards, optimized HPLC method with MS-MS detection was applied for the biotransformation study of azelastine enantiomers in rats after oral application. *S*-(+)-azelastine metabolic pathway is completely different compared to *R*-(-)-azelastine. Pihlainen and Kostiainen used Cyclobond I 2000 column for the enantioseparation of methorphan and amphetamine derivatives, *i.e.* 3,4-methylenedioxy-ethylamphetamine and 3,4-methylenedioxy-methamphetamine [33]. PO mode was the most convenient for methorphan, MP composed of MeOH/ACN/HAc/TEA 10/90/0.03/0.02 (*v/v/v/v*). On the other hand, RP mode gave the best results for enantioseparation of both amphetamine derivatives. Baseline enantioseparations were observed with MP composed of MeOH/1% triethylammonium acetate (TEAA) buffer, pH 4.0, 5/95 (*v/v*). All separations were performed at 15 °C. Simple RP HPLC method with native β-CD CSP (LichroCART 250-4 ChiraDex column) was developed and validated for simultaneous determination of eslicarbazepine acetate and its metabolites, oxcarbazepine, *S*-licarbazepine and *R*-licarbazepine in human plasma [34]. Chromatographic separation was achieved in 25 min by isocratic elution in MeOH/water 12/88 (*v/v*) as a MP, temperature 30 °C. Method for the enantioseparation and determination of perindopril erbumine, an ACE inhibitor, was developed and some validation parameters were determined [35]. Optimized chromatographic conditions were: Chiradex column (β-CD chemically bonded to spherical silica gel particles), ACN/50 mM phosphate buffer, pH 3.0, 55/45 (*v/v*), room temperature. Baseline separation was observed within 8 min. Optimized method was successfully applied for the analysis of *S*-enantiomer content in commercial tablets containing perindopril erbumine racemate. Enantiomers of oxazepam were successfully separated on native β-CD CSP (Cyclobond I 2000 column) in RP mode within 8 min with enantioselectivity $\alpha = 1.5$

[36]. Optimized chromatographic conditions were: MeOH/buffer (LiOH/HAc 9.90/60.0 mM), pH 4.01, 40/60 (*v/v*) as a MP, temperature 25 °C. 2D RPLC method with electrospray ionization-tandem MS was developed and validated to determine sertraline enantiomers in rat plasma to support pharmacokinetic studies [37]. Method was applied to separate and determine the diastereomers and enantiomers of sertraline simultaneously. System consisted of Hisep-RAM column in the first dimension for trapping proteineous part of plasma and a chiral Cyclobond I 2000 column in the second dimension. MP composed of ACN/0.1% trifluoroacetic acid (TFA), 86/14 (*v/v*).

3.1.2. Achiral separations

Native β-CD-based CSPs were also used for achiral separations of some drugs. Cyclobond I 2000 column was used for the separation of different organic acids in PO mode, MPs composed of ACN/MeOH/TEA/HAc (*v/v/v/v*) [38]. Analytes were eluted under gradient conditions and the elution order depended on the number, type and position of hydrogen bonding functional groups present in the molecule. Optimized conditions were used to evaluate the purity of potential pharmaceutical drug candidates that showed activity towards a kinase target vascular endothieal growth factor (Vegf). Using this method, purity estimates, which were difficult to determine by other HPLC methods, were obtained. HPLC method using Cyclobond I column for the simultaneous determination of ampicillin, sulbactam, and cefoperazone in pharmaceutical formulations was developed [39]. MP composed of MeOH/5 mM TEAA buffer, pH 4.5, 35/65 (*v/v*). Proposed method was reproducible and convenient for routine analyses of these drugs in sterilized water, saline, or 5% dextrose injection solutions. Cyclobond I β-CD column was applied for the separation of drug substance 2-[4-(1-hydroxy–4-[4-(hydroxydiphenylmethyl)–1-piperidinyl]-butyl)-phenyl]–2-methylpropionic acid (*para* isomer) and its *meta* isomer (positional isomeric impurity) [40]. The *para* isomer has antihistamine properties and is classified as a histamine H-1 receptor antagonist. Optimized separation conditions were: ACN/0.02 M ammonium acetate buffer as a MP, pH 4.0, flow rate 0.45 mL/min. Under these conditions, *meta* isomer impurity eluted prior to the parent peak. New HPLC method for the simultaneous determination of ticarcillin (TIC) and clavulanic acid (CA) in pharmaceutical formulations was designed and validated [41]. TIC, α-carboxypenicillin, is a semisynthetic β-lactam antibiotic, and CA works as a potent and irreversible β-lactamase inhibitor. Baseline separation was achieved on a Cyclobond I column with MeOH/16 mM ammonium acetate buffer, pH 6.0 50/50 (*v/v*) as a MP. Method was successfully applied for the simultaneous determination of these two drugs in sterilized water and 5% dextrose injection solutions. Separations of estrogen metabolites, *i.e.* sulfates and glucuronides, were performed in HILIC with native β-CD [42]. ACN and 5 mM ammonium acetate as a MP and flow rate 0.2 mL/min were used. Three estrogen glucuronides, *i.e.* estrone-3-glucuronide, 17β-estradiol-3-glucuronide and estriol-3-glucuronide were well resolved at 70% ACN. Baseline resolution was achieved for all estrogen metabolites studied (three glucuronides mentioned above and two sulfates, estrone sulfate and 17β-estradiol sulfate), but their strong retention resulted in a substantial band broadening.

3.2. Cyclodextrin derivatives-based CSPs

As already mentioned above, derivatives of CDs offer new interaction possibilities and thus increase the application potential of CD-based CSPs, in general and also in drug analysis. Many different CD derivatives-based CSPs were prepared and some of them are even commercialized.

3.2.1. Chiral separations

Enantiomers of lorazepam, a benzodiazepine drug, were baseline separated in RP mode with carboxymethyl β-CD CSP (Shodex ORpak CDBS-453 column) [43]. Optimized separation conditions were: ACN/0.2 M NaCl with 1% HAc 13/87 (*v/v*), temperature 15 °C. Optimized method was applied for the determination of lorazepam enantiomers in plasma after administration of a therapeutic dose of racemic drug. Under these conditions, no interconversion of enantiomers was observed. Series of norborn-2-ene-derivatized β-CD-based CSPs were prepared *via* ring-opening metathesis graft-polymerization and successfully used for the enantioseparation of proglumide, drug that inhibits gastrointestinal motility and reduces gastric secretions [44]. The best MP composition was ACN/MeOH/HAc/TEA 98/2/0.2/0.2 (*v/v/v/v*). Acetylated derivative of β-CD CSP (Cyclobond I 2000 Ac column) was used for the separation of citalopram and its metabolites, desmethylcitalopram and didesmethylcitalopram [45]. The best separation conditions were: MeOH/10 mM citric acid adjusted to pH 6.3 with TEA 55/45 (*v/v*) as a MP, temperature 30 °C. Individual analytes were baseline separated but only partial enantioseparation of all these enantiomers was achieved in one run. Separation of three stereoisomers of β-blocker nadolol was obtained using heptakis (6-azido-6-deoxy-2,3-di-*O*-phenylcarbamolyted) β-CD bonded CSP and MeOH/1% TEAA buffer, pH 5.5, 20/80 (*v/v*) as a MP at 20 °C [46]. Under optimal conditions, complete separation of the most active enantiomer (*R,S,R*)-nadolol was achieved. Similar results for nadolol enantiomers were obtained with perphenyl carbamoylated β-CD immobilized onto silica gel as a CSP and the same MP composition as mentioned before [47]. Tolperisone, a centrally acting muscle relaxant, was enantioseparated using heptakis(6-azido-6-deoxy)perphenylcarbamated β-CD chemically immobilized on silica gel as CSP [48]. Optimized MP composed of MeOH/1% TEAA buffer, pH 5.0, 60/40 (*v/v*) and temperature 22 °C. Baseline enantioseparation was observed within 13 minutes. New chemically bonded β-CD-based CSP was prepared in a "one pot" process by reaction of phenylated β-CD with silica gel [49]. This CSP was successfully applied for the separation of metoprolol, lorazepam and oxazepam enantiomers in NP mode. Optimized MPs composed of *n*-heptane/propane-2-ol (IPA) 90/10 (*v/v*) for oxazepam and lorazepam, and *n*-heptane/IPA/diethylamine 80/20/0.1 (*v/v/v*) for metoprolol. HPLC method with Cyclobond I-2000 RSP column ((*R,S*)-hydroxypropyl modified β-CD) for the enantioseparation of oxazepam, lorazepam and temazepam was developed [50]. Low temperature 12 °C was used during the separation to

avoid spontaneous chiral interconversion of enantiomers. Optimized MP composition was the same for all drugs, *i.e.* ACN/1% TEAA buffer, pH 4.5/water 19/8/73 (*v/v/v*). The same CSP but different MP composition, *i.e.* ACN/ethanol/TEA/HAc 100/1/0.001/0.001 (*v/v/v/v*), was used for the determination of oxazepam enantiomers in rabbit plasma. An EP/β-CDN$^+$ polymer column prepared by adsorption of epichlorhydrin/β-CDN$^+$ polymer (linkage of about one hydroxypropyl trimethyl ammonium group per β-CD unit) on Lichrospher Si-100 column was successfully applied for the enantioseparation of warfarin [51]. Separation conditions were: MeOH/0.1 M sodium acetate with HAc, pH 4.0, 30/70 (*v/v*) as a MP and temperature 22 °C. A novel CSP prepared by immobilization of heptakis(6-azido-6-deoxy-2,3-di-*O*-phenylcarbamoylated)-β-CD onto the surface of amino-functionalized silica gel was used for the enantioseparation of a variety of drugs in RP and NP modes [52]. Individual drugs, chromatographic conditions and parameters are summarized in Table 2. As expected, RP mode was favored for the enantioresolution. Brush-type 2-hydroxypropyl-β-CD CSP was developed using superficially porous particles (SPP), which are advantageous for ultrafast separations due to the high efficiencies and relatively low back pressures [53]. Using this CSP, enantiomers of pain-relievers nefopam and methadone were baseline separated in MPs composed of ACN/20 mM ammonium acetate 65/35 and ACN/0.1% acetic acid 78/22, respectively.

Table 2.

Cyclobond I-2000 RSP column was used for the enantioseparation of modafinil, a stimulant approved by the FDA to be used in the management of excessive sleepiness associated with narcolepsy [54]. To investigate the pharmacokinetics of modafinil in patients, HPLC method for the separation and quantitation of modafinil enantiomers in human serum was improved and validated. Gradient elution was used, MP A composed of 20 mM sodium phosphate adjusted to pH 3.0 with o-phosphoric acid, MP B composed of 30% ACN in MP A. MP B increased from 30% to 60% over a 30 min period and then returned to 30% B for 5 min. Various dihydrofurocoumarins (substituted psoralens and substituted angelicins) were baseline resolved on Cyclobond I RSP (hydroxypropyl β-CD as CS), Cyclobond I AC (acetyl β-CD as CS) and Cyclobond I DM (2,3-dimethyl β-CD as CS) columns in RP mode [55]. Simple MPs were used, *i.e.* MeOH/water or ACN/water in different volume ratios depending on the enantiomers separated. All the separations were carried out at a room temperature. Two CSPs, ph-α-CD and ph-γ-CD, were prepared from mono(6^A-azido-6^A-deoxy)perphenylcarbamoylated α- and γ-CDs immobilized onto silica gel [56]. Ph-γ-CD CSP showed good enantioselectivity for bendroflumethiazide and trichlormethiazide enantiomers under RP conditions. The best MP compositions were MeOH/1% TEAA buffer, pH 4.75, 30/70 (*v/v*) and MeOH/1% TEAA buffer, pH 4.75, 5/95 (*v/v*) for bendroflumethiazide and trichlormethiazide, respectively. Ph-α-CD CSP showed higher enantioselectivity for a broader spectrum of drugs. Enantiomers of bendroflumethiazide, trichlormethiazide, bupivacaine and chloroquine were baseline separated in MPs composed of MeOH and 1% TEAA buffer of various pH values in different volume ratios according to the structure of separated drugs. Proglumide and some β-blockers, propranolol, alprenolol and pindolol were baseline enantioresolved using a CSP prepared by immobilizing mono(6^A-N-allylamino-6^A-deoxy)-perphenylcarbamoylated β-CD onto the surface of silica gel *via* hydrosilylation [57]. Optimized chromatographic conditions for all these drugs were: MeOH/1% TEAA buffer, pH 5.5, 35/65 (*v/v*) as a MP and ambient temperature. Armstrong´s group synthesized and evaluated nine dinitrophenyl (DNP) substituted β-CD-based CSPs for the enantioseparation of various classes of chiral compounds [58]. Prepared CSPs differed at the degree of substitution of DNP group on β-CD and also at the position of nitro groups on phenyl ring. Three CSPs contained also additional trifluoromethyl group on phenyl ring and one had additional amino substituent on phenyl ring. All these CSPs were confirmed as multimodal, operating in RP, NP and PO modes. Baseline enantioseparations of different chiral drugs were observed, e.g. norephedrine hydrochloride, oxyphencyclimine hydrochloride, indapamide, ketoprofen, iophenoxic acid, mephenytoin, aminoglutethimide, oxazolidinone derivatives, oxazepam, nefopam hydrochloride, methyl trans-3-(4-methoxyphenyl)glycidate, naringin, flavanone, 5-(4-hydroxyphenyl)-5-phenylhydantoin and chlorthalidone under different separation conditions. RP mode was the most promising. New β-CD bonded silica called RAM-Chiral was designed for a direct determination of chiral drugs in biological fluids [59]. This RAM-chiral CSP was synthesized by a two step process: (i) introduction of β-CD carbamate on 70 Å pore size amino silica, (ii) coating of the external silica surface with bovine serum albumin (BSA). Drugs were retained and resolved by β-CD selector, while plasma proteins were excluded from RAM-chiral column by the BSA coated external silica surface. The drugs of interest were benzodiazepines, namely oxazepam, lorazepam, temazepam and lormetazepam. Plasma proteins were eluted with 100 mM sodium phosphate buffer, pH 7.0, followed by a step gradient elution with MP composed of the same buffer (80%) and MeOH (20%). The recovery of racemic drugs from plasma doped with benzodiazepines was almost 100%. Calix[4]arene-capped [3-(2-*O*-β-CD)-2-hydroxypropoxy]propylsilyl-appended silica particles were synthesized as a new CSP and applied for the enantioseparation of some drugs [60]. Baseline separations of tryptophan and indapamide enantiomers were achieved in RP mode at a room temperature. Optimized MP for separation of tryptophan enantiomers was ACN/water 40/60 (*v/v*). Excellent resolution of indapamide enantiomers, R = 5.14, was observed within 7 min in a simple MP composed of ACN/water 60/40 (*v/v*). Rao *et al*. developed RP HPLC method for the simultaneous separation of antidepressant sertraline and related enantiomeric impurities using Cyclobond I 2000 DM column (dimethyl β-CD CSP) [61]. Optimized chromatographic conditions were: ACN/0.4% TFA, pH 3.0, 20/80 (*v/v*) as a MP, temperature 30 °C. Optimized method allowed separation of cis (1*S*,4*S*), (1*R*,4*R*) and also trans (1*S*,4*R*), (1*R*,4*S*) enantiomers of sertraline HCl along with 5 other related enantiomers. Designed method

was validated for the determination of enantiomeric purity of sertraline HCl in drug substances and formulations. Cyclobond I 2000 DNP column (dinitrophenyl substituted β-CD CSP) was used for the enantioseparation of methanobenzazocines, compounds with analgesic activity, under RP conditions [62]. Two of the tested compounds belong to 1,5-methano-3-benzazocine group and three to 2,6-methano-3-benzazocine group. Four of five tested enantiomeric pairs were baseline separated in MPs composed of MeOH or ACN and ammonium acetate buffers, pH 5.0 or 7.0, in various volume ratios depending on the structure of enantiomers. Other β-CD-based columns, *i.e.* Cyclobond I 2000 and Cyclobond I 2000 DM, were also tested for the enantioselective recognition ability against methanobenzazocines. Both columns showed enantioselectivity only for compounds from 2,6-methano-3-benzazocine group. Permethyl-β-CD, β-CD and *R,S*-hydroxypropyl-β-CD CSPs prepared by a novel synthetic route were used for the enantioseparation of 19 β-lactams, which are the most widely used types of antibiotics [63]. Eleven of them were baseline separated on permethyl-β-CD, two on *R,S*-hydroxypropyl-β-CD and one on β-CD CSPs in a MP composed of ACN/0.1% TEAA, pH 5.0, 10/90 (*v/v*). Resolution higher than 5 was observed for some enantiomers on permethyl-β-CD CSP. Vancomycin-capped (3-(2-*O*-β-CD)-2-hydroxypropoxy)-propylsilyl-appended silica particles were prepared and used as a new type of β-CD-based CSP for the separation of some racemic drugs in RP mode [64]. This new CSP type combines vancomycin and β-CD CSs sites and thus can provide multiple interactions with analytes. Racemic proglumide, warfarin, pindolol and phenylalanine were successfully enantioseparated in MPs composed of ACN/aqueous part 10/90 (*v/v*). The aqueous part composed of water, 1% TEAA buffer, pH 7.0, for phenylalanine and pindolol, and 1% TEAA buffer, pH 4.0, for proglumide and warfarin. Ibuprofen enantiomers were baseline separated on a new β-CD functionalized organic polymer monolith in RP mode within 7 min [65]. CSP was prepared by covalently bonding ethylenediamine-β-CD to poly(glycidyl methacrylate-co-ethylene glycol dimethacrylate) monolith *via* ring opening reaction of epoxy groups. Optimized MP composed of MeOH/0.5% TEAA buffer, pH 4.0, 30/70 (*v/v*). Heptakis(6-deoxy-6-azido-phenylcarbamoylated)-β-CD CSP was prepared by a click chemistry and used for the enantioseparation of a variety of pharmaceutically active compounds [66]. Baseline enantioseparations of flavanone and some flavanoids (6-methoxyflavanone, 7-methoxyflavanone, 4′-hydroxyflavanone), β-blockers (alprenolol, propranolol), β-agonists (terbutaline, dobutamine, fenoterol) and atropine were achieved in RP mode. Different MPs compositions were suitable for these groups of pharmaceuticals. MP composed of MeOH/water 50/50 (*v/v*) for flavanone and flavonoids; MeOH/1% TEAA, pH 4.2, 35/65 (*v/v*) for β-blockers, fenoterol and atropine; MeOH/1% TEAA, pH 4.2, 5/95 (*v/v*) for terbutaline; MeOH/1% TEAA, pH 4.2, 50/50 (*v/v*) for dobutamine. The same research group prepared two new CSPs by immobilization of mono-6-azido-perphenylcarbamated-β-CD (CCP) and mono-6-azido-permethylated-β-CD (CCM) onto alkynyl modified silica *via* click chemistry [67]. CCM CSP showed good enantiorecognition for α-ionone and 3-methyl-α-ionone in a simple MP composed of MeOH/water 30/70 (*v/v*). CCP CSP exhibited wider enantioselectivity in RP mode. Baseline enantioseparations of flavanone and some flavonoids (6-methoxyflavanone, 7-methoxyflavanone, 4′-hydroxyflavanone), β-blocker nadolol, atropine, and thiazide diuretics (bendroflumethiazide, cyclothiazide) were achieved in MPs composed of MeOH/1% TEAA, pH 6.5, or ACN/water in different volume ratios. Flow rates 0.5 mL/min and 0.7 mL/min were used in case of CCM and CCP CSPs, respectively. Thamarai Chelvi *et al.* synthesized new BACD-HPS packed column and used it for the enantioseparation of drugs and intermediates in pharmaceutical synthesis in RP and NP modes [68]. BACD-HPS (bromoacetate-substituted [3-(2-*O*-β-CD)-2-hydroxypropoxy]propylsilyl-appended silica particles) was prepared by treatment of 3-(2-*O*-β-CD)-2-hydroxypropoxypropylsilyl-appended silica particles with bromoacetyl bromide. Enantiomers of benzyl mandelate, phenylalanine and nadolol were successfully resolved in simple MPs composed of ACN or MeOH and water in various volume ratios. Excellent resolution R = 5.54 of promethazine enantiomers was observed in NP mode, MP composed of IPA/HEX 20/80 (*v/v*), at a room temperature within 16 min. Racemic pindolol was baseline separated (R = 1.75) on a newly prepared CSP, rifamycin-capped (3-(2-*O*-β-CD)-2-hydroxypropoxy)-propylsilyl-appended silica particles, in RP mode [69]. Optimized chromatographic conditions were: ACN/1% TEAA buffer, pH 7.0, 10/90 (*v/v*) as a MP and a room temperature. This new type of CSP has CS with two recognition sites: rifamycin and β-CD. New chiral-CD-RAM CSP was synthesized and applied for the determination of certain chiral drugs in biological samples with direct injection [70]. Atom transfer radical polymerization (ATRP) initiator bound porous silica was synthesized to perform surface initiated polymerization. Then, CSP was synthesized by grafting poly(glycidyl methacrylate) (pGMA) on the surface of silica followed by β-CD immobilization. On the surface of this β-CD immobilized material, external pGMA layer was synthesized *via* second round ATRP. After hydrolysis, hydrophilic structure was formed to create a diffusion barrier for proteins. Good enantioseparation and recovery (for BSA) results were obtained for samples containing BSA and chiral drug, *i.e.* chlorthalidone, aminoglutethimide, amlodipine or chlorpheniramine. MP composition differed for individual samples. For the isocratic elution of sample containing chlorthalidone and BSA, MP composed of 1% TEAA buffer, pH 4.9. Gradient elution was necessary for other three samples: MeOH/0.3% TEAA buffer, pH 5.4, 0-50 min 1/99 to 30/70 (*v/v*) for BSA with aminoglutethimide; ACN/0.3% TEAA buffer, pH 6.8, 0-40 min 0/100 to 20/80 (*v/v*) for BSA with chlorpheniramine; ACN/0.3% TEAA buffer, pH 6.8, 0-20 min 0/100 to 20/80 (*v/v*) for BSA with amlodipine. Next year, the same research group prepared new type of CD-click-RAM CSP, also applicable for the determination of chiral drugs in biological samples directly injected to HPLC system [71]. A different synthetic procedure was used; poly(2-methyl-3-butyn-2-ol methacrylate) (pMBMA) was

grafted onto porous silica gel by a surface-initiated ATRP in order to synthesize an inner layer for β-CD immobilization. The azide-modified β-CD was bound to pMBMA by click chemistry. Then, second ATRP reaction was used to graft external pGMA layer onto the silica gel. The external hydrophilic layer was subsequently created by pGMA epoxy group hydrolysis. Samples of human plasma spiked with racemic mandelic acid or chlorthalidone were analyzed. Chromatographic results showed that proteins were excluded from the column in 6 min and the enantiomers were separated (α = 1.45 for mandelic acid and α = 1.30 for chlorthalidone). This CSP showed better enantioseparation ability than the material synthesized in their previous work. Three β-CD-based CSPs were developed applying novel bonding chemistry [72]. Separation performances of β-CD, (*R,S*)-2-hydroxypropyl-β-CD, and permethyl-β-CD-based CSPs were compared based on the resolution values of chiral compounds, including chiral drugs. Chromatographic parameters and conditions, which yielded baseline separations, are summarized in Table 3.

Table 3.

Permethylated-β-CD CSP proved to be the most effective among the CSPs prepared. Wang *et al.* prepared and successfully used two covalently bonded cationic β-CD CSPs by graft polymerization of 6^A-(3-vinylimidazolium)-6-deoxyperphenylcarbamate-β-CD chloride (IMPC) or 6^A-(*N,N*-allylmethylammonium)-6-deoxyperphenylcarbamoyl-β-CD (AMPC) chloride onto silica gel [73]. Baseline enantioseparations of diuretic thiazides, bendroflumethiazide on AMPC and trichlormethiazide and althiazide on IMPC CSPs in a MP composed of HEX/IPA 70/30 (*v/v*) were observed. IMPC CSP was also suitable for the enantioseparation of various pharmaceuticals, *i.e.* fenoterol, dobutamine, 6-methoxyflavanone, 7-methoxyflavanone or 4′-hydroxyflavanone in RP mode. MPs composed of MeOH and 0.1% TEAA buffer of different pH values and various volume ratios. Perera *et al.* used β-CD-based CSPs for the enantioseparation of 17 racemic tetrahydrobenzimidazoles, intermediates in a synthesis of pharmacologically important pyrrole-imidazole alkaloids [74]. Different β-CD-derivatives, *i.e.* dimethylated β-CD, dimethylphenyl carbamate β-CD, *R*- and *S*-naphthylethyl carbamate β-CD, acetylated β-CD, hydroxypropyl ether β-CD and native β-CD as CSs for CSPs, were tested to achieve baseline enantioseparations of the analytes of interest. MPs composed of ACN or MeOH and 20 mM ammonium acetate buffer, pH 4.1, in various volume ratios. Dimethylated β-CD CSP showed the best success ratio, the highest number of baseline separations. Enantiomers of different drugs, chloroquine, indapamide, indoprofen, proglumide and warfarin were baseline separated on a new type of CSP composed of 4-isopropylcalix[4]arene-capped (3-(2-*O*-β-CD)-2-hydroxypropoxy)propylsilyl appended silica particles [75]. All chiral drugs except indapamide were enantioresolved successfully in pure MeOH as a MP. Excellent resolution R = 6.54 for indapamide enantiomers was achieved in a MP composed of MeOH/water 20/80 (*v/v*) at room temperature. New restricted access CSPs (poly-CD-RAM) were synthesized for direct analysis of biological samples by HPLC [76]. Hydrophilic microparticles containing β-CD were prepared *via* one-pot synthesis using reversible addition-fragmentation chain-transfer precipitation polymerization, a "controlled/living" radical polymerization technique. Poly-CD-RAM I CSP was successfully used for direct separation of human plasma samples spiked with chlorthalidone, indapamide or ibuprofen enantiomers. During analysis, plasma proteins were totally excluded from the column (retention times 2.7 - 6.4 min) and the enantiomers were well separated from each other. MeOH and 0.3% TEAA buffer, pH 4.9 or 5.4, in different volume ratios were tested as MPs. Sets of drugs of various pharmaceutical groups, fluoroquinolones, proton pump inhibitors, β-blockers, 5-hydroxytryptamine receptor antagonists and oxazolidinone antibiotics were enantioseparated on two new types of β-CD derived CSPs with multiple urea linkages [77]. CSPs were prepared through the Staudinger reactions between aminopropyl silica gel and CD derivatives, namely heptakis(6-azido-6-deoxy-2,3-di-*O*-3,5-dimethylphenylcarbamoylated)-β-CD and heptakis(6-azido-6-deoxy-2,3-di-*O*-3,5-dichlorophenylcarbamoylated)-β-CD. Three separation modes were tested, *i.e.* NP, RP and PO. All chromatographic measurements were performed at a room temperature. Suitable chromatographic conditions for the individual drugs differed depending on their chemical structure. In this work, effects of MP composition were discussed in detail. Such study could be helpful for the development of chiral separation methods with modified CD-based CSPs. Enantiomers of pseudoephedrine, methylephedrine and methadone were baseline separated on Cyclobond I 2000 RSP column in RP mode [78]. Optimized MP compositions were: MeOH/ACN/0.5% TEAA buffer, pH 4.2, 2.5/2.5/95 (*v/v/v*); MeOH/ACN/0.5% TEAA buffer, pH 4.2, 0.5/0.5/99 (*v/v/v*); MeOH/0.5% TEAA buffer, pH 4.2, 5/95 (*v/v*) for pseudoephedrine, methylephedrine and methadone, respectively. SPP-based hydroxypropyl-β-CD (HPBCD) CSP was produced and successfully used for the enantioseparation of chlorthalidone [79]. HPBCD was bonded to 2.7 μm SPPs. Effective baseline separation was observed within 3 min with resolution R = 2.8 in a MP composed of ACN/10 mM ammonium acetate, pH 4.1, 15/85 (*v/v*). Lower resolution value, slightly higher analysis time and approximately three times lower efficiency were observed with the same CSs bonded to 3 μm or 5 μm fully porous particles used for comparison.

For the analyses of pharmaceuticals, miniaturized methods which require only small amounts of chemicals and analytes were also applied. However, quantity of papers that can be traced in the literature is not high.

Capillary HPLC, with heptakis(2,6-di-*O*-methyl-3-*O*-pentyl)-β-CD immobilized to aminopropyl silica as a CSP, was successfully used for the enantioseparation of various drugs in RP mode [80]. Drugs of different groups, *i.e.* barbiturates, profens (carprofen, ibuprofen, flurbiprofen, naproxen) and several heterocyclic drugs (tiberal, ethotoin, oxapadol, gluthethimide, ketorolac) were baseline separated. Separations were performed with ACN or MeOH and phosphate buffer, pH 4.0, in various volume ratios as MPs at room temperature and constant inlet pressure 350 bar.

Nano-LC technique was used for the enantioseparation of nomifensine, a norepinephrine-dopamine reuptake inhibitor [81]. A monolithic column containing covalently bonded hydroxypropyl β-CD as a CS was prepared by simple one-step in situ polymerization. The best MP composition was MeOH/0.1% TEAA buffer 20/80 (*v/v*). 2,3,6-Tris(phenylcarbamoyl)-β-CD-6-methacrylate was used as a functional monomer for the preparation of β-CD-based polymer [82] or β-CD-based silica monoliths for nano-LC [83]. Various groups of chiral pharmaceuticals were separated on these monoliths. Baseline separations were achieved for alprenolol, bufuralol, carbuterol, cizolirtine, desmethylcizolirtine, celiprolol, eticlopride, ifosfamide, 1-indanol, propranolol, tebuconazole and tertatolol under RP conditions using MP composed of MeOH and 0.1% TFA. The silica-based monoliths showed comparative enantioselectivity to the polymer monoliths.

3.2.2. Achiral separations

Some achiral separations of CD derivatives-based CSPs in drug purity control can be traced in the literature. Separations of estrogen metabolites, sulfates and glucuronides, were performed in HILIC with acetylated β-CD CSP [42]. ACN and 5 mM ammonium acetate as a MP and flow rate 0.2 mL/min were used. Three estrogen glucuronides, *i.e.* estrone-3-glucuronide, 17β-estradiol-3-glucuronide and estriol-3-glucuronide were well resolved at 80% ACN. Fluorinated active pharmaceutical ingredients (APIs) were achirally separated from their impurities - desfluoro analogs using SPP functionalized with hydroxylpropyl-β-CD [84]. Ofloxacin, ciprofloxacin and voriconazole were baseline separated from their desfluoro analogs in MPs composed of ACN/MeOH/TFA/TEA in various volume ratios. MP used for the separation of ezetimibe from its impurity composed of MeOH/5 mM ammonium acetate, pH 4.0, 50/50 (*v/v*).

3.3. Cyclofructan-based CSPs

Separations on CD-based CSPs are performed preferentially in RP mode, in which the CD hydrophobic cavity is employed. An opposite situation arises with CF-based CSPs, where the hydrophilic crown ether core determines that the majority of enantioseparations can be achieved in NP or PO modes.

3.3.1. Chiral separations

First synthesis and application of CF-based CSPs was presented in 2009 by the Armstrong´s group [16]. Various derivatives of CF6 differing in the derivatization group (four aliphatic and ten aromatic groups) and the degree of substitution were developed and applied for the enantioseparation of chiral compounds, including chiral drugs. For example, enantiomers of 1-aminoindan, α-methylbenzylamine, bendroflumethiazide, orphenadrine citrate salt, cromakalim, althiazide, lormetazepam, warfarin and thalidomide were baseline separated on CF-based CSPs in NP or PO modes. Results showed that these CSPs are able to separate a broad variety of enantiomers. CF-based CSP prepared by bonding isopropyl-carbamate functionalized CF6 (IP-CF6) to silica gel was successfully used for the enantioseparation of precursors, intermediates and chiral drugs containing primary amino group [85]. For example, enantiomers of normetanephrine hydrochloride, octopamine hydrochloride, phenylpropanolamine hydrochloride, 2-amino-1-phenylethanol, histidinol dihydrochloride, amlodipine, tocainide, trans-2-phenylcyclopropyl-amine and α-methylbenzylamine were baseline separated in PO mode. MPs mostly composed of ACN/MeOH/HAc/TEA in different volume ratios. Two aromatic-functionalized CF CSPs, *R*-naphthylethylcarbamate CF6 (RN-CF6) and dimethylphenyl-carbamate CF7 (DMP-CF7) were able to enantioresolve various classes of chiral drugs and intermediates in NP HPLC [86]. Baseline enantioseparations of α,α-diphenylprolinol, oxyphencyclimine hydrochloride, bendroflumethiazide, tolperisone hydrochloride, althiazide, phensuximide, warfarin and α-methyl-α-phenyl-succinimide were achieved on RN-CF6 CSP in various MPs in NP mode, depending on the compound structure. DMP-CF7 CSP was used for the enantioseparation of cromakalim, althiazide, 4-chlorophenyl-2,3-epoxypropyl ether, warfarin, α-methyl-α-phenyl-succinimide, methyl trans-3-(4-methoxyphenyl)glycidate and 5-methyl-5-phenyl hydantoin in different MPs. Excellent resolution $R = 5$ was obtained for 5-methyl-5-phenyl hydantoin on DMP-CF7 CSP in a MP composed of heptane/ethanol/TFA 95/5/0.1 (*v/v/v*), at temperature 20 °C. Baseline enantioresolution ($R = 2.25$) of hydrolytic decomposition product of fluridil was observed using RN-CF6 CSP in a MP composed of HEX/IPA/TFA 60/40/0.5 (*v/v/v*) [87]. Separation was performed at 25 °C. Direct enantioseparation of novel biologically active spiroindoline phytoalexins with potential anticancer and antimicrobial activity was achieved with RN-CF6 CSP [88]. All of the eighteen racemic mixtures were separated including cis- and trans-diastereoisomers in NP mode, *i.e.* in MPs composed of HEX/IPA 95/5 (*v/v*) or HEX/IPA/TFA 95/5/0.1 (*v/v/v*). Diuretic butizide was successfully enantioseparated on DMP-CF7 CSP in NP mode (Fig. 1) [89]. Using MPs composed of HEX/IPA 70/30 (*v/v*) or HEX/IPA/TFA 80/20/0.5 (*v/v/v*) resulted in resolution values $R = 1.98$ and $R = 2.59$, respectively.

Figure 1.

IP-CF6 CSP (commercial name Larihc CF6-P column) was used for the enantioseparation of primary amines including chiral biologically active compounds in NP and PO modes [90,91]. Simple MPs composed of HEX/ethanol (*v/v*) or ACN/MeOH (*v/v*), flow rate 2 mL/min and temperature 30 °C yielded successful enantioseparations. Enantiomers of 1-aminoindan, α-methylbenzylamine, norphenylepinephrine, normetanephrine, norephedrine, octapamine, phenylpropanolamine *etc*. were baseline separated under above mentioned conditions. IP-CF6 and DMP-CF7 CSPs were used for the enantioseparation of illicit drugs and controlled substances in PO and NP modes [78]. Baseline separated drugs and chromatographic conditions are summarized in Table 4. IP-CF6 CSP exhibited wider enantioselectivity for these compounds.

Table 4.

CSP prepared by chemical bonding of CF6, functionalized with isopropyl carbamate groups (IP-CF6) on SPPs (2.7 μm) was used for the enantioseparation of amlodipine [92]. Baseline enantioseparation was observed within 18 min in a MP composed of ACN/MeOH/HAc/TEA 80/20/0.3/0.2 (*v/v/v/v*). Lower resolution value, higher analysis time and significantly lower efficiency were observed with the same CSs bonded to 3 μm or 5 μm fully porous particles used for comparison. Larihc CF6-P column was applied for the determination of methionine enantiomers in a dietary supplement sample [93]. Optimized MP composed of MeOH/ACN/HAc/TEA 75/25/0.3/0.2 (*v/v/v/v*) and temperature 0 °C. Under the optimized conditions, some validation parameters were calculated. No interfering peaks were found in the chromatogram of real sample, which confirmed the specificity of the proposed HPLC method. Three CF-based columns, Larihc CF6-P, Larihc CF6-RN and Larihc CF7-DMP were used to develop HPLC methods for the enantioseparation of four racemic phenylisoserine analogues, intermediates in the synthesis of chemotherapy drug paclitaxel. The effects of (i) MP composition, (ii) nature and concentration of various additives (alcohols, amines and acids) in RP and NP modes, (iii) specific structural features of analytes, (iv) temperature on the retention were examined. Separations were carried out at constant MP compositions in the temperature range 5-35 °C. Changes in standard enthalpy, standard entropy, and free energy were calculated [94].

3.3.2. Achiral separations

CF-based CSPs also enabled achiral separations in HILIC. Sets of β-blockers, xanthines, salicylic acid and its derivatives or water soluble vitamins were successfully separated on silica-bonded sulfonated CF6 (SCF6) [95] or covalently bonded native CF6 CSPs [96]. Separation conditions differed according to CSP used and compounds separated. Biologically active enkephalin and vasopressin related peptides were separated using CF6 (Frulic-N CF6 column) and IP-CF6 CSPs [97]. Set of four enkephalin-based pentapeptides was baseline separated within 25 min and 36 min on Larihc CF6-P and Frulic-N CF6 columns, respectively. MP composed of ACN/20 mM ammonium acetate, pH 4.0, 88/12 (*v/v*). Five nonapeptides were also baseline separated on both CSPs used in a MP containing ACN/20 mM ammonium acetate, pH 4.0, 80/20 (*v/v*). Slightly higher analysis time was observed on Frulic-N CF6 column. Four classes of therapeutical peptides were separated on Frulic-N CF6 and Larihc CF6-P columns [98]. Peptides under investigation were: (i) cyclosporin A and C, microbial secondary metabolites used as immune suppressants; (ii) buserelin, leuprorelin, goserelin, and gonadorelin, synthetic gonadotropin hormones; (iii) oxytocin, octreotide, and desmopressin, synthetic cyclic disulfide linked hormone-regulating hormones and (iv) daptomycin, teicoplanin, and vancomycin, non-ribosomally derived polycyclic antibiotics utilized for penicillin- and other antibiotic-resistant infections. Optimized MP composed of ACN/20 mM ammonium acetate buffer, pH 4.1 or 6.5, in various volume ratios. Larihc CF6-P column provided the best resolution for the cyclosporins and Frulic-N CF6 column provided the best resolution for the cyclic hormone group. SPPs functionalized with native and IP-CF6 were used to separate three fluorinated APIs - antibiotics ofloxacin and ciprofloxacin, and antifungal agent voriconazole - from their desfluoro analogs in PO mode [84]. MPs composed of ACN/MeOH/TFA/TEA in volume ratios 90/10/0.3/0.2 and 95/5/0.3/0.2 for the separations with native and IP-CF6 CSPs, respectively.

4. SEPARATION/ANALYSIS IN SFC

Supercritical fluid chromatography is gaining its popularity in the field of enantioselective separations during the last years [10,99,100]. Supercritical fluids lying between gas and liquid states have unique features, such as liquid-like densities and dissolving power and gas-like viscosities and diffusion abilities. These properties make them ideal candidates for major MP components in SFC. In praxis, the main MP constituent is supercritical CO_2 because of easily achievable high flow rates. Therefore, much faster analyses can be obtained in SFC than in HPLC. Concerning CSPs, the same types as in LC can be applied in SFC.

4.1. Cyclodextrin-based CSPs

β-cyclose-6-OH-T and β-cyclose-6-OH CSPs were synthesized and used for the enantioseparation of aminoglutethimide in SFC [101]. The best separation conditions were: CO_2/MeOH + 0.2% DEA 70/30 (*v/v*) as a MP, flow rate 3 mL/min, Δp 15 bar, temperature 30 °C. Higher enantioselectivity (α = 1.44) for aminoglutethimide enantiomers showed β-cyclose-6-OH-T CSP. Diuretic thiazides, *i.e.* bendroflumethiazide, trichlormethiazide, althiazide, indapamide and chlorthalidone were baseline separated using cationic β-CD-based CSP (VIMPCCD-Poly) [102]. Stationary phase composed of 6^A-(3-vinylimidazolium)-6-deoxyperphenylcarbamate β-CD chloride immobilized on silica by co-polymerization. Optimized MP for the enantioseparation of diuretics composed of CO_2/MeOH 90/10 (*v/v*), flow rate 1 mL/min, back pressure 15 MPa, temperature 40 °C. Resolution of the enantiomers ranged from 1.87 to 3.94. The same CSP was suitable also for the enantioseparation of flavanone derivatives 4′-hydroxyflavanone and hesperetin. Optimized MPs were CO_2/IPA 97/3 (*v/v*) and CO_2/IPA 90/10 (*v/v*) for 4′-hydroxyflavanone and hesperetin, respectively. New chemically immobilized cationic β-CDs were applied as CSPs in packed column SFC for the enantioseparation of thiazide diuretics [103].Vinylene-functionalized cationic phenylcarbamoyl β-CDs were co-polymerized with vinylized silica in the presence of azobisisobutyronitrile and conjugated monomers. Baseline separation of bendroflumethiazide and trichlormethiazide enantiomers were achieved in a MP composed of CO_2/MeOH 90/10 (*v/v*), flow rate 1 mL/min, back pressure 15 MPa and temperature 40 °C.

4.2 Cyclofructan-based CSPs

Althiazide enantiomers were baseline resolved within 6 min on RN-CF6 CSP under gradient elution [16]. MP consisted of CO_2 and cosolvent composed of

MEOH/ethanol/IPA 1:1:1 and 0.2% diethylamine, flow rate 4 mL/min. Gradient MP composition was 5% cosolvent hold during 0-0.6 min, 5-60% during 0.6-4.3 min, 60% hold during 4.3-6.3 min, 60-5% during 6.3-6.9 min, and 5% hold during 6.9-8.0 min. DMP-CF7 CSP was used for the separation of butizide enantiomers [89]. Optimized separation conditions were: CO_2/IPA 70/30 (*v/v*) as a MP, flow rate 4 mL/min, temperature 40 °C, back pressure 120 bar. Under these conditions butizide enantiomers were resolved with resolution value R = 2.01. Enantiomers of 1-aminoindan, *α*-methylbenzylamine, norphenylepinephrine, normetanephrine, norephedrine, octapamine and phenylpropanolamine were baseline separated on Larihc CF6-P column [90,91]. MPs composed of CO_2 and 20, 25 or 40 volume % of MeOH with 0.3% TFA and 0.2% TEA, flow rate 4 mL/min and temperature 30 °C.

5. SEPARATION/ANALYSIS IN CEC

Capillary electrochromatography is another high performance separation technique suitable for pharmaceutical quality control, clinical chemistry or toxicology. CEC with CSPs represents a separation system similar to HPLC where the MP flow is created by electroosmotic flow instead of pressure or in addition to pressure.

5.1. Cyclodextrin-based CSPs

5.1.1. Chiral separations

Wistuba and Schurig used CEC with β-CD-based monolith for the enantioseparation of some profens and barbiturates [104]. Prepared monolith composed of permethylated β-CD covalently linked *via* an octamethylene spacer to dimethylpolysiloxane. MPs used composed of MeOH/20 mM MES (2-(*N*-morpholino)ethanesulfonic acid) buffer, pH 6.0, 3/7 (*v/v*). Separations with $R \geq 2.3$ for mephobarbital, 5-ethyl-1-methyl-5-(*n*-propyl)-barbituric acid and hexobarbital were obtained with 12 bar and positive voltage 20 kV and 15 kV, respectively. Similar separation conditions except voltage 25 kV and pressure 140 bar were used for the enantioseparation of carprofen and ibuprofen. Only enantiomers of carprofen were baseline separated under these conditions. Allyl carbamoylated β-CD derivatives were attached to a negatively charged polyacrylamide gel to form a CSP and successfully used for the enantioseparation of terbutaline, metaproterenol, propranolol, α-methyltryptamine and clenbuterol [105]. Optimized conditions for terbutaline, metaproterenol and propranolol were as follows: 200 mM Tris/300 mM boric acid buffer, pH 9.0, applied voltage 171-357 V/cm. The same buffer composition, pH 7.0, and the same voltage were used for baseline separation of α-methyltryptamine and clenbuterol enantiomers. Enantiomers of tropicamide and mephenytoin were successfully separated using negatively charged, and warfarin using positively charged polyacrylamide gels copolymerized with 2-hydroxy-3-allyloxy-propyl-β-CD (allyl β-CD) in 100 mM Tris/150 mM boric acid buffer, pH 8.2 [106]. These gels were covalently linked to a capillary wall. Excellent resolution, R = 8.18, was achieved for hydantoin mephenytoin enantiomers using anionic gel with 75 mM allyl β-CD. Drugs of different pharmacological groups, *i.e.* tryptophan, praziquantel, atropine and verapamil were baseline enantioseparated using dynamically modified sulfated β-CD-based stationary phase [107]. Capillary column was packed with strong anion exchange stationary phase, sulfated β-CD was added to the MP and dynamically adsorbed to the packing surface. MP consisted of 30% MeOH and 2 mg/mL sulfated β-CD in 30 mM TEAA buffer, pH 4.0, separation voltage was 10 kV and temperature 25 °C. High resolution, R = 11.23, was achieved for atropine enantiomers under these conditions. Four stereoisomers of labetalol, a mixed α/β adrenergic antagonist were baseline separated on bonded cyclam-capped β-CD-appended silica materials as CSP [108]. Optimized conditions were: ACN/10 mM Tris buffer, pH 8.6, containing 2 mM $Ni(ClO_4)_2$ 70/30 (*v/v*), voltage 15 kV. This CS contains three recognition sites, β-CD, cyclam and its side arm and exhibits excellent enantioselectivities in CEC. Previously described cyclam-capped β-CD-bonded silica particles were successfully used as a CSP for the enantioseparation of various chiral drugs in CEC [109]. For example, baseline separations of indapamide, isoproterenol, tolperisone, proglumide, oxprenolol, pindolol, metoprolol, indoprofen, bromopheniramine, propranolol or warfarin enantiomers were achieved in MPs composed of ACN and 10 mM Tris HCl buffer, pH 8.6, in different volume ratios. Other separation conditions were: voltage 10 or 15 kV, temperature 20 °C. Resolution values ranged from 1.75 to 7.86. Metoprolol enantiomers were baseline separated on CSP prepared of polyrotaxane monolith containing neutral β-CD and vinylsulfonic acid as ionic comonomer in the polymerization mixture [110]. Separation conditions were: ACN/0.05% TEAA buffer, pH 4.5, 50/50 (*v/v*) as a MP, separation voltage 5 kV. Enantioseparations of barbiturates hexobarbital and mephobarbital were performed on a permethylated β-CD CSP which was covalently bonded *via* thioether spacer to silica by pressure-supported CEC [111]. Optimized separation conditions for both compounds were: MeOH and 0.5 mM ammonium acetate as a MP, voltage 20.5 kV, additional pressure 10 bar. For on-line detection with ESI- and CIS- (coordination ion spray) MS was used modified sheath-liquid interface with 160 μg/mL of $CoCl_2$. High separation efficiency of CEC combined with high selectivity and sensitivity of CIS-MS offers a possibility to detect and identify enantiomers also in complex matrices. Two novel types of crown ether capped β-CD bonded silica, namely 4-´aminobenzo-X-crown-Y(X = 15, 18 and Y = 5, 6, resp.) capped [3-(2-*O*-β-CD)-2-hydroxypropoxy]propylsilyl-appended silica, were prepared and used as CSPs (AB15C5-CD-HPS and AB18C6-CD-HPS) for separation of chiral drugs [112]. Both prepared CSPs exhibited high selectivity for a wide range of chiral compounds. Chiral drugs, which were baseline separated on these CSPs, separation conditions and chromatographic data obtained are summarized in Table 5.

Table 5.

Barbiturates, *i.e.* mephobarbital, hexobarbital, thiopental and 5-ethyl-1-methyl-5-(*n*-propyl)-barbituric acid and profen carprofen were well enantioseparated using a chiral monolithic stationary phase containing permethyl β-CD

[113]. Stationary phase was prepared by fusion of permethyl β-CD-silica particles and linking them to the internal capillary wall. Separation voltage was 15 kV or 20 kV and MPs composed of MeOH/10 mM sodium acetate buffer, pH 4.5 in various volume ratios depending on the drugs separated. Lin *et al.* used a capillary packed with perphenylcarbamoylated β-CD bonded-silica particles for the separation of enantiomers of synephrine, propranolol and isoproterenol [114]. The best MP composition for propranolol and synephrine was MeOH/1% TEAA buffer, pH 4.6, 7/3 (*v/v*). Different buffer, *i.e*. 5 mM phosphate buffer, pH 6.3, but the same volume ratio of MeOH/buffer was required for the separation of isoproterenol enantiomers. Applied voltage and additional pressure were 15 kV and 55 bar, respectively. β-CD derivatized BSA covalently bonded to the inner surface of the capillary served as suitable CSP for the enantioseparation of antihistamine chlorpheniramine [115]. Fast baseline enantioseparation during 2 minutes was observed under the following conditions: 20 mM sodium phosphate, pH 3.5, as a MP, separation voltage -18 kV, column pressure 0.1 MPa. Enantiomers of baclofen, a muscle relaxer and antispastic agent were baseline separated on β-CD-bonded silica monolithic CSP prepared by a single-step sol-gel approach [116]. Separation conditions were: ACN/1% TEAA buffer, pH 7.0, 30/70 (*v/v*) as a MP, separation voltage 10 kV, additional pressure 2 bar. New capillary column coated with carbosilane dendrimers with peripheral Si-Cl groups and β-CD was prepared and applied for the enantioseparation of chlortrimeton (*i.e.* chlorpheniramine) enantiomers [117]. The best separation conditions were: 40 mM phosphate buffer, pH 3.5, separation voltage 16 kV. The enantioselective polymethacrylate-based monolithic column, prepared by ring-opening reaction of epoxy groups from poly(glycidylmethacrylate-*co*-ethylene dimethacrylate) monolith with a novel β-CD derivative bearing 4-dimethylamino-1,8-naphthalimide functionalities, was successfully used for a simultaneous separation of ibuprofen and naproxen enantiomers [118]. Baseline separation of all four enantiomers was obtained within 12 min under following conditions: mixture of 70% ACN and 30% MeOH, containing 350 mM HAc and 5 mM TEA as a MP, voltage -15 kV, additional pressure 50 psi, temperature 20 °C. Under these conditions, naproxen enantiomers eluted first. Perphenylcarbamated β-CD CSP prepared by a click chemistry was applied for the separation of bendroflumethiazide and 7-methoxyflavanone enantiomers [119]. Baseline enantioseparations were observed under following conditions: ACN/5 mM NaH_2PO_4, pH 7.0, 40/60 (*v/v*), applied voltage 10 kV for bendroflumethiazide, 5 kV for 7-methoxyflavanone, temperature 25 °C. CSPs prepared by immobilizing aspartate (Asp)-β-CD or NH_2-β-CD to the epoxy-activated poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) monolith were successfully used for the enantioseparation of mexiletine hydrochloride and/or oxybutynin chloride [120]. Asp-β-CD-based CSP showed higher enantioselectivity as both chiral drugs were baseline enantioseparated, while only oxybutynin chloride enantiomers were successfully separated on NH_2-β-CD-based CSP. Therefore, Asp-β-CD-based CSP was used for the separation of mexiletine hydrochloride in human plasma sample. Separation conditions were: 5 mM phosphate/trolamine buffer, pH 4.5, voltage -10 kV, temperature 20 °C. Migration times of the enantiomers were 3.46 and 3.84 min, respectively, and plasma components did not interfere. Gu and Shamsi synthesized new chiral monomer glycidyl methacrylate-bonded β-CD (GMA-β-CD) and subsequently prepared chiral monolith by polymerization of GMA-β-CD monomer with ethylene dimethacrylate [121]. GMA-β-CD2 (molar ratio GMA: β-CD 2.8:1, degree of substitution 2.0) monolithic column showed the highest enantioselectivity for tested chiral drugs. Baseline separated were (i) neutral drugs, *i.e*. chlorthalidone, flavanone, (ii) basic drugs, *i.e*. pseudoephedrine, propranolol, prilocaine, aminoglutethimide and (iii) acidic drugs, *i.e*. hexobarbital and catechin. The best separation conditions were slightly different for each group of drugs: neutral, acidic drugs and prilocaine: ACN/5 mM ammonium acetate buffer with 0.3% TEA, pH 4.0, 50/50 (*v/v*), separation voltage 20 kV, temperature 25 °C; for basic drugs (pseudoephedrine, propranolol, aminoglutethimide): ACN/5 mM ammonium acetate buffer with 0.3% TEA, pH 3.0 50/50 (*v/v*), separation voltage 20 kV, temperature 25 °C. Chu *et al*. synthesized new hyperbranched polycarbosilanes and coated on the inner surface of fused silica capillaries [122]. The end groups of hyperbranched polycarbosilanes were modified with 2-*O*-(2-hydroxypropyl)-β-CD. Antibiotic ofloxacin was baseline enantioseparated on these CSP types in 0.04 M phosphate buffer, pH 3.0, and applied voltage of 16 kV. Three drugs of different class of medications, *i.e*. zopiclone, chlorpheniramine and tropicamide were well enantioseparated using β-CD or thiolated β-CD modified gold nanoparticles as a CSP in CEC [123,124]. Separation conditions were as follows: 12.5 or 25 mM phosphate buffer, pH 3.0, temperature 20 °C, selected separation electric field strength 300 or 312.5 V/cm. Nie *et al.* prepared and applied a sulfobutyl ether-β-CD capillary CSP for enantioseparation of 10 dihydropyridine drugs (*e.g.* amlodipine, nimodipine, nicardipine) [125]. Optimal separation conditions were slightly different for particular drugs, MP composed of 20 mM NaH_2PO_4, pH 4.0, containing 4 mM sulfobutyl ether-β-CD and ACN (volume content ranged from 10% to 25%), separation voltage 15-25 kV and temperature 15 °C.

5.1.2. Achiral separations

CD-based CSPs showed also interesting capabilities for the achiral applications in pharmaceutical analyses in CEC. β-CD-bonded silica packed capillary column was used for the separation and quantitation of anti-HIV nucleosides [126]. Drugs of interests were nucleoside HIV reverse transcriptase inhibitors (NRTIs), *i.e*. zidovudine, lamivudine, didanosine and its administrated form, stavudine and hivid. Optimized separation conditions were as follows: ACN/20 mM TEAA buffer, pH 5.5, 10/90 (*v/v*) as a MP, temperature 20 °C and separation voltage -15 kV. Baseline separation of all six nucleosides was obtained within 7 min.

CONCLUSION

The importance of enantioselective methods for the analyses of pharmaceuticals is reflected by a high number of scientific papers. It is obvious from this review that the majority of chiral separations of drugs on the cyclic oligosaccharide-based CSPs were performed using HPLC and CEC methods. According to different physicochemical characteristics of CD- and CF-based CSPs, CD stationary phases were utilized in RP mode and CF stationary phases in NP or PO modes in HPLC in the majority of cases. CF-based stationary phases also found employment in HILIC for achiral separations of various pharmaceuticals and their impurities. Nevertheless, SFC and GC methods also found utilization in the enantioresolution of pharmaceutically active compounds. It can be predicted, that the use of SFC or UPLC methods will be favored with the growing interest in ultrafast analytical separations. A challenging area is the development of new chiral columns compatible/applicable in ultrafast separation methods. Recent research in the field of SPP-based CSPs is promising. Concerning the applicability of cyclic oligosaccharide-based stationary phases, the best results yielded derivatized CD-based CSPs, which is confirmed by the highest number of papers. However, native CD-based and derivatized CF-based CSPs also gave successful enantioresolution of various chiral drugs.

This review article gives an overview of chiral separation systems with CD- and CF-based CSPs that were used in the analyses of pharmaceutically active compounds. It can help in choosing proper chiral separation system for particular drugs, intermediates or impurities.

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REFERENCES

[1] Agranat, I.; Caner, H.; Caldwell, A. Putting chirality to work: The strategy of chiral switches. *Nat. Rev. Drug Discov.*, **2002**, *1*, 753-768.

[2] Hilts, P.J. Protecting America's Health: The FDA Business, and One Hundred Years of Regulation, University of North Carolina Press: New York, **2003**.

[3] Eriksson, T.; Björkman. S.; Roth, B.; Höglund, P. Intravenous formulations of the enantiomers of thalidomide: pharmacokinetic and initial pharmacodynamic characterization in man. *J. Pharm. Pharmacol*., **2000**, *52*, 807-817.

[4] Eriksson, T.; Björkman, S.; Roth, B.; Fyge, A.; Höglund, P. Stereospecific determination, chiral inversion in vitro and pharmacokinetics in humans of the enantiomers of thalidomide. *Chirality,* **1995***, 7,* 44-52.

[5] Ahuja, S. *Chiral separations by liquid chromatography*, American Chemical Society: Washington DC, **1991**.

[6] Beesley, T.E.; Scott, R.P.W. *Chiral chromatography*, Separation Science Series, John Wiley & Sons: Chichester, **1998.**

[7] Zhang, Y.W.; Yao, S.; Zeng, H.; Song, H. Chiral separation of pharmaceuticals by high performance liquid chromatography. *Curr. Pharm. Anal.*, **2010**, *6*, 114-130.

[8] Thompson, R. A practical guide to HPLC enantioseparations for pharmaceutical compounds. *J. Liq. Chromatogr. Relat. Technol.*, **2005**, *28*, 1215-1231.

[9] Mangelings, D.; Maftouh, M.; Vander Heyden, Y. Capillary electrochromatographic chiral separations with potential for pharmaceutical analysis. *J. Sep. Sci.*, **2005**, *28*, 691-709.

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

[11] Lämmerhofer, M.; Gargano, A. Monoliths with chiral surface functionalization for enantioselective capillary electrochromatography. *J. Pharm. Biomed. Anal.*, **2010**, *53*, 1091-1123.

[12] Kalíková, K.; Riesová, M.; Tesařová, E. Recent chiral selectors for separation in HPLC and CE. *Cent. Eur. J. Chem.*, **2012**, *10*, 450-471.

[13] Scriba, G.K.E. Pharmaceutical and biomedical applications of chiral capillary electrophoresis and capillary electrochromatography: An update. *Electrophoresis*, **2003**, *24*, 2409-2421.

[14] Duchene, D. *Cyclodextrins and their industrial uses*. Editions de Santé, Paris, **1987**.

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

[16] 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. *Anal. Chem.*, **2009**, *81*, 10215-10226.

[17] Immel, S.; Schmitt, G. E.; Lichtenthaler, F. W. Cyclofructins with six to ten β-(1 → 2)-linked fructofuranose units: Geometries, electrostatic profiles, lipophilicity patterns, and potential for inclusion complexation. *Carbohydr. Res.*, **1998**, *313*, 91-105.

[18] Schurig, V. Use of derivatized cyclodextrins as chiral selectors for the separation of enantiomers by gas chromatography, *Ann. Pharmaceut. Française*, **2010**, *68*, 82-98.

[19] Fox, S.; Strasdeit, H.; Haasmann, S.; Brückner, H. Gas chromatographic separation of stereoisomers of non-protein amino acids on modified γ-cyclodextrin stationary phase, *J. Chromatogr. A*, **2015**, *1411*, 101-109.

[20] Ramos, M.C.K.V.; Silva, E.F.; Neto, F.R.A.; Peçanha, E.P.; Rodrigues, C.R.; Barreiro, E.J.; Fraga, C.A.M. Chiral gas chromatographic separation of 2-oxabicyclo[3.3.0]octane derivatives and their synthetic precursors. *Anal. Chem.*, **2000**, *72*, 3056-3062.

[21] Williams, M.L.; Mager, D.E.; Parenteau, H.; Gudi, G.; Tracy, T.S.; Mulheran, M.; Wainer, I.W. Effects of protein calorie malnutrition on the pharmacokinetics of ketamine in rats. *Drug Metab. Dispos.*, **2004**, *32*, 786-793.

[22] Petrović, M.; Debeljak, Ž.; Blažević, N. Optimization of gas chromatographic method for the enantioseparation of arylpropionic non-steroidal anti-inflammatory drug methyl esters. *J. Pharm. Biomed. Anal.*, **2005**, *39*, 531-534.

[23] Drake, S.J.; Morrison, C.; Smith, F. Simultaneous chiral separation of methylamphetamine and common precursors using gas chromatography/mass spectrometry. *Chirality*, **2011**, *23*, 593-601.

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

[25] Płotka, J.M.; Simeonov, V.; Morrison, C.; Biziuk, M.; Namieśnik, J. Capillary gas chromatography using a γ-cyclodextrin for enantiomeric separation of methylamphetamine, its precursors and chloro intermediates after optimization of the derivatization reaction. *J. Chromatogr. A*, **2014**, *1347*, 146-156.

[26] Zhong, W.-Z.; Williams, M.G. Enantiospecific determination of PNU-83894 and its major metabolite, PNU-83892, in plasma, and its application to the characterization of the enantioselective pharmacokinetics of PNU-83894 in the dog. *J. Chromatogr. A*, **2000**, *871*, 201-206.

[27] VonVoigtlander, P.F.; Hall, E.D.; Ochoa, M.C.; Lewis, R.A.; Triezenberg, H.J. U-54494A: a unique anticonvulsant related to kapa-opioid agonists. *J. Pharmacol. Exp. Ther.*, **1987**, *243*, 542-547.

[28] Guillaume, M.; Jaulmes, A.; Sébille, B.; Thuaud, N.; Vidal-Madjar, C. Study of the retention properties of warfarin enantiomers on a β-

cyclodextrin polymeric support. *J. Chromatogr. B*, **2001**, *753*, 131-138.

[29] Čápka, V.; Xu, Y. Simultaneous determination of enantiomers of structurally related anticholinergic analogs in human serum by liquid chromatography-electrospray ionization mass spectrometry with on-line sample cleanup. *J. Chromatogr. B*, **2001**, *762*, 181-192.

[30] Naidong, W.; Ring, P.R.; Midtlien, C.; Jiang, X. Development and validation of a sensitive and robust LC–tandem MS method for the analysis of warfarin enantiomers in human plasma. *J. Pharm. Biomed. Anal.*, **2001**, *25*, 219-226.

[31] Lubda, D.; Cabrera, K.; Nakanishi, K.; Lindner, W. Monolithic silica columns with chemically bonded β-cyclodextrin as a stationary phase for enantiomer separations of chiral pharmaceuticals. *Anal. Bioanal. Chem.*, **2003**, *377*, 892-901.

[32] Heinemann, U.; Blaschke, G.; Knebel, N. Simultaneous enantioselective separation of azelastine and three of its metabolites for the investigation of the enantiomeric metabolism in rats I. Liquid chromatography–ionspray tandem mass spectrometry and electrokinetic capillary chromatography. *J. Chromatogr. B*, **2003**, *793*, 389-404.

[33] Pihlainen, K.; Kostiainen, R. Effect of the eluent on enantiomer separation of controlled drugs by liquid chromatography–ultraviolet absorbance detection–electrospray ionisation tandem mass spectrometry using vancomycin and native β-cyclodextrin chiral stationary phases. *J. Chromatogr. A*, **2004**, *1033*, 91-99.

[34] Alves, G.; Figueiredo, I.; Castel-Branco, M.; Loureiro, A.; Fortuna, A.; Falcão, A.; Caramona, M. Enantioselective HPLC-UV method for determination of eslicarbazepine acetate (BIA 2-093) and its metabolites in human plasma. *Biomed. Chromatogr.*, **2007**, *21*, 1127-1134.

[35] El-Gizawy, S.M.; Abdelmageed, O.H.; Derayea, S.M.; Omar, M.A.; Abdel-Megied, A.M. Chiral separation of perindopril erbumine enantiomers using high performance liquid chromatography and capillary electrophoresis. *Anal. Methods*, **2014**, *6*, 825-830.

[36] Kučerová, G.; Kalíková, K.; Riesová, M.; Tesařová, E. Effect of buffer constituents on retention and separation in achiral and chiral HPLC systems with β-cyclodextrin-based stationary phase. *Chromatographia*, **2015**, *78*, 917-921.

[37] Rao, R.N.; Kumar, K.N.; Shinde, D.D. Determination of rat plasma levels of sertraline enantiomers using direct injection with achiral–chiral column switching by LC–ESI/MS/MS, *J. Pharm. Biomed. Anal*., **2010**, *52*, 398-405.

[38] Simms, P.J.; Jeffries, C.T.; Zhao, X.; Huang, Y.; Arrhenius, T. Gradient elution of organic acids on a β-cyclodextrin column in the polar organic mode and its application to drug discovery. *J. Chromatogr. A*, **2004**, *1052*, 69-75.

[39] Tsou, T.-L.; Huang, Y.-C.; Lee, C.-W.; Lee, A.-R.; Wang, H.-J.; Chen, S.-H. Simultaneous determination of ampicillin, cefoperazone, and sulbactam in pharmaceutical formulations by HPLC with β-cyclodextrin stationary phase. *J. Sep. Sci.*, **2007**, *30*, 2407-2413.

[40] B´Hymer, C. Evaluation and validation of an HPLC procedure for the determination of the positional isomeric impurity in 2-[4-(1-hydroxy–4-[4-(hydroxydiphenylmethyl)-1-piperidinyl]-butyl)-phenyl]–2-methylpropionic acid. *J. Anal. Chem.*, **2008**, *63*, 376-380.

[41] Tsou, T.-L.; Lee, C.-W.; Wang, H.-J.; Cheng, Y.-C.; Liu, Y.-T.; Chen, S.-H. Development of a new HPLC method for the simultaneous determination of ticarcillin and clavulanic acid in pharmaceutical formulations. *J. AOAC Int.*, **2009**, *92*, 1089-1094.

[42] Nguyen, H.P.; Yang, S.H.; Wigginton, J.G.; Simpkins, J.W.; Schug, K.A. Retention behavior of estrogen metabolites on hydrophilic interaction chromatography stationary phases. *J. Sep. Sci.*, **2010**, *33*, 793-802.

[43] Kanazawa, H.; Kunito, Y.; Matsushima, Y.; Okubo, S.; Mashige, F. Stereospecific analysis of lorazepam in plasma by chiral column chromatography with a circular dichroism-based detector. *J. Chromatogr. A*, **2000**, *871*, 181-188.

[44] Mayr, B.; Sinner, F.; Buchmeiser, M.R. Chiral β-cyclodextrin-based polymer supports prepared via ring-opening metathesis graft-polymerization, *J. Chromatogr. A*, **2001**, *907*, 47-56.

[45] Carlsson, B.; Norlander, B. Optimization and characterization of the chiral separation of citalopram and its demethylated metabolites by response-surface methodology. *Chromatographia*, **2001**, *53*, 266-272.

[46] Wang, X.; Ching, C.B. Liquid chromatographic retention behavior and enantiomeric separation of three chiral center β-blocker drug (nadolol) using heptakis (6-azido-6-deoxy-2,3-di-O-phenylcarbamolyted) β-cyclodextrin bonded chiral stationary phase. *Chirality*, **2002**, *14*, 798-805.

[47] Wang, X.; Ching, C.B. Kinetic and equilibrium study of the separation of three chiral center drug, nadolol, by HPLC on a novel perphenyl carbamoylated β-cyclodextrin bonded chiral stationary phase. *Sep. Sci. Technol*., **2002**, *37*, 2567-2586.

[48] Velmurugan, T.; Ching, C.B.; Ng, S.C.; Bai, Z.W.; Ong, T.T. Optimization of the reversed-phase high-performance liquid chromatographic separation of the enantiomers of a cationic chiral drug (tolperisone) on a heptakis(6-azido-6-deoxy) perphenylcarbamated β-cyclodextrin column. *Chromatographia*, **2002**, *56*, 229-232.

[49] Tazerouti, F.; Badjah-Hadj-Ahmed, A.Y.; Meklati, B.Y.; Franco, P.; Minguillon, C. Enantiomeric separation of drugs and herbicides on a β-cyclodextrin-bonded stationary phase. *Chirality*, **2002**, *14*, 59-66.

[50] Pham-Huy, C.; Villain-Pautet, G.; Hua, H.; Chikhi-Chorfi, N.; Galons, H.; Thevenin, M.; Claude, J.R.; Warnet, J.M. Separation of oxazepam, lorazepam, and temazepam enantiomers by HPLC on a derivatized cyclodextrin-bonded phase: application to the determination of oxazepam in plasma. *J. Biochem. Biophys. Methods*, **2002**, *54*, 287-299.

[51] Thuaud, N.; Sebille, B.; Renard, E. Insight into the chiral recognition of warfarin enantiomers by epichlorhydrin/β-cyclodextrin polymer-based supports: determination of stoichiometry and stability of warfarin/β-cyclodextrin polymer complexes. *J. Biochem. Biophys. Methods*, **2002**, *54*, 327-337.

[52] Chen, L.; Zhang, L.-F.; Ching, C.-B.; Ng, S.-C. Synthesis and chromatographic properties of a novel chiral stationary phase derived from heptakis(6-azido-6-deoxy-2,3-di-*O*-phenylcarbamoylated)- β-cyclodextrin immobilized onto amino-functionalized silica gel via multiple urea linkages. *J. Chromatogr. A*, **2002**, *950*, 65-74.

[53] Patel, D.C.; Breitbach, Z.S.; Wahab, M.F.; Barhate, C.L.; Armstrong, D.W. Gone in seconds: Praxis, performance, and peculiarities of ultrafast chiral liquid chromatography with superficially porous particles, *Anal. Chem*., **2015**, *87*, 9137-9148.

[54] Donovan, J.L.; Malcolm, R.J.; Markowitz, J.S.; DeVane, C.L. Chiral analysis of d- and l-modafinil in human serum: application to human pharmacokinetic studies. *Ther. Drug Monit*., **2003**, *25*, 197-202.

[55] Schumacher, D.D.; Mitchell, C.R.; Xiao, T.L.; Rozhkov, R.V.; Larock, R.C.; Armstrong, D.W. Cyclodextrin-based liquid chromatographic enantiomeric separation of chiral dihydrofurocoumarins, an emerging class of medicinal compounds. *J. Chromatogr. A*, **2003**, *1011*, 37-47.

[56] Lai, X.-H.; Bai, Z.-W.; Ng, S.-C.; Ching, C.-B. Preparation and enantioseparation characteristics of two chiral stationary phases based on mono(6^A-azido-6^A-deoxy)-perphenylcarbamoylated α- and γ-cyclodextrin. *Chirality*, **2004**, *16*, 592-597.

[57] Lai, X.-H.; Ng, S.-C. Preparation and chiral recognition of a novel chiral stationary phase for high-performance liquid chromatography, based on mono(6^A-*N*-allylamino-6^A-deoxy)-perfunctionalized β-cyclodextrin and covalently bonded silica gel. *J. Chromatogr. A*, **2004**, *1031*, 135-142.

[58] Zhong, Q.; He, L.; Beesley, T.E.; Trahanovsky, W.S.; Sun, P.; Wang, C.; Armstrong, D.W. Development of dinitrophenylated cyclodextrin derivatives for enhanced enantiomeric separations by high-performance liquid chromatography. *J. Chromatogr. A*, **2006**, *1115*, 19-45.

[59] Félix, G.; Campèse, D. Development of a new RAM-chiral column for determination of chiral drugs in biological fluids by HPLC. *Chromatographia*, **2007**, *66*, 159-164.

[60] Thamarai Chelvi, S.K.; Yong, E.L.; Gong, Y. Preparation and evaluation of calix[4]arene-capped β-cyclodextrin-bonded silica particles as chiral stationary phase for high-performance liquid chromatography. *J. Chromatogr. A*, **2008**, *1203*, 54-58.

[61] Rao, R.N.; Talluri, M.V.N.K.; Maurya, P.K. Separation of stereoisomers of sertraline and its related enantiomeric impurities on a dimethylated β-cyclodextrin stationary phase by HPLC. *J. Pharm. Biomed. Anal.*, **2009**, *50*, 281-286.

[62] Barker, W.M.; Worm, K.; Dolle, R.E. High-performance liquid chromatographic enantioseparation of methanobenzazocines. *J. Chromatogr. A*, **2009**, *1216*, 7708-7714.

[63] Fodor, G.; Ilisz, I.; Szemán, J.; Iványi, R.; Szente, L.; Varga, G.; Forró, E.; Fülöp, F.; Péter, A. LC enantioseparation of β-lactam stereoisomers through the use of β-cyclodextrin-based chiral stationary phases. *Chromatographia*, **2010**, *71*, S29-S34.

[64] Zhao, J.; Thamarai Chelvi, S.K.; Tan, D.; Yong, E.L.; Lee, H.K.; Gong, Y. Development of vancomycin-capped β-CD-bonded silica particles as chiral stationary phase for LC. *Chromatographia*, **2010**, *72*, 1061-1066.

[65] Lv, Y.; Mei, D.; Pan, X.; Tan, T. Preparation of novel β-cyclodextrin functionalized monolith and its application in chiral separation. *J. Chromatogr. B*, **2010**, *878*, 2461-2464.

[66] Wang, Y.; Young, D.J.; Tan, T.T.Y.; Ng, S.-C. "Click" preparation of hindered cyclodextrin chiral stationary phases and their efficient resolution in high performance liquid chromatography. *J. Chromatogr. A*, **2010**, *1217*, 7878-7883.

[67] Wang, Y.; Young, D.J.; Tan, T.T.Y.; Ng, S.-C. "Click" immobilized perphenylcarbamated and permethylated cyclodextrin stationary phases for chiral high-performance liquid chromatography application. *J. Chromatogr. A*, **2010**, *1217*, 5103-5108.

[68] Thamarai Chelvi, S.K.; Yong, E.L.; Gong, Y. Application of bromoacetate-substituted β-CD-bonded silica particles as chiral stationary phase for HPLC. *J. Sep. Sci.*, **2010**, *33*, 74-78.

[69] Zhao, J.; Tan, D.; Thamarai Chelvi, S.K.; Yong, E.L.; Lee, H.K.; Gong, Y. Preparation and application of rifamycin-capped (3-(2-O-β-cyclodextrin)-2-hydroxypropoxy)-propylsilyl-appended silica particles as chiral stationary phase for high-performance liquid chromatography. *Talanta*, **2010**, *83*, 286-290.

[70] Wang, H.; Xu, D.; Jiang, P.; Zhang, M.; Dong, X. Novel restricted access chiral stationary phase synthesized via atom transfer radical polymerization for the analysis of chiral drugs in biological matrices. *Analyst*, **2010**, *135*, 1785-1792.

[71] Wang, H.; Jiang, P.; Zhang, M.; Dong, X. Synthesis of a novel restricted access chiral stationary phase based on atom transfer radical polymerization and click chemistry for the analysis of chiral drugs in biological matrices. *J. Chromatogr. A*, **2011**, *1218*, 1310-1313.

[72] Varga, G.; Fodor, G.; Ilisz, I.; Szemán, J.; Visy, J.; Szente, L.; Péter, A. Comparison of separation performances of novel β-cyclodextrin-based chiral stationary phases in high-performance liquid chromatographic enantioseparation. *J. Pharm. Biomed. Anal.*, **2012**, *70*, 71-76.

[73] Wang, R.-Q.; Ong, T.-T.; Tang, W.; Ng, S.-C. Cationic cyclodextrins chemically-bonded chiral stationary phases for high-performance liquid chromatography. *Anal. Chim. Acta*, **2012**, *718*, 121-129.

[74] Perera, S.; Na, Y.-C.; Doundoulakis, T.; Ngo, V.J.; Feng, Q.; Breitbach, Z.S.; Lovely, C.J.; Armstrong, D.W. The enantiomeric separation of tetrahydrobenzimidazoles cyclodextrins- and cyclofructans. *Chirality*, **2013**, *25*, 133-140.

[75] Thamarai Chelvi, S.K.; Zhao, J.; Chen, L.; Yan, S.; Yin, X.; Sun, J.; Yong, E.L.; Wei, Q.; Gong, Y. Preparation and characterization of 4-isopropylcalix[4]arene-capped (3-(2-O-β-cyclodextrin)-2-hydroxypropoxy)-propylsilyl-appended silica particles as chiral stationary phase for high-performance liquid chromatography. *J. Chromatogr. A*, **2014**, *1324*, 104-108.

[76] Song, W.-J.; Wei, J.-P.; Wang, S.-Y.; Wang, H.-S. Restricted access chiral stationary phase synthesized via reversible addition-fragmentation chain-transfer polymerization for direct analysis of biological samples by high performance liquid chromatography. *Anal. Chim. Acta*, **2014**, *832*, 58-64.

[77] Lin, C.; Fan, J.; Liu, W.; Tan, Y.; Zhang, W. Comparative HPLC enantioseparation on substituted phenylcarbamoylated cyclodextrin chiral stationary phases and mobile phase effects. *J. Pharm. Biomed. Anal.*, **2014**, *98*, 221-227.

[78] Padivitage, N.L.T.; Dodbiba, E.; Breitbach, Z.S.; Armstrong, D.W. Enantiomeric separations of illicit drugs and controlled substances using cyclofructan based (LARIHC) and Cyclobond I 2000 RSP HPLC chiral stationary phases. *Drug Test Anal.*, **2014**, *6*, 542-551.

[79] Spudeit, D.A.; Breitbach, Z.S.; Dolzan, M.D.; Micke, G.A.; Armstrong, D.W. Superficially porous particle based hydroxypropyl-β-cyclodextrin stationary phase for high-efficiency enantiomeric separations. *Chirality*, **2015**, *27*, 788-794.

[80] Dittmann, H.; König, W.A. Synthesis and silica-based immobilization of monofunctionalized heptakis(2,6-di-O-methyl-3-O-pentyl)-β-cyclodextrin for enantioselective capillary-HPLC. *J. High Resol. Chromatogr.,* **2000**, *23*, 583-589.

[81] Rocco, A.; Maruška, A.; Fanali, S. Enantioseparation of drugs by means of continuous bed (monolithic) columns in nano-liquid chromatography. *CHEMIJA*, **2012**, *23*, 294-300.

[82] Ahmed, M.; Ghanem, A. Chiral β-cyclodextrin functionalized polymer monolith for the direct enantioselective reversed phase nano liquid chromatographic separation of racemic pharmaceuticals. *J. Chromatogr. A*, **2014**, *1345*, 115-127.

[83] Ghanem, A.; Ahmed, M.; Ishii, H.; Ikegami, T. Immobilized β-cyclodextrin-based silica *vs* polymer monoliths for chiral nano liquid chromatographic separation of racemates. *Talanta*, **2015**, *132*, 301-314.

[84] Barhate, C.L.; Breitbach, Z.S.; Pinto, E.C.; Regalado, E.L.; Welch, C.J.; Armstrong, D.W. Ultrafast separation of fluorinated and desfluorinated pharmaceuticals using highly efficient and selective chiral selectors bonded to superficially porous particles, *J. Chromatogr. A*, **2015**, *1426*, 241-247.

[85] Sun, P.; Armstrong, D.W. Effective enantiomeric separations of racemic primary amines by the isopropyl carbamate-cyclofructan6 chiral stationary phase. *J. Chromatogr. A*, **2010**, *1217*, 4904-4918.

[86] 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.

[87] Vozka, J.; Kalíková, K.; Janečková, L.; Armstrong, D.W.; Tesařová, E. Chiral HPLC separation on derivatized cyclofructan versus cyclodextrin stationary phases. *Anal. Lett.*, **2012**, *45*, 2344-2358.

[88] Gondová, T.; Petrovaj, J.; Kutschy, P.; Armstrong, D.W. Stereoselective separation of spiroindoline phytoalexins on *R*-naphthylethyl cyclofructan 6-based chiral stationary phase. *J. Chromatogr. A*, **2013**, *1272*, 100-105.

[89] Vozka, J.; Kalíková, K.; Roussel, C.; Armstrong, D.W.; Tesařová, E. An insight into the use of dimethylphenyl carbamate cyclofructan 7 chiral stationary phase in supercritical fluid chromatography: The basic comparison with HPLC. *J. Sep. Sci.,* **2013**, *36*, 1711-1719.

[90] Woods, R.M.; Breitbach, Z.S., Armstrong, D.W. Comparison of enantiomeric separations and screening protocols for chiral primary amines by SFC and HPLC. *LC GC North America*, **2014**, *32*, 742-751.

[91] Woods, R.M.; Breitbach, Z.S., Armstrong, D.W. Comparison of enantiomeric separations and screening protocols for chiral primary amines by SFC and HPLC. *LC GC Europe*, **2015**, *28*, 26-33.

[92] Spudeit, D.A.; Dolzan, M.D.; Breitbach, Z.S.; Barber, W.E.; Micke, G.A.; Armstrong, D.W. Superficially porous particles vs. fully porous particles for bonded high performance liquid chromatographic chiral stationary phases: Isopropyl cyclofructan 6. *J. Chromatogr. A*, **2014**, *1363*, 89-95.

[93] Hroboňová, K.; Moravčík, J.; Lehotay, J.; Armstrong, D.W. Determination of methionine enantiomers by HPLC on the cyclofructan chiral stationary phase. *Anal. Methods*, **2015**, *7*, 4577-4582.

[94] Ilisz, I.; Grecsó, N.; Forró, E.; Fülöp, F.; Armstrong, D.W.; Péter, A. High-performance liquid chromatographic separation of paclitaxel intermediate phenylisoserine derivatives on macrocyclic glycopeptide and cyclofructan-based chiral stationary phases, *J. Pharm. Biomed. Anal.*, **2015**, *114*, 312-320.

[95] Padivitage, N.L.T.; Armstrong, D.W. Sulfonated cyclofructan 6 based stationary phase for hydrophilic interaction chromatography. *J. Sep. Sci.*, **2011**, *34*, 1636-1647.

[96] Qiu, H.; Loukotková, L.; Sun, P.; Tesařová, E.; Bosáková, Z.; Armstrong, D.W. Cyclofructan 6 based stationary phases for hydrophilic interaction liquid chromatography. *J. Chromatogr. A*, **2011**, *1218*, 270-279.

[97] Kozlík P.; Šímová, V.; Kalíková, K.; Bosáková, Z.; Armstrong, D.W.; Tesařová, E. Effect of silica gel modification with cyclofructans on properties of hydrophilic interaction liquid chromatography stationary phases. *J. Chromatogr. A*, **2012**, *1257*, 58-65.

[98] Shu, Y.; Lang, J.C.; Breitbach, Z.S.; Qiu, H.; Smuts, J.P.; Kiyono-Shimobe, M.; Yasuda, M.; Armstrong, D.W. Separation of therapeutic peptides with cyclofructan and glycopeptide based columns in hydrophilic interaction liquid chromatography. *J. Chromatogr. A*, **2015**, *1390*, 50-61.

[99] Desfontaine, V.; Guillarme, D.; Francotte, E.; Nováková, L. Supercritical fluid chromatography in pharmaceutical analysis. *J. Pharm. Biomed. Anal.*, **2015**, *113*, 56-71.

[100] Lesellier, E.; West, C. The many faces of packed column supercritical fluid chromatography – A critical review. *J. Chromatogr. A*, **2015**, *1382*, 2-46.

[101] Duval, R.; Lévêque, H.; Prigent, Y.; Aboul-Enein, H.Y. Enantioseparation of aminoglutethimide and thalidomide by high performance liquid chromatography or supercritical fluid chromatography on mono-2 and mono-6-O-pentenyl-*β*-cyclodextrin-based chiral stationary phases. *Biomed. Chromatogr.*, **2001**, *15*, 202-206.

[102] Wang, R.-Q.; Ong, T.-T.; Ng, S.-C. Chemically bonded cationic β-cyclodextrin derivatives and their applications in supercritical fluid chromatography. *J. Chromatogr. A*, **2012**, *1224*, 97-103.

[103] Wang, R.-Q.; Ong, T.-T.; Ng, S.-C. Chemically bonded cationic β-cyclodextrin derivatives as chiral stationary phases for enantioseparation applications. *Tetrahedron Lett.*, **2012**, *53*, 2312-2315.

[04] Wistuba, D.; Schurig, V. Enantiomer separation by capillary electrochromatography on a cyclodextrin-modified monolith. *Electrophoresis*, **2000**, *21*, 3152-3159.

[105] Koide, T.; Ueno, K. Enantiomeric separations of cationic and neutral compounds by capillary electrochromatography with monolithic chiral stationary phases of β-cyclodextrin-bonded negatively charged polyacrylamide gels. *J. Chromatogr. A*, **2000**, *893*, 177-187.

[106] Végvári, A.; Földesi, A.; Hetényi, C.; Kocnegarova, O.; Schmid, M.G.; Kudirkaite, V.; Hjertén, S. A new easy-to-prepare homogeneous continuous electrochromatographic bed for enantiomer recognition. *Electrophoresis*, **2000**, *21*, 3116-3125.

[107] Ye, M.; Zou, H.; Lei, Z.; Wu, R.; Liu, Z.; Ni, J. Enantiomer separation by strong anion-exchange capillary electrochromatography with dynamically modified sulfated β-cyclodextrin. *Electrophoresis*, **2001**, *22*, 518-525.

[108] Gong, Y.; Xue, G.; Xiang, Y.; Bradshaw, J.S.; Lee, M.L.; Lee, H.K. Synthesis of cyclam-capped β-cyclodextrin-bonded silica particles for use as chiral stationary phases in capillary electrochromatography. *Tetrahedron Lett.*, **2002**, *43*, 2463-2466.

[109] Gong, Y.; Lee, H.K. Application of cyclam-capped β-cyclodextrin-bonded silica particles as a chiral stationary phase in capillary electrochromatography for enantiomeric separations. *Anal. Chem.*, **2003**, *75*, 1348-1354.

[110] Kornyšova O.; Šurna, R.; Snitka, V.; Pyell, U.; Maruška, A. Polyrotaxane approach for synthesis of continuous beds for capillary electrochromatography. *J. Chromatogr. A*, **2002**, *971*, 225-235.

[111] von Brocke, A.; Wistuba, D.; Gfrörer, P.; Stahl, M.; Schurig, V.; Bayer, E. On-line coupling of packed capillary electrochromatography with coordination ion spray-mass spectrometry for the separation of enantiomers. *Electrophoresis*, **2002**, *23*, 2963-2972.

[112] Gong, Y.; Lee, H.K. Enantiomeric separations in capillary electrochromatography with crown ether-capped *β*-cyclodextrin-bonded silica particles as chiral stationary phase. *Helv. Chim. Acta*, **2002**, *85*, 3283-3293.

[113] Wistuba, D.; Banspach, L.; Schurig, V. Enantiomeric separation by capillary electrochromatography using monolithic capillaries with sol-gel-glued cyclodextrin-modified silica particles. *Electrophoresis*, **2005**, *26*, 2019-2026.

[114] Lin, B.; Shi, Z.-G.; Zhang, H.-J.; Ng, S.-C.; Feng, Y.-Q. Perphenylcarbamoylated β-cyclodextrin bonded-silica particles as chiral stationary phase for enantioseparation by pressure-assisted capillary electrochromatography. *Electrophoresis*, **2006**, *27*, 3057-3065.

[115] Dai, R.; Tang, L.; Li, H.; Deng, Y.; Fu, R.; Parveen, Z. Synthesis and characterization of β-CD derivatized bovine serum albumin protein as chiral selector in pressurized capillary electrochromatography. *J. Appl. Polym. Sci.*, **2007**, *106*, 2041-2046.

[116] Hsieh, M.-L.; Li, G.-Y.; Chau, L.-K.; Hon, Y.-S. Single-step approach to β-cyclodextrin-bonded silica as monolithic stationary phases for CEC. *J. Sep. Sci.*, **2008**, *31*, 1819-1827.

[117] Shou, C.-Q.; Kang, J.-F.; Song, N.-J. Preparation and evaluation of capillary electrophoresis column bonded with carbosilane dendrimers. *Chin. J. Anal. Chem.*, **2008**, *36*, 297-300.

[118] Tian, Y.; Zhong, C.; Fu, E.; Zeng, Z. Novel β-cyclodextrin derivative functionalized polymethacrylate-based monolithic columns for enantioselective separation of ibuprofen and naproxen enantiomers in capillary electrochromatography. *J. Chromatogr. A*, **2009**, *1216*, 1000-1007.

[119] Wang, Y.; Xiao, Y.; Tan, T.T.Y.; Ng, S.-C. Application of click-chemistry-based perphenylcarbamated β-CD chiral stationary phase in CEC. *Electrophoresis*, **2009**, *30*, 705-711.

[120] Li, Y.; Song, C.; Zhang, L.; Zhang, W.; Fu, H. Fabrication and evaluation of chiral monolithic column modified by β-cyclodextrin derivatives. *Talanta*, **2010**, *80*, 1378-1384.

[121] Gu, C.; Shamsi, S.A. Evaluation of a methacrylate-bonded cyclodextrins as a monolithic chiral stationary phase for capillary electrochromatography (CEC)-UV and CEC coupled to mass spectrometry. *Electrophoresis*, **2011**, *32*, 2727-2737.

[122] Chu, Y.; Xu, W.; Edgar, J.S.; Shou, C. Synthesis of hyperbranched polycarbosilane modified with cyclodextrin derivatives and its application in coated capillary electrophoresis columns. *J. Appl. Polym. Sci.*, **2011**, *122*, 2167-2173.

[123] Li, M.; Liu, X.; Jiang, F.; Guo, L.; Yang, L. Enantioselective open-tubular capillary electrochromatography using cyclodextrin-modified gold nanoparticles as stationary phase. *J. Chromatogr. A*, **2011**, *1218*, 3725-3729.

[124] Li, M.; Tarawally, M.; Liu, X.; Liu, X.; Guo, L.; Yang, L.; Wang, G. Application of cyclodextrin-modified gold nanoparticles in enantioselective monolith capillary electrochromatography. *Talanta*, **2013**, *109*, 1-6.

[125] Nie, G.; Li, L.; Cheng, B.; Zhou, R.; Zhang, H. Enantioseparations of dihydropyridine drugs by sulfobutyl ether-β-cyclodextrin modified capillary electrochromatography. *Chem. J. Chinese Univ.*, **2014**, *35*, 1414-1422.

[126] Mesplet, N.; Morin, P.; François, C.; Agrofoglio, L.A. Simultaneous quantitation of nucleoside HIV-1 reverse transcriptase inhibitors by short-end injection capillary electrochromatography on a β-cyclodextrin-bonded silica stationary phase. *J. Chromatogr. A*, **2001**, *927*, 161-168.

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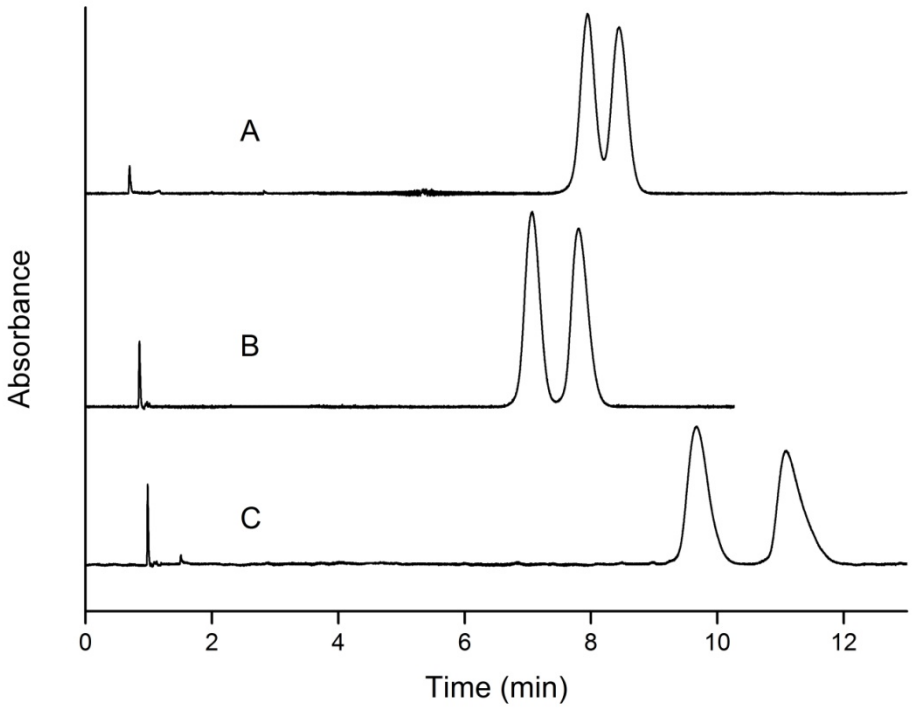

Figure (1). The influence of the organic modifier in MP on the separation of butizide enantiomers using SFC [80]. MP composed of CO_2/organic modifier 80/20 (*v/v*); A - MeOH, B - EtOH, C - IPA.

Table 1. GC methods using CD-based CSPs applicable in pharmaceutical analyses.

Selector/capillary column	Analyte	Separation conditions	Note	Ref.
2,3,6-tri-*O*-methyl-β-CD **2,3-di-*O*-methyl-6-*O*-(*tert*-butyldimethylsilyl)-β-CD** **2,3-di-*O*-acetyl-6-*O*-(*tert*-butyldimethylsilyl)-β-CD** In-house immobilized columns	2-Oxabicyclo[3.3.0]octane derivatives and its precursors	H_2 as a carrier gas various temperature gradients constant linear velocity 50 cm/s	analytes derivatized with acetic anhydride and/or trifluoracetic anhydride cyclopentanic and bicyclic cyclopentanic rings are structural features of many important natural products and pharmaceutical drugs	[14]
2,6-di-*O*-pentyl-3-trifluoroacetyl-γ-CD Astec Chiraldex™ G-TA	Ketamine Norketamine 5,6-Dehydronorketamine	He as a carrier gas 40 °C (0 min) at 20 °C/min to 140 °C (10 min) at 0.5 °C/min to 150 °C (14 min) at 10 °C /min to 170 °C (4 min) flow rate 1.7 mL/min	analytes derivatized with trifluoroacetic acid anhydride study of protein calorie malnutrition on the pharmacokinetics of ketamine enantiomers in rats	[15]
heptakis-(2,3-di-*O*-methyl-6-*O*-*t*-butyldimethyl-silyl)-β-CD Hydrodex β-6-TBDM	Ibuprofen methyl ester Fenoprofen methyl ester Ketoprofen methyl ester	H_2 as a carrier gas various temperature gradients pressure 83 kPa	separation of all six enantiomers in one run	[16]
2,6-di-*O*-pentyl-3-propionyl-γ-CD Astec Chiraldex™ G-PN	Methylamphetamine Ephedrine Pseudoephedrine	He as a carrier gas 100 °C (1 min) at 5 °C/min to 140 °C (9 min) at 10 °C/min to 170 °C (4 min) constant linear velocity 47.5 cm/s	analytes derivatized with trifluoroacetic anhydride MS detection separation of all six enantiomers in one run	[17]
2,3-di-*O*-methyl-6-*t*-butyl-silyl β-CD Astec Chiraldex™ B-DM	Methylamphetamine + its precursors and intermediates Pseudoephedrine Chlorinated intermediates Ephedrine	He as a carrier gas PFP derivatives: 115 °C (28 min) at 5 °C/min to 150 °C (5 min) TFA derivatives: isothermal temperature 110 °C flow of carrier gas 1 mL/min injector and detector temperatures 200 °C	methylamphetamine purity control 3 analytes derivatized with pentafluoropropanoic anhydride ephedrine derivatized with trifluoroacetic anhydride	[18]

2,6-di-*O*-pentyl-3-propionyl-γ-CD Astec Chiraldex™ G-PN	Methylamphetamine + its precursors and intermediates Pseudoephedrine Chlorointermediates Ephedrine	He as a carrier gas 65 °C (3 min) at 10 °C/min to 126 °C (13min) at 4 °C/min to 140 °C (9 min) flow gradient: 2.9 mL/min (23 min) at 1.1 mL/min to 1.8 mL/min (4 min) at 2.2 mL/min to 4.5 mL/min(6 min)	analytes derivatized with trifluoroacetic anhydride simultaneous separation of all enantiomers in one run	[19]

PFP pentafluoropropanoic derivatives, TFA trifluoroacetyl derivatives

Table 2. The enantioseparations of chiral drugs on a novel CSP prepared by immobilization of heptakis(6-azido-6-deoxy-2,3-di-*O*-phenylcarbamoylated)-β-CD on amino-functionalized silica gel under optimized conditions [45].

Drug	MP (*v/v*)	k_1	α	*R*
Brompheniramine	MeOH/1% TEAA, pH 5.33 30/70	2.27	1.25	1.77
Pindolol	MeOH/1% TEAA, pH 5.33 30/70	0.91	1.34	1.43
Isoproterenol	MeOH/1% TEAA, pH 4.33 20/80	0.22	5.06	3.88
Acebutolol HCl	MeOH/1% TEAA, pH 4.33 20/80	1.39	1.30	3.00
Alprenolol	MeOH/1% TEAA, pH 4.33 20/80	2.02	1.50	2.18
Bendroflumethiazide	ACN/water 30/70	5.80	1.49	3.76
Propranolol	*n*-hexane/IPA 80/20	4.29	1.43	1.79

IPA, propane-2-ol; k_1, retention factor of the first eluted enantiomer; α, separation factor; *R*, resolution of enantiomers

Table 3. Enantioseparation values of pharmaceutically active compounds on three different CD-based CSPs under optimized separation conditions. MP composed of MeOH/1% triethylammonium phosphate buffer, pH 3.5 (*v/v*) at ambient temperature [64].

Compound	CSP	MP (*v/v*)	k_1	α	*R*
Acenocoumarol	PMBCD	66/34	6.12	1.14	2.15
Warfarin	PMBCD	66/34	2.29	1.22	2.94
Tropic acid	HPBCD	74/26	4.33	4.18	4.18
	PMBCD	74/26	1.52	2.06	2.06
Terbutaline	BCD	66/34	1.76	1.78	1.78
	HPBCD	66/34	1.76	2.54	2.54
Fluoxetine	HPBCD	90/10	16.71	1.80	1.80
	PMBCD	90/10	2.67	2.33	2.33

CSP: BCD, β-CD-bonded CSP; HPBCD, (*R*,*S*)-hydroxypropyl-β-CD-bonded CSP; PMBCD, permethyl-β-CD-bonded CSP

Table 4. Chromatographic data of chiral illicit drugs and controlled substances baseline separated on IP-CF6 and DMP-CF7 CSPs under optimized conditions [70].

Compound	CSP	MP (volume ratio)	α	*R*
Lysergic acid diethylamide	IP-CF6	90ACN/10MeOH/0.3HAc/0.2TEA	1.15	2.5
Stanozolol	IP-CF6	80HEP/20EtOH/0.1TFA	1.51	2.0
Cathinone HCl	IP-CF6	70HEP/30EtOH/0.1TFA	1.09	1.5
3,4-Methylenedioxyphenyl-2-butanamine hydrochloride	IP-CF6	60ACN/40MeOH/0.3HAc/0.2TEA	1.08	1.5
Phenylpropanolamine	IP-CF6	60ACN/40MeOH/0.3HAc/0.2TEA	1.08	1.5
Clenbuterol	DMP-CF7	80HEP/20EtOH/0.1TFA	1.19	2.0
Temazepam	DMP-CF7	80HEP/20EtOH/0.1TFA	1.10	1.8

ACN, acetonitrile; MeOH, methanol; HAc, acetic acid; TEA, triethylamine; HEP, heptane; EtOH, ethanol; TFA, trifluoroacetic acid

Table 5. Separation data of chiral drugs studied on CSPs AB15C5-CD-HPS and AB18C6-CD-HPS [102].

Drug	CSP	MP (*v/v*)	Voltage (kV)	k_1	α	R
Fenipentol	A	ACN/phosphate 60/40	12	1.08	1.67	2.11
Isoproterenol	A	ACN/phosphate 60/40	12	0.63	1.31	2.53
Indapamide	A	ACN/phosphate 40/60	14	0.76	1.34	2.76
Pindolol	A	ACN/phosphate 40/60	16	0.45	1.98	7.74
Bromopheniramine	B	ACN/phosphate 60/40	12	0.92	1.54	2.42
5-Methyl-5-phenylhydantoin	B	ACN/phosphate 60/40	12	1.24	2.44	9.58
Proglumide	B	ACN/phosphate 40/60	16	1.06	1.66	4.05
Metoprolol	B	ACN/phosphate 40/60	12	1.18	1.59	4.95
Labetalol	B	ACN/phosphate 40/60	16	1.16	1.21	4.85
Promethazine	B	ACN/Tris HCl 50/50	10	0.54	1.56	5.91
Indoprofen	B	ACN/Tris HCl 50/50	10	0.23	1.80	2.64

CSP A: AB15C5-CD-HPS; CSP B: AB18C6-CD-HPS; buffer composed of Tris HCl (10 mM, pH 8.8) with H_3PO_4.NaOH (5 mM, pH 8.8) for CSP A or with H_3PO_4.KOH (5 mM, pH 8.8) for CSP B

Publikace V – Stanovení poměru enantiomerů aminokyselin chromatografickými metodami pro určení stáří a v diagnostice některých chorob

Výskyt D-enantiomerů aminokyselin v organických materiálech se využívá k indikaci změn nebo poruch v různých vědních oborech, ať už z lékařského nebo technologického hlediska. D-aminokyseliny vznikají v buňkách a tkáních při jejich morfologickém vývoji a stárnutí, jsou důležitými ukazateli při vývoji některých chorob a jejich diagnostice, při biologickém a geologickém datování a podílely se na vývoji života na Zemi. **Publikace V** shrnuje chromatografické metody vyvinuté za účelem stanovení poměru enantiomerů aminokyselin v různých biologických materiálech, jídle i v meteoritech. Příprava vzorků je poměrně náročná, je nutné vyvarovat se podmínek, za kterých by mohlo dojít k izomerizaci studovaných aminokyselin. Přímé separační metody vyžadují použití CSP, při nepřímých metodách se aplikuje vhodné chirální derivatizační činidlo. Derivatizace se používá především při stopových analýzách biologických vzorků ke zvýšení citlivosti separačních metod. Matrice analyzovaných vzorků jsou různorodé. Mohou to být biologické materiály, jako jsou biologické tekutiny a tkáně, oční čočky, dentin (zubovina) a rostlinné buňky, dále také jídlo nebo farmaceutika [2,44]. Detekce izomerizace aminokyselin v proteinech je instrumentálně náročná, obvykle se používá HPLC nebo GC s hmotnostní detekcí. Obdržená spektra se pak porovnávají se spektry syntetických peptidů pro lokalizaci specifických míst, na kterých dochází k izomerizaci aminokyselin [45]. K detekci izomerizace volných aminokyselin se obvykle používá derivatizace vhodným činidlem a fluorescenční detekce.

„Screening“ biomarkerů provázejících neléčitelná onemocnění, jako je např. *Diabetes mellitus*, schizofrenie, roztroušená skleróza nebo Alzheimerova choroba, nabývá v dnešní době velkého významu s přibývajícím počtem pacientů a vývojem vhodných separačních metod [46,47,48]. Vývoj těchto onemocnění lze sledovat z izomerizace aminokyselin v proteinech, především z poměru D- a L-enantiomerů serinu a kyseliny asparagové v mozku, mozkomíšním moku, moči, séru nebo plazmě. Ke změnám v poměru enantiomerů kyseliny asparagové dochází také procesem stárnutí, jak u lidí, tak i u ostatních živočichů [49]. Jako vhodný materiál k této analýze se používají oční čočky nebo dentin ze zubů. Klíčovým bodem stanovení stáří je také vhodně zvolená metoda, při které nesmí docházet k dalším racemizačním procesům. Determinace stáří fosilních materiálů dle racemizace aminokyselin se používá především pro paleontologické, archeologické a biogeochemické účely [50]. Na základě těchto měření se získávají

informace potřebné nejen k porozumění geologického vývoje planety, ale také vzniku života na Zemi [51]. Studovanými materiály jsou nejčastěji mořské i suchozemské sedimenty, speleotémy a fosilní schránky měkkýšů. Méně často se takto analyzují kosterní pozůstatky, u kterých se spíše využívá radiouhlíkové analýzy [52,53].

Publikace V

Enantiomeric ratio of amino acids as a tool for determination of aging and disease diagnostics by chromatographic measurements

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Zasláno do periodika *Separations*

separations

MDPI

Review

Enantiomeric ratio of amino acids as a tool for determination of aging and disease diagnostics by chromatographic measurements

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Abstract: Occurrence of D-amino acids in organic matter is a useful indicator of various changes or disorders in different fields of science, technology and human life in general. Determination of amino acid enantiomers, namely enantiomeric ratio of amino acids or excess of certain D-amino acids, represents a useful tool in the studies of aging processes, fossils or biomarkers in disease/disorder diagnosis. The amount of D-amino acids is usually very low. Therefore, suitable sample pretreatment and highly selective and sensitive separation methods are essential for D-amino acid analysis. Chromatographic techniques offer appropriate choices for solving these tasks. This review covers the advances in methodology and development of improved instrumental chromatographic methods focused on D,L-amino acids determination. New findings are also briefly commented.

Keywords: chromatography; amino acid; enantiomeric ratio; aging; dating; biomarker

1. Introduction

Formerly, it was believed that the proteins in living organisms are composed exclusively of L-amino acids (AAs). Later on, it has been recognized that some tissues contain also D-AAs, namely D-aspartic acid which is accumulated during aging. The formation of D-Asp was explained as a result of spontaneous racemization in different tissues [1,2]. In 1991, a protein (relative molecular weight 22,500) containing D-Asp was isolated from bovine lens and characterized as αA-crystallin by SDS-PAGE method and immunoblot analysis [2]. Other simple procedure used at that time for the determination of optical activity and purity was polarimetry. Since then, it was a great challenge to develop new fast and sensitive separation methods. Among the possible methods used for the enantioselective separation/determination of AAs in different matrices, gas chromatography, micellar electrokinetic chromatography (MEKC) and high- or ultra-performance liquid chromatography mostly prevail [3-7]. Other methods are used less frequently. Direct or indirect chromatographic methods can be used for the separation of AA enantiomers [8]. Advantages and disadvantages of indirect methods as kinetic resolution or possibility of racemization are clearly summarized in work of Ilisz *et al.* [9]. In order to improve the detection sensitivity and separation selectivity, AAs were often derivatized. Matrices in which AAs were analyzed were rather different, ranging from biological materials such as various biological fluids and tissues, lens, dentin, through plant cells, to meteorites [10,11], food stuff and pharmaceutical formulations [3,12,13]. The importance of D-AAs is mapped for the formation and differentiation of cells and tissues, biological

and geological dating, the origin of life on Earth, diagnostics, in food control and clinical biochemistry.

A general problem which must be taken into account is a possible AA racemization during acidic hydrolysis of proteins or peptides [14,15]. This procedure is a usual part of sample pretreatment before analysis. Realization of more exact D/L-analysis, which eliminates racemization generated by hydrolysis, was demonstrated [16] and subsequently optimized [17]. Hydrolysis should be performed in deuterated solvents, usually in deuterated hydrochloric acid [16,18,19]. Any molecule that is inverted during this step automatically becomes labelled with deuterium. This method differentiates D-isomers formed during protein acid hydrolysis from the initial contribution of the sample.

Post-translational modifications of proteins do not always result in a change of their mass, thus they are difficult to detect by mass spectrometry (MS). For example, chiral residue epimerization within a peptide does not lead to mass shifts, but can be identified by independently acquired tandem MS or by LC-MS, where the separated isomers are treated separately. To evaluate the isomeric species, multiple tandem MS spectra at the same m/z must be acquired to confirm epimerization [20]. Specific sites of AA isomerization within each peptide can be identified by comparison with synthetic peptides. Disordered regions of α-crystallin proteins were demonstrated to undergo the highest degree of isomerization, which could have important implications on chaperone functionality within the context of aging. Equation 1 [20,21] can be used for the evaluation of isomerization/epimerization by MS.

$$S_{CID/RDD} = R_{isomer} = R_A/R_B \quad (1)$$

R_A and R_B represent ratios of the relative intensities of a pair of fragment ions which varies the most between two isomers; $R_{isomer} = 1$ indicates that the two tandem MS spectra are exactly identical, *i.e.* no isomerization occurs; $R_{isomer} > 1$, a larger number reflects a higher probability of isomerization.

The aim of this work is to show and explain the importance of determination of D- and L- AAs in various fields, which represents a useful and irreplaceable tool in the studies of aging processes, fossils or biomarkers in disease/disorder diagnosis. Since the chromatographic methods are appropriate for this purpose, detailed description of separation conditions and chromatographic advances are also included.

2. D/L amino acid ratio as a tool for the determination of aging and dating

2.1 Food and beverage analysis

Enantiomeric ratio of AAs (and other compounds) in food is a parameter that gives valuable information about its quality. The ratio of enantiomers can indicate contamination, wrong treatment, adulteration, aging, different flavors or geographical origin [22,23]. Interesting reviews dealing with separation of enantiomers in food analysis, where the authors consider also D/L-AAs, were published by Fanali and coworkers [5,24]. D-Ala, D-Val, D-Thr, D-Leu, D-Ser, D-Asx, D-Met, D-Phe and D-Glx present in wines (Roupeiro white) bottled during the period 1978-1989 were shown to be suitable markers for the biotechnological process used in vinification [25]. However, it was not possible to correlate D/L AA ratios to the age of wine. GC analysis of pentafluoropropyl AA propyl esters was carried out on a capillary column Chirasil-Val (Chirasil-Val columns were developed by the team of Bayer [26] and are still widely used in chiral GC). Detailed study of the content of D-Pro and some other D-AA enantiomers in 26 wine samples did not provide evidence for correlation between storage time of bottled wine and quantity of D-AAs [27]. The separation method used was similar to that published 15 years earlier. AAs were converted into *N*(O)-pentafluoropropionyl-(2)-propyl esters and chiral stationary phase Chirasil-L-Val was used for enantioselective separation by GC. A substantial improvement in sensitivity was achieved by MS detection. Non-proteinogenic AAs in date fruits were examined by following steps: ion-exchange chromatography of hydrolyzed samples; derivatization with

6-aminoquinoyl-carbamyl-*N*-hydroxysuccinimidyl carbamate; separation of derivatives by RP HPLC with a C18 stationary phase under gradient elution; GC-MS analysis of total hydrolysates using Chirasil-L-Val or Lipodex E (3-butyl-2,6-pentyl γ-cyclodextrin) chiral stationary phases (CSPs) [28]. Chemical composition, sensorial properties, and nutritional value of fruit depended on the respective cultivar as well as the stage of ripening, time of harvesting and processing. Relatively large amounts of non-proteinogenic AAs, such as (2*S*,5*R*)-5-hydroxypipecolic acid, 1-aminocyclopropane-1-carboxylic acid or γ-amino-*n*-butyric acid were detected besides common proteinogenic AAs. Very low amounts of D-Ala, D-Asp, D-Glu, D-Ser and D-Phe (1.2–0.4 %, relative to the corresponding L-enantiomers) were determined in Saudi Arabian date fruits. The characteristic taste of sake is attributed to contents of specific D- and L-AAs [29]. The long aging process "*Choukijukusei*" effectively increases the D-AA content in sakes, but the mechanism of this increase is still unknown. Different derivatization procedures were used for the determination of D- and L-AAs in 141 sake bottles by HPLC with fluorescence detection. Two precolumn derivatization methods with *o*-phthalaldehyde and *N*-acetyl-L-cysteine or with (+)-1-(9-fluorenyl)ethyl chloroformate/1-aminoadamantane and one postcolumn derivatization method with *o*-phthalaldehyde and N-acetyl-L-cysteine were used. RP HPLC with C18 stationary phase under gradient elution was employed, but different enantiomers required different separation conditions. Shim-pac Amino-Na column was used to separate D,L-cysteine with gradient elution. Sakes contained following D-AAs: Ala, Asn, Asp, Arg, Glu, Gln, His, Ile, Leu, Lys, Ser, Tyr, Val, Phe, and Pro while D-Met, D-Thr or D-Trp were not found in any of the samples analyzed. D-AAs found in the highest amount were D-Ala, D-Asp, and D-Glu ranging from 34.4%, over 12.0 % to 14.6%, respectively. Content of D-AAs in the sake samples brewed with deep-sea water and with the long aging process are higher compared to other sake samples. Two dimensional (2D) HPLC was used for the enantioselective AA metabolome analysis of the Japanese traditional black vinegars [30]. This method combines an achiral separation on a microbore-monolithic octadecylsilica (ODS) column in the first dimension and a narrowbore-enantioselective Pirkle-type or quinine-based (Sumichiral OA-2500 or 3200 or Chiralpak QN-AX) columns in the second dimension. Pre-column fluorescence derivatization with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) was performed before analysis. High amounts of D-Ala, D-Asp and D-Glu were found, while relatively small amounts of D-Ser, D-Leu and D-allo-Ile were observed. Developmental changes in the amount of D-AAs during fermentation and aging processes were also investigated. High amounts of D-Ala (4000–5000 nmol/mL) were present in aged Kurozu vinegars, and the values were almost similar to those observed in Kurozu vinegars fermented for 1 year. The amount of L-Glu significantly decreased. On the other hand, the amount of D-Ser was likely to increase after long-term aging processes. D-enantiomers of Ala, Asp, and Glu were shown to be useful indicators of cheese ripening [31]. Free AAs were extracted from the cheese and achiral ion-paring RP HPLC method was optimized in order to completely separate the AAs of interest from all other AA species present in extracts. Dowex anion-exchange resin-based chromatographic method was also optimized to simplify the sample mixture before chiral ligand-exchange chromatography analysis with a CSP based on *S*-trityl-L-cysteine. Cu(II) nitrate solution was used as a mobile phase (MP) for complexation. The highest difference between enantiomeric excess (ee) values in ripened and fresh cheese was found for Ala (ee 83.0% in ripened sample while 20.5% in fresh cheese) and the least difference showed Glu (ee values changed from 91.8 to 99.0 %).

2.2 Age determination of humans and animals

The D-Asp to L-Asp ratio in proteins obtained from eye lenses [2,32,33] or dentine from teeth [34] can be used for the age estimation of humans or animals, since these materials are well preserved from additional racemization. Special focus is dedicated to the determination of D/L-Asp in dentin. Methods for the determination of Asp racemization are discussed in the review by Yekkala *et al*. [35]. Many of these methods used GC analysis. However, in this review special attention was given to efficiency and sensitivity of HPLC coupled with fluorescence detection, since this method revealed higher ratios of Asp racemization. Effects of sample preparation and buffer

conditions were studied. Usual approach to estimate the age from human or animal teeth is by D/L-Asp ratio from hydrolyzed dentin [36-40]. Many papers regarding this topic were published. Some articles concentrate just on the determination of D/L-Asp using a previously described method [36]. AAs from dentin were derivatized with *o*-phthalaldehyde-*N*-acetyl-L-cysteine and separated by RP HPLC with fluorescence detection. Advantages of this method are 15 min short analysis time (including derivatization and chromatographic analysis) and good sensitivity of detection (1 pmol). Another group of papers deals with the method improvement and proper evaluation of the results. The authors tested different separation conditions, *e.g.* effect of pH [37] and temperature [40], in order to improve method accuracy and reliability. Consequently, conditions for the analysis under which no additional racemization occurs were optimized. The racemization rate can differ depending on the protein structure [33]. The reliability of age estimation based on Asp enantiomeric ratio from different tissues depends on the way of sample preservation, preparation and analysis. Racemization must not proceed during the analysis. Teeth were found to fulfill these requirements. Despite the well-preserved collagen in dentin, some racemization, *i.e.* higher D/L-Asp ratio, was observed at higher temperatures [40]. Benesova *et al*. performed an interesting study [39], in which two well-established routinely used methods were compared. (i) AAs from hydrolyzed collagen from dentin derivatized with *o*-phthaldialdehyde-*N*-acetyl-L-cysteine (OPA-NAC) were separated by RP HPLC on C8 column, (ii) AAs for GC analysis were converted into trifluoroacetic acid isopropyl esters. Chiral XE-60-S-VAL-SA-PEA fused silica column was used for GC measurement. Both methods were shown to be accurate and reliable. However, the HPLC method provided lower racemization values for all samples tested. This result could be attributed to the derivatization procedure in GC, where racemization can occur. New GC method for the enantioselective separation of derivatized AAs was introduced using novel six chiral selectors, *S*-(–)-*t*-Leu-cyclopropylamide, *S*-(–)-*t*-Leu-cyclopentylamide, *S*-(–)-*t*-Leu-cyclohexylamide, *S*-(–)-*t*-Leu-cycloheptylamide, *S*-(–)-*t*-Leu-cyclooctylamide, *S*-(–)-*t*-Leu-cyclododecylamide, anchored to polydimethylsiloxane. *S*-(–)-*t*-Leu-cyclooctylamide was found to be suitable for the age estimation from the Asp racemization extent in human dentin [38]. Increased detection sensitivity for the determination of polar AAs was achieved by the selective ion monitoring (SIM) mode (m/z 134) with zero needle potential [41]. The utility of zero needle voltage ESI was demonstrated on the age determination of human teeth by the Asp racemization method. Enantioseparation of Asp from tooth extract was performed on a teicoplanin-based Chirobiotic T column with MP composed of 90% methanol, 9% water, and 1% of a 30% NH_3 (by volume) and detection by zero voltage ESI-MS. The gain in signal-to-noise ratio was 40-50 times higher than detection at 4 kV. Racemization of L-Asp to D-Asp in eye lens was used for the age estimation of narwhals [32]. After protein hydrolysis a modified analysis method which provided better separation of diastereomers was carried out. Derivatization with OPA-NAC was performed in 0.1 M sodium borate buffer, pH 9.4. The use of C18 stationary phase and mobile phase containing sodium citrate, pH 5.6, as the buffering agent instead of sodium acetate [42] improved the resolution between the diastereomeric derivatives of Asp. Longevity of different whale species could be estimated from the results obtained. Some relation between age and adaptation to a variable climate was suggested.

2.3 Analysis of fossil matter

In general, diverse racemization (or epimerization) rates of different AAs serve for dating of organic matter originating in different geological periods [43]. AA racemization in proteins from fossil material was used for the age determination in archaeometry, biogeochemistry, aquatic and even terrestrial surroundings. Different mathematical models were proposed to describe the extent of AA racemization *vs*. time that could be used in geochronology. The simplest linear equation resulting from the first order kinetics of D/L racemization is valid under isothermal conditions. A more thorough understanding of the temperature sensitivity would be necessary to achieve accurate equations taking into account all factors affecting the time dependent racemization. A detailed summary of the different expressions was made by Clarke and Murray-Wallace [44]. A comprehensive review published by Schweitzer *et al.* covers some recent powerful analytical tools,

196 their advantages and limitations, and how can they be applied to paleontological and archaeological
197 sciences in order to elucidate the characteristics of extinct organisms and their paleoecological
198 environments. Among various techniques/methods (their principles are described in detail) applied
199 to fossils, collagen AAs from tissues including their L- and D-enantiomers are mentioned just briefly
200 [45]. The potential of AA racemization usage along with luminescence and lipid biomarkers in
201 organic matter analysis relevant to speleothems (chemically precipitated cave deposits) offers a tool
202 to record changes in the surrounding environment. Methods used were luminescence
203 spectrophotometry, HPLC and GC-MS. Advantages and disadvantages of these approaches were
204 summarized in a huge review [46]. Chromatographic determination of D/L AA ratios in geological
205 material, *e.g.* fossil molluscs, suffered from wide discordance attributed to differences in chemical
206 procedures and instrumentation [47]. However, Dahl and Meyer pointed out that other uncertainties
207 potentially arise directly from the chromatograms, incomplete resolution of adjacent AA peaks or
208 low signal-to-noise ratio. Although the peak-size measurement method yielded highly inaccurate
209 results when applied to marginal chromatograms, accurate peak-size ratios were recovered from the
210 same chromatogram by first deconvolving peaks by chemometric algorithms before applying the
211 traditional graphical methods. This procedure represents a convenient approach to recover useful
212 age information (relative or absolute) from important geological samples. A fully automated
213 procedure for racemization study of AAs in fossils was introduced by Kaufman and Manley [48]. RP
214 HPLC method allowing baseline separation of at least nine pairs of DL-AAs was developed. Method
215 consisted of online pre-column derivatization of DL-AAs with OPA together with the chiral thiol,
216 *N*-isobutyryl-L-cysteine (IBLC) to yield fluorescent diastereomeric derivatives of chiral primary
217 AAs. Derivatization procedure developed by Brückner *et al.* [49] for food analysis was modified and
218 optimized. Chromatographic separation was performed on C18 column and MPs composed of
219 acetonitrile (ACN), methanol (MeOH) and acetate buffer with gradient elution. Fluorescent
220 detection of the AA derivatives attained detectability in a sub-picomole range, which was sufficient
221 for milligram-size molluscan samples. Hydrolysis time and temperature differed for older and
222 younger molluscs. Sodium azide was added to protect the sample solution from bacteria. Method
223 proposed by Kaufman and its modification [50] is still used in this field. Determination of AA
224 racemization in fossil invertebrates serves as a suitable tool in geochronology [51-65]. Examples of
225 invertebrates, AAs and analytical methods used are summarized in Table 1. Kinetic studies of
226 ostrich eggshell (OES) confirmed that the intra-crystalline protein fraction in OES is useful for AA
227 geochronology [66]. The intra-crystalline fraction in ostrich eggshell is more challenging to isolate
228 than that from mollusc shells (frequently/usually used). The intra-crystalline AAs approximate a
229 closed system and follow predictable patterns of hydrolysis and racemization for all AAs. A new
230 scaling method of relative reaction rates determination estimated by the application of scaling
231 factors to overlap observed rates of racemization (or hydrolysis) at different temperatures was
232 introduced. Chromatographic method described by Kaufman and Manley was used for the
233 determination of D- and L-AAs. L-homo-Arg was used as an internal standard. Since the rate of
234 hydrolysis and racemization of Ile, Phe, Val, Ala, Asx, Glx and Ser was predictable, these AAs could
235 be used for the relative age estimation of OES samples in the archaeological and geological contexts.
236 The determination of racemization extent of Asp, Glu and Val was used to assign relative and
237 numeric ages, and examine the taphonomy of the sedimentary infill of Apollo 11 Rockshelter from
238 Namibia [67]. Results were obtained by HPLC analysis [47] of 37 ostrich eggshell samples from the
239 rockshelter sedimentary succession. The racemization of AAs in fossil bones is less frequently used
240 for age determination. Hydrolyzed bone samples were precolumn derivatized and the enantiomeric
241 ratio was measured by HPLC [68]. Different reagents leading to fluorometrically active derivatives
242 of α-AAs and imino acids were formed. His, Phe, Asp, Glu, Ala, Ile, and Val were the best indicators
243 for the age determination. Based on half-lives of racemization and plotting the D/L ratio as a
244 function of time for various AAs, the calibration curves were obtained; results correlated with the
245 first order kinetics. Using this method, the age of fossil bone samples could be determined in the
246 range of 2000-500 000 years.

247 **Table 1**: Fossil invertebrates, AAs and analytical methods used in geochronology.

Taxon	AAs ratio	Derivatization	Method	Note	Ref.
Saxidomus *Mercenaria*	Asp, Glu,Ala, Phe Leu, Ile/Allo-Ile	OPA-IBC	RP HPLC	geochronology	[51]
Ostracoda	Asp, Glu	OPA-IBLC	RP HPLC	temperature history of Bonneville Basin, Utah geochronology, paleothermometry	[52]
Pulleniatina	Asp, Glu, Ser	OPA-IBLC	RP HPLC	deep marine sediments temperature sensitivity of AA racemization, geochronology	[53,54]
Bouchardia, Semele	Asp	*N*-TFA isopropyl der.	GC	geochronology	[55]
Mercenaria	Ala, Leu, Glu, Phe, Ile/Allo-Ile	procedure to convert Asn to Asp; Gln to Glu	GC/MS	stable carbon isotope composition of AAs in modern and fossil *Mercenaria*	[56]
Bythinia spp., Corbicula, Valvata	Asp, Glu, Val,	OPA-IBLC	RP HPLC	racemization/epimerization of AAs from the intra-crystalline fraction (biomineral) of river shells, geochronology	[57]
Candona	Asp, Glu	OPA-IBLC	RP HPLC	effect of oxidizing pre-treatment on AA composition	[58]
Bithynia, Saxidomus	Tyr, Leu, Phe, Val, Ala, Arg, Ser, Glx, Asx, Ile/Allo-Ile	various, depending on the separation method	RP HPLC; GC	interlab proficiency study modern heated ostrich eggshell samples for comparison, geochronology	[59]
Ostracoda (Herpetocypris Candona, Ilyocypris, Cyprideis)	Asp, Glu	OPA-IBLC	RP HPLC	AA racemization rate of four main ostracoda species	[60]
Liloa, Scissulina, Pinguitellina	Asp, Glu, Val, Ser, Ala, Phe, Leu, Ile/Allo-Ile	OPA-IBLC	RP HPLC	including radiocarbon dating geochronology	[61]
Patella, Strombus, Tibia, Chicoreus Trochus,Anadara	Asx, Glx, Ser, Ala, Val	OPA-IBLC	RP HPLC	intra-crystalline proteins for geochronology	[62,63]
Globigerinoides spp. Neogloboquadrina Pulleniatina	Asp, Glu, Ser	OPA-IBLC	RP HPLC	deep sea sediments geochronology	[64]
Glycymeris	Asx, Glx, Ser, Ala, Val	OPA-IBLC	RP HPLC	effect of bleaching and high temperature, intra-crystalline protein geochronology	[65]

However, factors that affect racemization, *i.e.* temperature, pH, or soil composition, must be strictly controlled in order to get reliable results. The most frequently used methods for dating were radiocarbon analysis and D/L-AAs ratio determination and their results usually correlated. RP-HPLC method with fluorescence detection for the determination of Asp and Ala enantiomeric ratios in fossil bones was developed [69]. The D/L ratio served as a marker of DNA preservation. Hydrolyzed bone samples were derivatized with OPA-NAC and the diastereomers were separated on Atlantis dC18 column, MP composed of MeOH and acetate buffer, pH 5.5, under gradient elution. Developed method provided a simple and effective tool for rapid identification of ancient bone samples suitable for DNA extraction and amplification. AA chronology of fossils in sediments can provide the estimation of geological age. Harada *et al.* examined the applicability of AA chronology to the age determination of siliceous sediments from northwestern North Pacific over a 300-kyr time span [70]. Reversible first-order kinetic model and a parabolic model were used to determine the relationship of D/L ratio of Asp and reference ages. Asp is suitable for the age determination of sediments in the order of 10^5 years, since its racemization reaction rate is one of highest of all AAs. Large differences were observed between Asp ages estimated by the first-order kinetic model and the reference ages, while the parabolic estimates were consistent with the reference ages. Individual steps for the analysis of AA enantiomers were: protein hydrolysis, purification *via* cation exchange resin column, AA conversion to *N*-trifuoroacetyl isopropyl ester derivatives, GC separation on a chiral fused silica Chirasil-L-Val column (CHROMPAC) and detection by flame ionization detector. The application of whole-rock AA racemization offers a new dating approach for previously undatable bioclastic grainstones, calcarenites and oolites in a similar way as marine shells and land snails [71,72]. The ratio of D-Allo-Ile/L-Ile determined by ion exchange HPLC measures the extent of Ile epimerization. RP HPLC method [48] focused particularly on D/L Asp, Glu, and Ser. Racemization of these AAs occurs at moderately faster rates compared to D-Allo-Ile/L-Ile. Ser was used as an indicator of contamination in the deposits or during the laboratory preparation. Study of coastal Chilean sediments showed that the decomposition of organic matter proceeds faster at greater water depth [73]. Estimates of mineralization of total hydrolysable AAs (THAA) showed that the reactivity of sedimentary AAs decreased with increasing water depth and also as a result of progressive degradation of organic matter incorporated in the sediment. Concentrations of D-Asp, D-Glu, D-Ser and D-Ala served for the estimation of contribution of AAs from bacterial cell walls (peptidoglycan AAs) to THAA. The contribution of peptidoglycan AAs to THAA increased with increased sediment depth and age. The origin of sedimentary organic matter and the influence of microbial reworking in the Hauraki Gulf, New Zealand, were determined using the stable carbon isotopic composition of sedimentary D- and L-AAs [74]. GC/MS method with Chirasil-Val fused silica capillary column was used to resolve the TFA isopropyl esters of AAs. Using the same column and GC-isotope ratio MS, TFA isopropyl esters of the AAs were directly analyzed for their stable carbon isotopic compositions. Obtained data strongly suggested extensive microbial reworking of sediments in the Hauraki Gulf. The racemization rate constants of chiral AAs such as Asx, Glx and Ala were determined in terrestrial sediments from Rikubetsu, Hokkaido, Japan [75]. The racemization rate constants were plotted as ln [(1 + D/L)/(1 - D/L)] versus sediment age. The RP HPLC determination of AA enantiomers was obtained using precolumn derivatization with OPA-NAC, followed by SPE of hydrophobic impurities on C18 cartridge and separation on C18 analytical column under gradient elution conditions. In a different study, the amount of Asp adsorbed on a mineral layer was analyzed by HPLC [76]. Phenomenex Chirex 3126 (D)-penicillamine column and mobile phase composed of 85% 2 mM $CuCl_2$ solution and 15% MeOH (*v*/*v*) were used. The determination of Asp can add valuable information about the conditions on the early Earth and possibly help to explain the origin of life. Combined experimental approach and computer modeling can bring some insight to the processes in the early Earth life. The potential of layered double hydroxide minerals to act as hosts for Asp was studied and the host guest structure elucidated. Analytical methods (including sample collection, extraction, treatment/conditioning, preconcentration and determination) dealing with characterization and determination of organic nitrogen and phosphorus are clearly summarized in a

review by Worsfold *et al.* [77]. Amelung *et al.* focused on the soil characterization by changes in organic nitrogen content and enantiomeric AA composition [78]. GC technique using Chirasil-L-Val capillary column was used for the separation of AA derivatives. Correlation among racemization, climate changes and microbial activity was difficult to define. Processes taking place in arable soil were also studied [79]. The assessment of AA enantiomers was used to gain insight into the origin and relative age of soil organic nitrogen. Derivatized AAs, *N*-pentafluoropropionyl-AA isopropyl esters, were separated on a chiral capillary column using GC with flame ionization detector. GC/MS analysis of AA esters on a capillary Chirasil-Val column was used to investigate synthesis and dynamics of AA enantiomers in soil [80]. Mass spectrometer was set in negative ion chemical ionization mode with CH_4 as reaction gas. The incorporation of ^{15}N or ^{13}C isotopes into AA enantiomers was traced. The key point was to differentiate newly biosynthesized and inherent AAs.

3. D-amino acids as biomarkers of diseases/disorders

Proteins are subjected to numerous forms of damage during aging. McCudden *et al.* reviewed the biochemistry of AA racemization in proteins and its clinical application to musculoskeletal diseases [81]. Since the racemization occurs slowly, the quantification of D-AAs is particularly useful when directed at long-lived proteins, such as those found within musculoskeletal tissues. As many of these diseases are caused by dysregulation of protein turnover rates, it is supposed that the determination of the racemization level in particular biomarkers could improve the diagnostic and prognostic information value. The concentrations of many D-AAs in organs and tissues alter during various diseases. Chervyakov *et al.* summed up the role of D-AAs in normal aging and pathogenesis of neurodegenerative diseases [82]. Distribution and physiological role of D-AAs during normal aging, occurrence of D-AAs in pathology, relation between D-AAs and conformational diseases of brain and some methods for the determination of D-AAs and their metabolites are also included. D-aspartyl residues were detected in various proteins from diverse tissues of elderly individuals [83]. Moreover, detection of D-β-Asp is associated with some age-related diseases. Fujii *et al.* described a method for the detection of D-β-Asp at specific sites in particular proteins, proposed a mechanism by which Asp residues invert and isomerize to D-β-form with age under physiological conditions and discussed factors that favor such a reaction. Ultra-high performance liquid chromatography methods for the determination and analysis of disease biomarkers including some chiral methods for AAs were clearly reviewed [84]. Several review papers focused on the presence of D-AAs in mammals, their function, diagnostic value as biomarkers and also HPLC methods for their determination [85-90]. The enantioseparation of D,L-AAs continues to be a subject of immense study due to its significant importance in various areas. Despite recent improvements in analytical techniques which facilitated the accuracy of AA enantiomer analysis at the trace level, there is still a need to develop new, more sensitive, rapid and robust methods [4]. Next section focus on chromatographic methods developed for the determination of L- and D-AAs in physiological fluids and tissues. Some of these methods were applied for the determination of D,L-AAs in samples from patients with different diseases. Certain new findings were made in the role of D,L-AAs in various diseases/disorders.

3.1 Chromatographic methods for the AA determination in biological samples

Recent discovery of specific functions of D-AAs in humans will inevitably lead to the elucidation of D-AA abnormalities in various diseases/disorders. Therefore, high-throughput analysis techniques are necessary to determine D-AAs in biological fluids and tissues [91]. Fuchs *et al.* developed and validated two chromatographic methods, *i.e.* GC-MS and LC-MS for the determination of D-Ser, L-Ser, and Gly in cerebrospinal fluid (CBF) [91]. Pentafluoropropionic anhydride was used for sample derivatization before CG analysis. Analysis was carried out on a Chirasil-L-Val (*N*-propionyl-L-valine-*tert*-butylamide polysiloxane) fused-silica capillary column under programmed temperature elution with helium as a carrier gas. Samples were derivatized with Marfey's reagent [92] before LC analysis. Gradient elution of AA derivatives was performed on

Atlantis dC18 column, linear gradient composed of 100% MP A (250 mg ammonium formiate in 1 L of water; pH adjusted to 4.6 with formic acid) to 50% MP B (ACN) in 15 min. Using these methods, age-dependent concentration ranges of studied AAs in human were determined. MEKC with laser-induced fluorescence (LIF) detection was developed for the determination of chiral AAs in CBF and urine [93]. AAs were derivatized with chiral reagent, *i.e.* (+)- or (-)-1-(9-anthryl)-2-propyl chloroformate before analysis. Separation electrolyte consisted of 20 mM borax buffer pH 9.8. The micellar system consisted of 20 mM sodium dodecyl sulfate (SDS) and 7.5 mM sodium deoxycholate (SDC). D-Ala, D-Gln, and D-Asp were detected in CBF and D-Ala and D-Glu in urine**.** Grant *et al.* developed a simple and versatile methodology using HPLC with fluorimetric detection to simultaneously determine D-Ser along with other metabolically related neuroactive AAs in the glutamatergic system: L-Ser, L-Glu, L-Gln, and Gly in human plasma [94]. AAs were derivatized by a combination of two chiral thiol reagents, OPA and IBLC. Separation was carried out on Symmetry C18 column by a concave MP gradient. This methodology is proposed for studying the role of AAs in the glutamatergic system in the pathophysiology and treatment of neurological and psychiatric disorders. A straightforward approach for the determination of D-AA trace levels in mammals utilizes 2D on-line column-switching system consisting of non-enantioselective and enantioselective separation parts in combination with sensitive pre-column or post-column derivatization [95-97]. Hamase *et al*. [95] described a 2D HPLC method for the comprehensive analysis of small quantities of branched aliphatic D-AAs in the presence of large amounts of their L-congeners in mammalian tissues and physiological fluids. Target analytes were determined as their fluorescent derivatives, pre-column labeled with NBD-F. Authors established a multi-loop 2D column-switching HPLC system, combining on-line a reversed-phase system (Capcell pak C18 MG II column) as the first dimension with an enantioselective column (Chiralpak QN-AX or Chiralpak QD-AX) in the second dimension for the simultaneous determination of D-Val, D-*allo*-Ile, D-Ile, and D-Leu. Chiral columns contain weak anion exchange-type CSP with quinine (or quinidine) *tert*-butylcarbamate moiety as chiral selectors and provide reversal elution order of the enantiomers. Significant amounts of D-Leu were found in rats in all the tissues and physiological fluids tested. High amount of D-*allo*-Ile was determined in urine. D-*allo*-Ile was also found in the urine of dogs and mice. This scientific group also developed micro 2D HPLC method for the determination of hydrophilic AA enantiomers (His, Asn, Ser, Gln, Arg, Asp, allo-Thr, Glu and Thr) in mammalian tissues and physiological fluids [98]. This method combines a microbore-monolithic ODS column in the first dimension and narrowbore-Pirkle-type chiral column (Sumichiral OA-2500S or Sumichiral OA-2500R) in the second dimension. Derivatization of AAs with NBD-F was performed before analysis. D-isomers of all investigated AAs were found in rat urine but at various enantiomeric ratios. Developed procedure was also successfully used for the determination of D- and L-Ser in urine and serum of mice after renal ischemia-reperfusion injury (IRI), known as a mouse model of acute kidney injury [99]. The level of D-Ser gradually increased in serum after renal IRI in parallel with creatinine, whereas the L-Ser level decreased sharply in the early phase after IRI. The work provides a novel understanding of the AA imbalance during renal failure and offers a new potential biomarker for the early detection of acute kidney injury. Column-switching chiral HPLC system using similar column types and derivatization agent as mentioned above was formerly applied for the determination of minute amounts of D-Ala in rat tissues [100]. It was revealed that the amount of D-Ala is highest at the age of 6 weeks and then significantly decreases, and the amount of D-Ala is significantly higher during the daytime than the nighttime. Han *et al.* used 2D HPLC method for the determination of D-AAs in biological samples [101]. This method combines columns and derivatization agents mentioned above; monolithic ODS column in the first dimension, Chiralpak QD-1-AX column in the second dimension and NBD-F for derivatization. This method was applied to analyze the aging model senescence accelerated mouse prone 1 (SAMP1) mice which had low immunocompetence. There was an obvious increase of D-Asp in thymus and spleen of SAMP1 mice compared to the accelerated senescence resistant mice. Visser *et al.* developed and validated sensitive, fast and simple UPLC/MS/MS method for the quantification of both enantiomers of proteinogenic AAs in body fluids [102]. Seven different chiral derivatization agents for the D-AA analysis were compared.

(*S*)-NIFE (*N*-(4-nitrophenoxycarbonyl)-L-phenylalanine 2-methoxyethyl ester) reagent offered an outstanding performance in the terms of sensitivity and enantioselectivity. The diastereoisomers were separated on Acquity BEH C18 column under gradient elution. MP composed of ACN and 10 mM ammonium hydrogencarbonate, pH 9.5. Baseline separation ($R > 2.45$) was achieved for all 19 chiral proteinogenic AA isomers. It was shown that D-AAs in stable isotope tracers could result in erroneous estimates of enrichment, particularly if urine is used as a surrogate for plasma enrichment [103]. To describe the effects of D-AA content of less than 0.2% in 3 different AA tracers on the isotope enrichment in urine and plasma, Arg, Pro, and Phe tracers were given enterally to human neonates. The enrichment was measured in urine and plasma by HPLC with teicoplanin-based Chirobiotic T column and tandem MS. Labeled D-Arg resulted in an enrichment overestimate of 20% in plasma and 87% in urine. Smaller effect was obtained for D-Phe, where the overestimate was 5% in plasma and 40% in urine. D-Pro had no significant effect. The same Phe tracer was tested also on children and adults. A reduction in the overestimate in children compared with infants and no effect on enrichment in adults were observed. Stable isotope-labeled AA studies should be performed, particularly in children, to ensure that this potential error is identified and eliminated. Reischl *et al.* developed an enantioselective LC-MS/MS method applicable for the analysis of proteinogenic AAs in urine [104]. This method utilizes a derivatization step on the amino group with an iron ferrocenyl propionate hydroxy succinimide ester followed by enantioselective anion exchange chromatography with cinchona alkaloid-based CSP. Homemade HALO-QD-AX-CSP contains *t*-butyl-carbamoylquinidine immobilized on a highly efficient fused core chromatographic support material. MP composed of 90% MeOH with 10% water and 100 mM ammonium formate/0.1% formic acid (*v*/*v*). Baseline enantioseparation for all proteinogenic AAs except for Pro, Arg and His was achieved. Cinchona alkaloid-based zwitterionic ion-exchange type enantioselective column, Chiralpak ZWIX(+) was successfully used for a simultaneous analysis of D-Ala, D-Asp, and D-Ser besides their L-forms in rat plasma and tissues [105]. Sensitive and selective chiral LC-MS/MS method was developed and validated for this purpose. AAs were derivatized with 6-aminoquinolyl-*N*-hydroxysuccinimidylcarbamate or *p*-*N*,*N*,*N*-trimethylammonioanilyl *N*′-hydroxysuccinimidyl carbamate iodide before analysis. MP composed of MeOH/aqueous solution containing 50 mM ammonium formate and 50 mM formic acid 98/2 (*v*/*v*) ratio. Three D-AAs and their L-enantiomers were simultaneously determined within 20 min in biological samples. Selective and high-throughput LC–MS/MS method using a surrogate analyte and an authentic matrix to determine D-Ser in mouse brain was developed and validated [106]. To ensure the validity of D-Ser determination, [2,3,3-^{2}H]D-serine and [^{15}N]D-serine were used as a surrogate analyte and an internal standard, respectively. Enantiomeric separation was performed within 6 min on a chiral crown ether column (CROWNPAK CR(+)), MP composed of 0.3% trifluoroacetic acid at 5°C without derivatization. Results demonstrated that the surrogate analyte method is valid for the accurate measurement of D-Ser in mouse brain and is time-saving in the means of sample preparation and analysis. Furthermore, method successfully provided D-Ser levels in brain of normal mice. Sugimoto *et al.* developed new, highly sensitive and specific LC/ESI-MS/MS method with the same chiral column for the determination of L- and D-Ser in human plasma [107]. Phosphate buffered saline (PBS) was used as a surrogate matrix. D- and L-Ser in human plasma and PBS were treated by cationic exchange solid phase extraction (CE SPE) and no sample derivatization. Elution was isocratic, MP composed of 0.3% trifluoroacetic acid in 10% ACN and the separation temperature was below 4°C. This method offers a direct enantioseparation of D- and L-Ser without a time-consuming and/or poor qualitative derivatization step. The same scientific group also developed LC/ESI-MS/MS method for the determination of L- and D-Leu in human and animal plasma [108]. PBS was used as a surrogate matrix for the preparation of calibration curves and quality control samples. CE SPE was applied for the extraction of D- and L-Leu from plasma. The enantioseparation was performed on CHIRALPAK ZWIX(−) column, MP composed of MeOH/ACN/1 M ammonium formate/formic acid 50/50/2.5/0.2 (*v*/*v*/*v*/*v*). Baseline enantioseparation was accomplished without a derivatization step to accurately determine the D- and L-Leu in plasma. The fragmentation at m/z 43 for D- and L-Leu enabled the discrimination of D,L-Ile and D,L-*allo*-Ile by MS. Mochizuki *et al.* synthesized new chiral

labeling reagents, *i.e.* L-pyroglutamic acid succinimidyl ester (L-PGA-OSu) and its isotopic variant (L-PGA[d5]-OSu), for the derivatization and consequent enantioseparation of AA derivatives by UPLC/ESI-MS/MS [109]. Nine pairs of proteolytic AAs were completely separated by RP chromatography using ACQUITY UPLC BEH C18 column under isocratic elution conditions; MP composed of ACN-water mixture containing 0.1% (*v*/*v*) formic acid. Method efficiency was demonstrated by analyzing AAs in human serum samples. Proposed procedure using L-PGA-OSu and L-PGA[d5]-OSu was intended for D,L-AA chiral metabolome study. A novel triazine-based chiral derivatization agent (*S*)-2,5-dioxopyrrolidin-1-yl-1-(4,6-dimethoxy-1,3,5-triazin-2-yl) pyrrolidine-2-carboxylate was synthesized and applied for the determination of chiral amines and AAs in saliva of healthy volunteers by UPLC/ESI-MS/MS [110]. Diastereomers derived from proteolytic AAs except serine were well separated under isocratic elution conditions by RP chromatography using an ACQUITY UPLC BEH C18 column. D,L-Ser was separated on a Capcell Core ADME (core-shell type column with adamantyl functional group bonded on the polymer coated silica). Although the simultaneous separation of proteolytic D,L-AAs by a single chromatographic run was not possible, the enantiomeric separations of 19 AAs were performed using several elution profiles and different columns. The amount of the D-isomer in saliva was negligible for most AAs, except Ala and Pro.

3.1.1 Chromatographic determination of AAs as possible biomarkers of diseases/disorders

Nowadays, the importance of chiral AA analysis for the screening of new biomarkers is arising in the diagnostic applications. *N*-methyl-D-aspartate (NMDA) receptors are involved in learning and memory processes. It was shown that in Alzheimer´s disease (AD) there is a reduction of NMDA receptors [111]. NMDA also play an important role in pathophysiology of schizophrenia. D-Ser may function as an endogenous agonist of the Gly site in the NMDA receptor [112,113]. A reduction in serum levels of D-Ser, an endogenous co-agonist of the NMDA receptor, was reported in schizophrenia [112]. D´Aniello *et al.* supposed there is a relation between reduced levels of D-Asp in brain and reduction of NMDA receptor signal transduction system, since D-Asp is an endogenous agonist of NMDA receptor [111]. Using an HPLC method, regional distribution of free D-Asp levels in post mortem brain samples from patients with AD were determined. Significantly lower D-Asp levels in Alzheimer´s patients compared to controls were found. Thorsén *et al.* focused on AA enantiomeric composition of β-amyloid peptides from deceased AD patients [114]. Peptides were hydrolyzed with mineral acid, free AAs were derivatized with chiral reagent, *i.e.* (+)- or (-)-1-(9-anthryl)-2-propyl chloroformate, and subsequently separated using MEKC-LIF method. The separation electrolyte consisted of 20 mM borate buffer adjusted to pH 9.8 with 0.1 M NaOH. The micellar system consisted of 20 mM SDS and 7.5 mM SDC. Method allowed simultaneous determination of nine AA enantiomers. Samples revealed impurities which could overshadow or dilute racemization events inherent to the β-amyloid peptides. Thus, more rigorous peptide purification protocols must be designed. The role of D-Ser in the pathophysiology of AD was also studied [115]. The reason of the study was that D-Ser may function as an endogenous agonist of the Gly site on the NMDA receptor which was implicated in the pathophysiology of AD. Previously described HPLC column-switching method with fluorimetric detection was used [116]. AAs were derivatized with 4-nitro-7-piperazino-2,1,3-benzoxadiazole fluorescent reagent. At first, step gradient elution was performed on ODS column; initial MP composed of ACN, MeOH and 0.1% trifluoroacetic acid. Then the analysis method was switched to chiral, using phenylcarbamoylated β-cyclodextrin (CD) column and MP composed of ACN/MeOH/water 75/20/5 (*v*/*v*/*v*). D-Ser serum levels determined in patients with AD were slightly lower than those of controls. In contrast, serum levels of L-Ser in patients were slightly higher than those of controls. This study suggested that reduced activity of serine racemase, an enzyme catalyzing the formation of D-Ser from L-Ser, can play a role in the pathophysiology of AD. Samakashvili *et al.* used chiral MEKC-LIF method for the determination of D- and L-AA content in CBF samples related to different AD stages [117]. Samples were derivatized with fluorescein isothiocyanate (FITC) before analysis. Running buffer composed of 100 mM sodium tetraborate, 80 mM SDS and 20 mM β-CD at pH 10.0. Using this method, L-Arg,

L-Leu, L-Gln, γ-aminobutyric acid, L-Ser, D-Ser, L-Ala, Gly, L-Lys, L-Glu and L-Asp were detected in all the CBF samples. Some of the results shown in this work seem to disagree with those shown in literature (*e.g.* not significant variation in D-Ser level was observed depending on the AD stage), while some of them agree with the results obtained by others (*e.g.* L-Asp occurs at significantly lower concentrations in AD CBF than normal CBF). The post-translational AA racemization (AAR) and AA isomerisation (AAI) are typical markers of protein aging and could significantly induce the density and localization of plaque deposition in brain tissues [118]. AD is related to the formation and aggregation of amyloid-β peptide plaques in human brain. Inoue *et al.* developed a covalent chiral derivatized ultra performance liquid chromatography tandem mass spectrometry (CCD-UPLC-MS/MS) method for the determination of post-translational AAR and AAI of *N*-terminal amyloid-β peptide (*N*-Aβ_{1-5}) in human brain tissues [118]. Covalent chiral derivatization reagent (*R*)-(-)-4-(*N*,*N*-dimethylaminosulfonyl)-7-(3-isothiocyanatopyrrolidin-1-yl)-2,1,3-benzoxadiazole was used for the derivatization of amyloid-β peptides. Separation was performed by ACQUITY UPLC BEH C18 column; MP composed of 50 mM aqueous ammonium formate with 0.01% formic acid and 0.01% formic acid in MeOH. The CCD-UPLC/MS/MS assay of potential *N*-Aβ_{1-5} discovered the presence and ratio levels of these *N*-Aβ_{1-5} sequences with L-Asp, D-Asp, L-isoAsp, and D-isoAsp in AD patients. A novel UPLC/ESI-MS/MS method for the determination of biomarker candidates, chiral amines and carboxyls in AD brain homogenates, was developed [119]. Solutions of carboxyls or amines were derivatized with a pair of enantiomers of chiral derivatization reagents, *i.e.* (*S* and *R*)-1-(4,6-dimethoxy-1,3,5-triazin-2-yl)pyrrolidin-3-amine or (*S* and *R*)-2,5-dioxopyrrolidin-1-yl-1-(4,6-dimethoxy-1,3,5-triazin-2-yl) pyrrolidine-2-carboxylate. Derivatives were separated by gradient elution using water/ACN containing 0.1% formic acid on ACQUITY UPLC BEH C18 column. As a result, only L-Phe and L-lactic acid were identified as decreased and increased biomarker candidates in the AD brain, respectively. This strategy should be helpful for the identification and extraction of chiral metabolomics in samples from different environments. The determination of chiral metabolites in different sample groups is currently in progress in author´s laboratory.

Xie *et al.* developed and fully validated LC/MS/MS method for the determination of D-Ser levels in human plasma [120]. Method was successfully applied to sequential changes in plasma D-Ser and L-Ser levels in 6 CRPS (complex regional pain syndrome) patients receiving a continuous 5-day intravenous infusion of (*R*,*S*)-ketamine. Method utilizes pre-column derivatization using (*R*)-1-Boc-2-piperidine carbonyl chloride. Separation was performed on Zorbax Eclipse XDB-C18 column under gradient elution; MP composed of water with 0.3% TFA (eluent A) and MeOH with 0.3% TFA (eluent B). Interesting results were observed. In three patients (*R*,*S*)-ketamine administration produced a continuous drop in D-Ser levels with a maximum decrease of 20%. This treatment also produced increased D-Ser levels in two patients, with a 35% and 21% increase, respectively. But one patient had an initial 17% increase in circulating D-Ser levels on the third day followed by 8% reduction at the end of the treatment. These results indicate the importance in determining the D-Ser levels in plasma and their potential role in physiological response. Above mentioned method was also applied for the determination of D-Ser plasma levels in patients with (*R*,*S*)-ketamine treatment-resistant depression [121]. Baseline (before infusion of ketamine) D-Ser and also L-Ser plasma concentrations were significantly lower in ketamine treatment responder than in ketamine treatment non-responder. Obtained data suggested that the pre-treatment determination of baseline D-Ser levels in major depressive disorder patients could be used as effective, rapid, and simple method for the prediction of antidepressant response to ketamine treatment.

Pålsson *et al*. used previously reported HPLC methods with fluorescence detection [122-124] to explore glutamate hypothesis of bipolar disorder by examining peripheral and central levels of AAs related to glutamate signaling [125]. Study included 215 patients with bipolar disorder and 112 healthy controls. Serum levels of Glu, Gly and D-Ser were significantly higher whereas L-Ser levels were lower in patients as compared to controls. No differences between the patient and control

group in AA levels were observed in CBF. Obtained results could be interpreted as a systemic aberration in AA metabolism which affects several AAs related to glutamate signaling.

2D HPLC method with electrochemical detection was developed and used for the determination of homo-cysteine, methionine and cysteine enantiomers in human serum [126]. AAs were separated from each other by achiral column (Purospher RP-18) in the first dimension and their enantiomers were separated on teicoplanin-aglycone based column (Chirobiotic TAG) in the second dimension by on-line system. MP composed of ACN/MeOH/25 mM phosphate buffer, 1 mM 1-octanesulfonic acid sodium salt, pH 2.7 3/3/94 (*v*/*v*/*v*). Method was applied to the analysis of human serum of healthy volunteers and patients with multiple sclerosis. D-enantiomers of all tested AAs were not detected in serum samples. Preliminary results showed that patients with multiple sclerosis had higher levels of Met in comparison to healthy volunteers. Nevertheless, no generalization can be made, since only three samples from patients were tested.

GC/MS method employing RT-γDEXsa (2,3-di-acetoxy-6-*O*-tert-butyl-dimethylsilyl γ-CD doped into 14% cyanopropylphenyl/86% dimethyl polysiloxane) column for AA determination in human serum and urine was developed and applied to urine samples from patients with renal insufficiency [127]. Method required protein removal by precipitation before AA derivatization with methyl chloroformate/methanol and reaction performance at neutral pH. In comparison to healthy volunteers, D-ratios of Ala, Val, Pro, Thr, Asp, and Asn significantly increased in patients. The differences in D-AA ratios were mainly result of significant decrease in L-AA concentrations normalized by creatinine levels.

Free D-AAs can also serve as biomarkers of *diabetes mellitus* (DM). Min *et al.* developed UPLC/ESI-TOF-MS method for the determination of D,L-AAs derivatized with *R*(−)-4-(3-isothiocyanatopyrrolidin-1-yl)-7-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole in nails of diabetic patients [128,129]. Separations of derivatives were performed by gradient elution on ACQUITY UPLC BEH C18 column, MPs contained water and ACN with 0.1% formic acid or 5 mM ammonium acetate. Method provided trace detection of D,L-AAs and enabled the detection of D-Ala, D-Pro, D-Val, D-Ile and D-Leu from nails of diabetic patients and healthy volunteers. No significant difference in the content of L-AAs was observed. However, statistically significant and strong correlation between the D/L-AA concentration ratios for Ala, Val, Ile and Leu was observed. Chiral GC-MS method for the determination of free D-AAs in urine was developed, validated and applied to a gestational DM study [130]. Pentafluoropropionic anhydride was used for sample derivatization before analysis. Chiral separations were carried out on a Chirasil-L-Val fused-silica capillary column under programmed temperature elution with helium as a carrier gas. % D-relative amounts were determined for Ala, Val, Thr, Ser, Leu, Asx (Asp+Asn), Glx (Glu+Gln), Met, Phe, Tyr, Orn and Lys. Statistically significant differences were observed only for D-Phe and higher values were found in the gestational DM group in comparison with pregnant women with normal glucose tolerance.

4. Conclusions

This review article gives an overview of AA enantiomeric ratio utilization in various fields as well as analytical separation methods suitable for the determination. The growing interest in the determination of chiral AAs from biological samples is obvious from the number of novel papers including improved and/or new chromatographic methods. New findings in AA analyses of fossil matters used in geochronology are usually achieved by well-established chromatographic methods. On the other hand, significant improvement of chromatographic methods is actual especially for the determination of D-AAs in various biological fluids and tissues. Progress in this field was enabled by the introduction of 2D HPLC and fast and sensitive UPLC/MS methods. Both direct and indirect chiral separation methods are used. Indirect LC methods require appropriate chiral derivatization agent while direct methods suitable chiral stationary phase. Also non-chiral derivatization agents are frequently used in direct methods to increase selectivity and sensitivity. Certainly, racemization of AAs during sample preparation or derivatization must be avoided.

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Abbreviations

The following abbreviations are used in this manuscript:

AA: amino acid
MEKC: micellar electrokinetic chromatography
MS: mass spectrometry
CSP: chiral stationary phase
2D: two dimensional
ODS: octadecylsilica
NBD-F: 4-fluoro-7-nitro-2,1,3-benzoxadiazole
MP: mobile phase
ee: enantiomeric excess
OPA-NAC: *o*-phthaldialdehyde-*N*-acetyl-L-cysteine
SIM: selective ion monitoring
IBLC: *N*-isobutyryl-L-cysteine
ACN: acetonitrile
MeOH: methanol
OES: ostrich eggshell
THAA: total hydrolysable AAs
CBF: cerebrospinal fluid
LIF: laser-induced fluorescence
SDS: sodium dodecyl sulfate
SDC: sodium deoxycholate
L-PGA-OSu: L-pyroglutamic acid succinimidyl ester
NMDA: *N*-methyl-D-aspartate
AD: Alzheimer´s disease
FITC: fluorescein isothiocyanate
AAR: AA racemization
AAI: AA isomerisation
CCD-UPLC-MS/MS: covalent chiral derivatized ultra performance liquid chromatography tandem mass spectrometry
DM: *diabetes mellitus*

References

1. Helfman, P.M.; Bada, J.L. Aspartic acid racemization in dentine as a measure of aging. *Nature* **1976**, *262*, 279-281.
2. Muraoka, S.; Fujii, N.; Ueda, Y.; Mitsui, Y.; Satoh, K.; Harada, K. Racemization of aspartic acid in αA-crystallin of bovine lens. *Biomed. Res.* **1991**, *12*, 61-64.
3. Iadarola, P.; Ferrari, F.; Fumagalli, M.; Viglio, S. Determination of amino acids by micellar EKC: Recent advances in method development and novel applications to different matrices. *Electrophoresis* **2008**, *29*, 224-236.
4. Tanwar, S.; Bhushan, R. Enantioresolution of amino acids: a decade's perspective, prospects and challenges. *Chromatographia* **2015**, *78*, 1113-1134.
5. Rocco, A.; Aturki, Z.; Fanali, S. Chiral separations in food analysis. *Trends Anal. Chem*. **2013**, *52*, 206-225.
6. Kalíková, K.; Riesová, M.; Tesařová, E. Recent chiral selectors for separation in HPLC and CE. *Cent. Eur. J. Chem.* **2012**, *10*, 450-471.
7. König, W.A.; Rahn, W. Gas chromatographic separation of diastereoisomeric amino acid derivatives on glass capillaries: The use of pentafluoropropionyl-amino acid (+)-3-methyl-2-butyl esters. *J. Chromatogr. A* **1977**, *133*, 141-146.
8. Ilisz, I.; Péter, A.; Lindner, W. State-of-the-art enantioseparations of natural and unnatural amino acids by high-performance liquid chromatography. *Trends Anal. Chem*. **2016**, doi: 10.1016/j.trac.2016.01.016.
9. Ilisz, I.; Aranyi, A.; Péter, A. Chiral derivatizations applied for the separation of unusual amino acid enantiomers by liquid chromatography and related techniques. *J. Chromatogr. A* **2013**, *1296*, 119-139.
10. Monroe, A.A.; Pizzarello, S. The soluble organic compounds of the Bells meteorite: Not a unique or unusual composition. *Geochim. Cosmochim. Ac.* **2011**, *75*, 7585-7595.
11. Pizzarello, S.; Yarnes, C.T. Enantiomeric excesses of chiral amines in ammonia-rich carbonaceous meteorites. *Earth Planet. Sci. Lett*. **2016**, *443*, 176-184.
12. Zahradníčková, H.; Hartvich, P.; Holoubek, I. History and significance of chiral analysis of amino acids in biological matrices and environment. *Chem. Listy* **2005**, *99*, 703-710.
13. Weiner, S.; Kustanovich, Z.; Gil-Av, E.; Traub, W. Dead Sea Scroll parchments: unfolding of the collagen molecules and racemization of aspartic acid. *Nature* **1980**, *287*, 820-823.
14. Nagata, Y.; Kubota, K. A trial to determine D-amino acids in tissue proteins of mice. *Amino Acids* **1993**, *4*, 121-125.
15. Miyamoto, T.; Sekine, M.; Ogawa, T.; Hidaka, M.; Homma, H.; Masaki, H. Origin of D-amino acids detected in the acid hydrolysates of purified *Escherichia coli* β-galactosidase. *J. Pharm. Biomed. Anal.* **2015**, *116*, 105-108.
16. Frank, H.; Woiwode, W.; Nicholson, G.J.; Bayer, E. *Determination of optical purity of amino acids in proteins*; In *Stable isotopes*; Kein, E.; Kein, P.P., Eds.; Academic Press: New York, USA, 1979; pp. 165-172.
17. Gerhardt, J.; Nicholson, G.J. *Unambiguous determination of the optical purity of peptides via GC-MS*; In *Peptides: chemistry, structure and biology*; Hodges, R.S.; Smith J.A., Eds.; Escom Science Publishers BV: Leiden, Netherlands, 1994; pp. 241-243.
18. Liardon, R.; Ledermann, S.; Ott, U. Determination of D-amino acids by deuterium labelling and selected ion monitoring. *J. Chromatogr. A* **1981**, *203*, 385-395.
19. Nokihara, K.; Gerhardt, J. Development of an improved automated gas-chromatographic chiral analysis system: Application to non-natural amino acids and natural protein hydrolysates. *Chirality* **2001**, *13*, 431-434.
20. Tao, Y.; Julian, R. R. Identification of amino acid epimerization and isomerization in crystallin proteins by tandem LC-MS. *Anal. Chem*. **2014**, *86*, 9733-9741.
21. Tao, W.A.; Zhang, D.; Nikolaev, E.N.; Cooks, R.G. Copper(II)-assisted enantiomeric analysis of D,L-amino acids using the kinetic method: Chiral recognition and quantification in the gas phase. *J. Am. Chem. Soc*. **2000**, *122*, 10598-10609.
22. Marchelli, R.; Dossena, A.; Palla, G. The potential of enantioselective analysis as a quality control tool. *Trends Food Sci. Technol.* **1996**, *71*, 113-119.
23. Srkalová, S.; Kalíková, K.; Tesařová, E. The presence and importance of enantiomers in food. *Chem. Listy* **2008**, *102*, 480-486.
24. Herrero, M.; Simó, C.; García-Cañas, V.; Fanali, S.; Cifuentes, A. Chiral capillary electrophoresis in food analysis, *Electrophoresis* **2010**, *31*, 2106-2114.

25. Chaves das Neves, H.J.; Noronha, J.P. Recognition of vinification technology through gas chromatographic data on enantiomeric purity of free amino acids. *Fresenius J. Anal. Chem*. **1995**, *352*, 783-787.
26. Frank, H.; Nicholson, G.J.; Bayer, E. Rapid gas chromatographic separation of amino acid enantiomers with a novel chiral stationary phase. *J. Chromatogr. Sci.* **1977**, *15*, 174-176.
27. Ali, H.S.M.; Pätzold, R.; Brückner, H. Gas chromatographic determination of amino acid enantiomers in bottled and aged wines. *Amino Acids* **2010**, *38*, 951-958.
28. Ali, H.S.M.; Alhaj, O.A.; Al-Khalifa, A.S.; Brückner, H. Determination and stereochemistry of proteinogenic and non-proteinogenic amino acids in Saudi Arabian date fruits. *Amino Acids* **2014**, *46*, 2241-2257.
29. Gogami, Y.; Okada, K.; Oikawa, T. High-performance liquid chromatography analysis of naturally occurring D-amino acids in sake. *J. Chromatogr. B* **2011**, *879*, 3259-3267.
30. Miyoshi, Y.; Nagano, M.; Ishigo, S.; Ito, Y.; Hashiguchi, K.; Hishida, N.; Mita, M.; Lindner, W.; Hamase, K. Chiral amino acid analysis of Japanese traditional Kurozu and the developmental changes during earthenware jar fermentation processes. *J. Chromatogr. B* **2014**, *966*, 187-192.
31. Sardella, R.; Lisanti, A.; Marinozzi, M.; Ianni, F.; Natalini, B.; Blanch, G.P.; Ruiz del Castillo, M.L. Combined monodimensional chromatographic approaches to monitor the presence of D-amino acids in cheese. *Food Control* **2013**, *34*, 478-487.
32. Garde, E.; Heide-Jørgensen, M.P.; Hansen, S.H.; Nachman, G.; Forchhammer, M.C. Age-specific growth and remarkable longevity in natahal *(Monodon monoceros*) from west greenland as estimated by aspartic acid racemization. *J. Mammalogy*, **2007**, *88*, 49-58.
33. Fujii, N.; Muraoka, S.; Satoh, K.; Hori, H.; Harada, K. Racemization of aspatic acids at specific sites in αA-crystallin from aged human lens. *Biomed. Res.* **1991**, *12*, 315-321.
34. Ohtani, S.; Yamamoto, T.; Sugimoto, H.; Sashima, M.; Satoh, M. Age-related changes in the D-aspartic acid content of the teeth of the senescence-accelerated mouse. *Arch. Oral Biol.* **2000**, *45*, 13-18.
35. Yekkala, R.; Meers, C.; Van Schepdael, A.; Hoogmartens, J.; Lambrichts, I.; Willems, G. Racemization of aspartic acid from human dentin in the estimation of chronological age. *Forensic Sci. Int.* **2006**, *159S*, S89-S94.
36. Fu, S.-J.; Fan Ch.-Ch.; Song, H.-W.; Wei, F.-Q. Age estimation using a modified HPLC determination of ratio of aspartic acid in dentin. *Forensic Sci. Int.* **1995**, *73*, 35-40.
37. Ohtani, S. Estimation of age from dentin by utilizing the racemization of aspartic acid: influence of pH. *Forensic Sci. Int.* **1995**, *75*, 181-187.
38. Abe, I.; Ohtani, S. Novel chiral selectors anchored on polydimethylsiloxane as stationary phases for separation of derivatized amino acid enantiomers by capillary gas chromatography. *J. Sep. Sci*. **2006**, *29*, 319-324.
39. Benešová, T.; Hozátko, A.; Pilín, A.; Votruba, J.; Flieger, M. A modified HPLC method for the determination of aspartic acid racemization in collagen from human dentin and its comparison with GC. *J. Sep. Sci*. **2004**, *27*, 330-334.
40. Dobberstein, R.C.; Huppertz, J.; von Wurmb-Schwark, N.; Ritz-Timme, S. Degradation of biomolecules in artificially and naturally aged teeth: Implications for age estimation based on aspartic acid racemization and DNA analysis. *Forensic Sci. Int.* **2008**, *179*, 181-191.
41. Sørensen, M.B.; Aaslo, P.; Egsgaard, H.; Lund, T. Determination of D/L-amino acids by zero needle voltage electrospray ionization. *Rapid Commun. Mass Spectrom.* **2008**, *22*, 455-461.
42. Zhao, M.; Bada, J.L. Determination of α-dialkylamino acids and their enantiomers in geological samples by high performance liquid chromatography after derivatization with a chiral adduct of *o*-phthaldialdehyde. *J. Chromatogr. A* **1995**, *690*, 55-63.
43. Engel, M.H.; Goodfriend, G.A.; Qian, Y.; Macko, S.A. Indigeneity of organic matter in fossils: A test using stable isotope analysis of amino acid enantiomers in Quaternary mollusk shells. *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 10475-10478.
44. Clarke, S.J.; Murray-Wallace, C.V. Mathematical expressions used in amino acid racemisation geochronology–A review. *Quat. Geochronol*. **2006**, *1*, 261-278.
45. Schweitzer, M.H.; Avci, R.; Collier, T.; Goodwin, M.B. Microscopic, chemical and molecular methods for examining fossil preservation. *C. R. Palevol* **2008**, *7*, 159-184.

46. Blyth, A.J.; Baker, A.; Collins, M.J.; Penkman, K.E.H.; Gilmour, M.A.; Moss, J.S.; Genty, D.; Drysdale, R.N. Molecular organic matter in speleothems and its potential as an environmental proxy. *Quaternary Sci. Rev.* **2008**, *27*, 905-921.
47. Dahl, P.S.; Meyer, V.R. Overcoming peak overlap and low signal intensity in chromatography: Implications for amino acid geochronology of Quaternary shell materials. *Quaternary Sci. Rev*. **1999**, *18*, 1549-1559.
48. Kaufman, D.S.; Manley, W.F. A new procedure for determining DL amino acid ratio in fossils using reversed phase liquid chromatography. *Quaternary Sci. Rev.* **1998**, *17*, 987-1000.
49. Brückner, H.; Wittner, R.; Godel, H. Fully automated high-performance liquid chromatographic separation of DL amino acids derivatized with o-phthaldialdehyde together with *N*-isobutyryl-cysteine, application to food samples. *Chromatographia* **1991**, *32*, 383-388.
50. Penkman, K.E.H.; Kaufman, D.S.; Maddy, D.; Collins, M.J. Closed-system behaviour of the intra-crystalline fraction of amino acids in mollusc shells. *Quat. Geochronol.* **2008**, *3*, 2-25.
51. Fitznar. H.P.; Lobbes, J.M.; Kattner, G. Determination of enantiomeric amino acids with high-performance liquid chromatography and pre-column derivatisation with *o*-phthaldialdehyde and *N*-isobutyrylcysteine in seawater and fossil samples (mollusks). *J. Chromatogr. A* **1999**, *832*, 123-132.
52. Kaufman, D.S. Amino acid paleothermometry of Quaternary ostracodes from the Bonneville Basin, Utah. *Quaternary Sci. Rev*. **2003**, *22*, 899-914.
53. Hearty, P.J.; O'Leary, M.J.; Kaufman, D.S.; Page, M.C.; Bright, J. Amino acid geochronology of individual foraminifer (*Pulleniatina obliquiloculata*) tests, north Queensland margin, Australia: A new approach to correlating and dating Quaternary tropical marine sediment cores. *Paleoceanography* **2004**, *19*, PA 402.
54. Kaufman, D.S. Temperature sensitivity of aspartic and glutamic acid racemization in the foraminifera *Pulleniatina. Quat. Geochronol.* **2006**, 1, 188-207.
55. Barbour Wood, S.L.; Krause Jr., R.A.; Kowalewski, M.; Wehmiller, J.; Simões, M.G. Aspartic acid racemization dating of Holocene brachiopods and bivalves from the southern Brazilian shelf, South Atlantic. *Quaternary Res.* **2006**, *66*, 323-331.
56. O'Donnell, T.H.; Macko, S.A.; Wehmiller, J.F. Stable carbon isotope composition of amino acids in modern and fossil *Mercenaria*. *Org. Geochem.* **2007**, *38*, 485-498.
57. Penkman, K.E.H.; Preece, R.C.; Keen, D.H.; Maddy, D.; Schreve, D.C.; Collins, M.J. Testing the aminostratigraphy of fluvial archives: the evidence from intra-crystalline proteins within freshwater shells. *Quaternary Sci. Rev.* **2007**, *26*, 2958-2969.
58. Bright, J.; Kaufman, D.S. Amino acid racemization in lacustrine ostracodes, part I: effect of oxidizing pre-treatments on amino acid composition. *Quat. Geochronol.* **2011**, *6*, 154-173.
59. Powell, J.; Collins, M.J.; Cussens, J.; MacLeod, N.; Penkman, K.E.H. Results from an amino acid racemization inter-laboratory proficiency study; design and performance evaluation. *Quat. Geochronol.* **2013**, *16*, 183-197.
60. Ortiz, J.E.; Torres, T.; Pérez-González, A. Amino acid racemization in four species of ostracodes: Taxonomic, environmental, and microstructural controls. *Quat. Geochronol.* **2013**, *16*, 129-143.
61. Kosnik, M.A.; Kaufman, D.S.; Hua, Q. Radiocarbon-calibrated multiple amino acid geochronology of Holocene molluscs from Bramble and Rib Reefs (Great Barrier Reef, Australia). *Quat. Geochronol.* **2013**, *16*, 73-86.
62. Demarchi, B.; Williams, M.G.; Milner, N.; Russell, N.; Bailey, G.; Penkman, K. Amino acid racemization dating of marine shells: A mound of possibilities. *Quatern. Int.* **2011**, *239*, 114-124.
63. Demarchi, B.; Rogers, K.; Fa, D.A.; Finlayson, C.J.; Milner, N.; Penkman, K.E.H. Intra-crystalline protein diagenesis (IcPD) in *Patella vulgata*. Part I: Isolation and testing of the closed system. *Quat. Geochronol.* **2013**, *16*, 144-157.
64. Kaufman, D.S.; Cooper, K.; Behl, R.; Billups, K.; Bright, J.; Gardner, K.; Hearty, P.; Jakobsson, M.; Mendes, I.; O'Leary, M.; Polyak, L.; Rasmussen, T.; Rosa, F.; Schmidt, M. Amino acid racemization in mono-specific foraminifera from Quaternary deep-sea sediments. *Quat. Geochronol.* **2013**, *16*, 50-61.
65. Demarchi, B.; Clements, E.; Coltorti, M.; van de Locht, R.; Kröger, R.; Penkman, K.; Rose, J. Testing the effect of bleaching on the bivalve Glycymeris: A case study of amino acid geochronology on key Mediterranean raised beach deposits. *Quat. Geochronol.* **2015**, *25*, 49-65.

66. Crisp, M.; Demarchi, B.; Collins, M.; Morgan-Williams, M.; Pilgrim, E.; Penkman, K. Isolation of the intra-crystalline proteins and kinetic studies in *Struthio camelus* (ostrich) eggshell for amino acid geochronology. *Quat. Geochronol*. **2013**, *16*, 110-128.
67. Murray-Wallace, C.V.; Richter, J.; Vogelsang, R. Aminostratigraphy and taphonomy of ostrich eggshell in the sedimentary infill of Apollo 11 Rockshelter, Namibia. *J. Archaeol. Sci. Reports* **2015**, *4*, 143-151.
68. Csapó, J.; Csapó-Kiss, Z.; Csapó Jr., J. Use of amino acids and their racemization for age determination in archaeometry. *Trends Anal. Chem.* **1998**, *17*, 140-148.
69. Iuliani, P.; Di Federico, L.; Fontecchio, G.; Carlucci, G. RP-HPLC method with fluorescence detection for amino acids D/L ratio determination in fossil bones as a marker of DNA preservation. *J. Sep. Sci.* **2010**, 33, 2411-2416.
70. Harada, N.; Kondo, T.; Fukuma, K.; Uchida, M.; Nakamura, T.; Iwai, M.; Murayama, M.; Sugawara, T.; Kusakabe, M. Is amino acid chronology applicable to the estimation of the geological age of siliceous sediments? *Earth Planet. Sci. Lett.* **2002**, *198*, 257-266.
71. Hearty, P.J., Kaufman, D.S. Whole-rock Aminostratigraphy and Quaternary sea-level history of the Bahamas. *Quaternary Res*. **2000**, *54*,163-173.
72. Hearty, P., O'Leary, M.; Donald, A.; Lachlan, T. The enigma of 3400 years BP coastal oolites in tropical northwest Western Australia. . . why then, why there? *Sediment. Geol.* **2006**, *186*, 171-185.
73. Lomstein, B.A.; Jørgensen, B.B.; Schubert, C.J.; Niggemann, J. Amino acid biogeo- and stereochemistry in coastal Chilean sediments. *Geochim. Cosmochim. Ac.* **2006**, *70*, 2970-2989.
74. Uhle, M.E.; Sikes, E.L.; Nodder, S.D.; Pilditch, C.A. Sources and diagenetic status of organic matter in the Hauraki Gulf, New Zealand: Evidence from the carbon isotopic composition of D- and L-amino acids. *Org. Geochem.* **2007**, *38*, 440-457.
75. Takano, Y.; Kobayashi, K.; Ishikawa, Y.; Marumo, K. Emergence of the inflection point on racemization rate constants for D- and L-amino acids in the early stages of terrestrial diagenesis. *Org. Geochem.* **2006**, *37*, 334-341.
76. Grégoire, B.; Erastova, V.; Geatches, D.L.; Clark, S.J.; Greenwell, H.C.; Fraser, D.G. Insights into the behaviour of biomolecules on the early Earth: The concentration of aspartate by layered double hydroxide minerals. *Geochim. Cosmochim. Ac.* **201**6, *176*, 239-258.
77. Worsfold, P.J.; Monbet, P.; Tappin, A.D.; Fitzsimons, M.F.; Stiles, D.A.; McKelvie, I.D. Characterisation and quantification of organic phosphorus and organic nitrogen components in aquatic systems: A Review. *Anal. Chim. Acta* **2008**, *624*, 37-58.
78. Amelung, W.; Zhang, X.; Flach, K.W. Amino acids in grassland soils: Climatic effects on concentrations and chirality. *Geoderma* **2006**, *130*, 207-217.
79. Brodowski, S.; Amelung, W.; Lobe, I.; Du Preez, C.C. Losses and biogeochemical cycling of soil organic nitrogen with prolonged arable cropping in the South African Highveld - evidence from D- and L-amino acids. *Biogeochemistry* **2004**, *71*, 17-42.
80. Zhang, X.; He, H.; Amelung, W. A GC/MS method for the assessment of ^{15}N and ^{13}C incorporation into soil amino acid enantiomers. *Soil Biol. Biochem.* **2007**, *39*, 2785-2796.
81. McCudden, C.R.; Kraus, V.B. Biochemistry of amino acid racemization and clinical application to musculoskeletal disease. *Clin. Biochem.* **2006,** *39*, 1112-1130.
82. Chervyakov, A.V.; Gulyaeva, N.V.; Zakharova, M.N. D-amino acids in normal ageing and pathogenesis of neurodegenerative diseases. *Neurochem. J.* **2011**, *5*, 100-114.
83. Fujii, N.; Kaji, Y.; Fujii, N. D-Amino acids in aged proteins: Analysis and biological relevance. *J. Chromatogr. B* **2011**, *879*, 3141-3147.
84. Denoroy, L.; Zimmer, L.; Renaud, B.; Parrot, S. Ultra high performance liquid chromatography as a tool for the discovery and the analysis of biomarkers of diseases: A review. *J. Chromatogr. B* **2013**, *927*, 37-53.
85. Imai, K.; Fukushima, T.; Santa, T.; Homma, H.; Hamase, K.; Sakai, K.; Kato, M. Analytical chemistry and biochemistry of D-amino acids. *Biomed. Chromatogr.* **1996**, *10*, 303-312.
86. Hamase, K.; Morikawa, A.; Zaitsu, K. D-Amino acids in mammals and their diagnostic value. *J. Chromatogr. B* **2002**, *781*, 73-91.
87. Hamase, K. Sensitive two-dimensional determination of small amounts of D-amino acids in mammals and the study on their functions. *Chem. Pharm. Bull.* **2007**, *55*, 503-510.
88. Miyoshi, Y.; Koga, R.; Oyama, T.; Han, H.; Ueno, K.; Masuyama, K.; Itoh, Y.; Hamase, K. HPLC analysis of naturally occurring free D-amino acids in mammals. *J. Pharm. Biomed. Anal.* **2012**, *69*, 42-49.

89. Miyoshi, Y.; Oyama, T.; Itoh, Y.; Hamase, K. Enantioselective two-dimensional high-performance liquid chromatographic determination of amino acids; analysis and physiological significance of D-amino acids in mammals. *Chromatography* **2014**, *35*, 49-57.
90. Fujii, N.; Takata, T.; Fujii, N. Quantitative analysis of isomeric (L-α-, L-β-, D-α-, D-β-) aspartyl residues in proteins from elderly donors. *J. Pharm. Biomed. Anal.* **2015**, *116*, 25-33.
91. Fuchs, S.A.; de Sain-van der Velden, M.G.M.; de Barse, M.M.J.; Roeleveld, M.W.; Hendriks, M.; Dorland, L.; Klomp, L.W.J.; Berger, R.; de Koning, T.J. Two mass-spectrometric techniques for quantifying serine enantiomers and glycine in cerebrospinal fluid: Potential confounders and age-dependent ranges. *Clin. Chem.* **2008**, *54*, 1443-1450.
92. Goodlett, D.R.; Abuaf, P.A.; Savage, P.A.; Kowalski, K.A.; Mukherjee, T.K.; Tolan, J.W.; Corkum, N.; Goldstein, G.; Crowther, J.B. Peptide chiral purity determination: hydrolysis in deuterated acid, derivatization with Marfey's reagent and analysis using high-performance liquid chromatography-electrospray ionization-mass spectrometry. *J. Chromatogr. A* **1995**, 707, 233-244.
93. Thorsén, G.; Bergquist, J. Chiral separation of amino acids in biological fluids by micellar electrokinetic chromatography with laser-induced fluorescence detection. *J. Chromatogr. B* **2000**, *745*, 389-397.
94. Grant, S.L.; Shulman, Y.; Tibbo, P.; Hampson, D.R.; Baker, G.B. Determination of D-serine and related neuroactive amino acids in human plasma by high-performance liquid chromatography with fluorimetric detection. *J. Chromatogr. B* **2006**, *844*, 278-282.
95. Hamase, K.; Morikawa, A.; Ohgusu, T.; Lindner, W.; Zaitsu, K. Comprehensive analysis of branched aliphatic D-amino acids in mammals using an integrated multi-loop two-dimensional column-switching high-performance liquid chromatographic system combining reversed-phase and enantioselective columns. *J. Chromatogr. A* **2007**, *1143*, 105-111.
96. Long, Z.; Nimura, N.; Adachi, M.; Sekine, M.; Hanai, T.; Kubo, H.; Homma, H. Determination of D- and L-aspartate in cell culturing medium, within cells of MPT1 cell line and in rat blood by a column-switching. *J. Chromatogr. B* **2001**, *761*, 99-106.
97. Armstrong, D.W.; Gasper, M.P.; Lee, S.H.; Ercal, N.; Zukowski, J. Factors controlling the level and determination of D-amino acids in the urine and plasma of laboratory rodents. *Amino Acids* **1993**, *5*, 299-315.
98. Hamase, K.; Miyoshi, Y.; Ueno, K.; Han, H.; Hirano, J.; Morikawa, A.; Mita, M.; Kaneko, T.; Lindner, W.; Zaitsu, K. Simultaneous determination of hydrophilic amino acid enantiomers in mammalian tissues and physiological fluids applying a fully automated micro-two-dimensional high-performance liquid chromatographic concept. *J. Chromatogr. A* **2010**, *1217*, 1056-1062.
99. Sasabe, J.; Suzuki, M.; Miyoshi, Y.; Tojo, Y.; Okamura, C.; Ito, S.; Konno, R.; Mita, M.; Hamase, K.; Aiso, S. Ischemic acute kidney injury perturbs homeostasis of serine enantiomers in the body fluid in mice: Early detection of renal dysfunction using the ratio of serine enantiomers. *PLOS ONE* **2014**, *9*, e. 86504.
100. Morikawa, A.; Hamase, K.; Zaitsu, K. Determination of D-alanine in the rat central nervous system and periphery using column-switching high-performance liquid chromatography. *Anal. Biochem.* **2003**, *312*, 66-72.
101. Han, H.; Wang, Q.-Q.; Wu, H.-H.; Wang, H. Establishment and application of an automated chiral two-dimensional high performance liquid chromatography method for bio-analysis of *D*-acidic amino acids. *Chinese J. Anal. Chem.* **2014**, *42*, 891-898.
102. Visser, W.F.; Verhoeven-Duif, N.M.; Ophoff, R.; Bakker, S.; Klomp, L.W.; Berger, R.; de Koning, T.J. A sensitive and simple ultra-high-performance-liquid chromatography–tandem mass spectrometry based method for the quantification of D-amino acids in body fluids. *J. Chromatogr. A* **2011**, *1218*, 7160-7136.
103. Tomlinson, C.; Rafii, M.; Ball, R.O.; Pencharz, P. The significance of D-isomers in stable isotope studies in humans is dependent on the age of the subject and the amino acid tracer. *Metabolism* **2010**, *59*, 14-19.
104. Reischl, R.J.; Hartmanova, L.; Carrozzo, M.; Huszar, M.; Frühauf, P.; Lindner, W. Chemoselective and enantioselective analysis of proteinogenic amino acids utilizing N-derivatization and 1-D enantioselective anion-exchange chromatography in combination with tandem mass spectrometric detection. *J. Chromatogr. A* **2011**, *1218*, 8379-8387.
105. Karakawa, S.; Shimbo, K.; Yamada, N.; Mizukoshi, T.; Miyano, H.; Mita, M.; Lindner, W.; Hamase, K. Simultaneous analysis of D-alanine, D-aspartic acid, and D-serine using chiral high-performance liquid chromatography-tandem mass spectrometry and its application to the rat plasma and tissues. *J. Pharm. Biomed. Anal.* **2015**, *115*, 123-129.

106. Kinoshita, K.; Jingu, S.; Yamaguchi, J. A surrogate analyte method to determine D-serine in mouse brain using liquid chromatography–tandem mass spectrometry. *Anal. Biochem.* **2013**, *432,* 124-130.
107. Sugimoto, H.; Kakehi, M.; Jinno, F. Bioanalytical method for the simultaneous determination of D- and L-serine in human plasma by LC/MS/MS. *Anal. Biochem.* **2015**, *487*, 38-44.
108. Sugimoto, H.; Kakehi, M.; Jinno, F. Method development for the determination of D- and L-isomers of leucine in human plasma by high-performance liquid chromatography tandem mass spectrometry and its application to animal plasma samples. *Anal. Bioanal. Chem.* **2015**, *407*, 7889-7898.
109. Mochizuki, T.; Todoroki, K.; Inoue, K.; Min, J.Z.; Toyo'oka, T. Isotopic variants of light and heavy L-pyroglutamic acid succinimidyl esters as the derivatization reagents for DL-amino acid chiral metabolomics identification by liquid chromatography and electrospray ionization mass spectrometry. *Anal. Chim. Acta* **2014**, *811*, 51-59.
110. Mochizuki, T.; Takayama, T.; Todoroki, K.; Inoue, K.; Min, J.Z.; Toyo'oka, T. Towards the chiral metabolomics: Liquid chromatography–mass spectrometry based DL-amino acid analysis after labeling with a new chiral reagent, (*S*)-2,5-dioxopyrrolidin-1-yl-1-(4,6-dimethoxy-1,3, 5-triazin-2-yl)pyrrolidine-2-carboxylate, and the application to saliva of healthy volunteers. *Anal. Chim. Acta* **2015**, *875*, 73-82.
111. D'Aniello, A.; Lee, J.M.; Petrucelli, L.; Di Fiore, M.M. Regional decreases of free D-aspartate levels in Alzheimer's disease. *Neurosci. Lett.* **1998**, *250*, 131-134.
112. Hashimoto, K.; Fukushima, T.; Shimizu, E.; Komatsu, N.; Watanabe, H.; Shinoda, N.; Nakazato, M.; Kumakiri, C.; Okada, S.; Hasegawa, H.; Imai, K.; Iyo, M. Decreased serum levels of D-serine in patients with schizophrenia. Evidence in support of the N-methyl-D-aspartate receptor hypofunction hypothesis of schizophrenia. *Arch. Gen. Psychiatry* **2003**, *60*, 572-576.
113. Yamada, K.; Ohnishi, T.; Hashimoto, K.; Ohba, H.; Iwayama-Shigeno, Y.; Toyoshima, M.; Okuno, A.; Takao, H.; Toyota, T.; Minabe, Y.; Nakamura, K.; Shimizu, E.; Itokawa, M.; Mori, N.; Iyo, M.; Yoshikawa, T. Identification of multiple serine racemase (*SRR*) mRNA isoforms and genetic analyses of *SRR* and *DAO* in schizophrenia and D-serine levels. *Biol. Psychiatry* **2005**, *57*, 1493-1503.
114. Thorsén, G.; Bergquist, J.; Westlind-Danielsson, A.; Josefsson, B. Stereoselective determination of amino acids in β-amyloid peptides and senile plaques. *Anal. Chem.* **2001**, *73*, 2625-2631.
115. Hashimoto, K.; Fukushima, T.; Shimizu, E.; Okada, S.; Komatsu, N.; Okamura, N.; Koike, K.; Koizumi, H.; Kumakiri, C.; Imai, K.; Iyo, M. Possible role of D-serine in the pathophysiology of Alzheimer's disease. *Prog. Neuro-Psychopharmacol. Biol. Psych*. **2004**, *28*, 385-388.
116. Fukushima, T.; Lee, J.-A.; Korenaga, T.; Ichihara, H.; Kato, M.; Imai, K. Simultaneous detemination of D-lactic acid and 3-hydroxybutyric acid in rat plasma using a column-switching HPLC with fluorescent derivatization with 4-nitro-7-piperazino-2,1,3-benzoxadiazole (NBD-PZ). *Biomed. Chromatogr.* **2001**, *15*, 189-195.
117. Samakashvili, S.; Ibáñez, C.; Simó, C.; Gil-Bea, F.J.; Winblad, B.; Cedazo-Mínguez, A.; Cifuentes, A. Analysis of chiral amino acids in cerebrospinal fluid samples linked to different stages of Alzheimer disease. *Electrophoresis* **2011**, *32*, 2757-2764.
118. Inoue, K.; Hosaka, D.; Mochizuki, N.; Akatsu, H.; Tsutsumiuchi, K.; Hashizume, Y.; Matsukawa, N.; Yamamoto, T.; Toyo'oka, T. Simultaneous determination of post-translational racemization and isomerization of *N*-terminal amyloid-β in Alzheimer's brain tissues by covalent chiral derivatized ultraperformance liquid chromatography tandem mass spectrometry. *Anal. Chem.* **2014**, *86*, 797-804.
119. Takayama, T.; Mochizuki, T.; Todoroki, K.; Min, J.Z.; Mizuno, H.; Inoue, K.; Akatsu, H.; Noge, I.; Toyo'oka, T. A novel approach for LC-MS/MS-based chiral metabolomics fingerprinting and chiral metabolomics extraction using a pair of enantiomers of chiral derivatization reagents. *Anal. Chim. Acta* **2015**, *898*, 73-84.
120. Xie, Y.; Alexander, G.M.; Schwartzman, R.J.; Singh, N.; Torjman, M.C.; Goldberg, M.E.; Wainer, I.W.; Moaddel, R. Development and validation of a sensitive LC–MS/MS method for the determination of D-serine in human plasma. *J. Pharm. Biomed. Anal.* **2014**, *89*, 1-5.
121. Moaddel, R.; Luckenbaugh, D.A.; Xie, Y.; Villaseñor, A.; Brutsche, N.E.; Machado-Vieira, R.; Ramamoorthy, A.; Lorenzo, M.P.; Garcia, A.; Bernier, M.; Torjman, M.C.; Barbas, C.; Zarete Jr., C.A.; Wainer, I.W. D-serine plasma concentration is a potential biomarker of (*R,S*)-ketamine antidepressant response in subjects with treatment-resistant depression. *Psychopharmacology* **2015**, *232*, 399-409.

122. Fukushima, T.; Kawai, J.; Imai, K.; Toyo'oka, T. Simultaneous determinationof D- and L-serine in rat brain microdialysis sample using a column-switching HPLC with fluorimetric detection. *Biomed. Chromatogr.* **2004**, *18*, 813-819.
123. Hashimoto, K.; Engberg, G.; Shimizu, E.; Nordin, C.; Lindström, L.H.; Iyo, M. Reduced D-serine to total serine ratio in the cerebrospinal fluid of drug naive schizophrenic patients. *Prog. Neuro-Psychopharmacol. Biol. Psychiatry* **2005**, *29*, 767-769.
124. Hashimoto, K.; Engberg, G.; Shimizu, E.; Nordin, C.; Lindström, L.H.; Iyo, M. Elevated glutamine/glutamate ratio in cerebrospinal fluid of first episode and drug naive schizophrenic patients. *BMC Psychiatry* **2005**, 5:6.
125. Pålsson, E.; Jakobsson, J.; Södersten, K.; Fujita, Y.; Sellgren, C.; Ekman, C.-J.; Ågren, H.; Hashimoto, K.; Landén, M. Markers of glutamate signaling in cerebrospinal fluid and serum from patients with bipolar disorder and healthy controls. *Eur. Neuropsychopharmacol.* **2015**, *25*, 133-140.
126. Deáková, Z.; Ďuračková, Z.; Armstrong, D.W.; Lehotay, J. Two-dimensional high performance liquid chromatography for determination of homocysteine, methionine and cysteine enantiomers in human serum. *J. Chromatogr. A* **2015**, *1408*, 118-124.
127. Waldhier, M.C.; Dettmer, K.; Gruber, M.A.; Oefner, P.J. Comparison of derivatization and chromatographic methods for GC–MS analysis of amino acid enantiomers in physiological samples. *J. Chromatogr. B* **2010**, *878*, 1103-1112.
128. Min, J.Z.; Hatanaka, S.; Yu, H.; Higashi, T.; Inagaki, S.; Toyo'oka, T. First detection of free D-amino acids in human nails by combination of derivatization and UPLC-ESI-TOF-MS. *Anal. Methods* **2010**, *2*, 1233-1235.
129. Min, J.Z.; Hatanaka, S.; Yu, H.; Higashi, T.; Inagaki, S.; Toyo'oka, T. Determination of DL-amino acids, derivatized with *R*(−)-4-(3-isothiocyanatopyrrolidin-1-yl)-7-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole, in nail of diabetic patients by UPLC–ESI-TOF-MS. *J. Chromatogr. B* **2011**, *879*, 3220-3228.
130. Lorenzo, M.P.; Dudzik, D.; Varas, E.; Gibellini, M.; Skotnicki, M.; Zorawski, M.; Zarzycki, W.; Pellati, F.; García, A. Optimization and validation of a chiral GC–MS method for the determination of free D-amino acids ratio in human urine: Application to a Gestational Diabetes Mellitus study. *J. Pharm. Biomed. Anal.* **2015**, *107*, 480-487.

Publikace VI – Enantioselektivní separace v superkritické fluidní chromatografii

Rostoucí obliba SFC v chirálních separacích je dána především lepší konstrukcí SFC přístrojů a zvýšenou robustností této metody. **Publikace VI** přibližuje mechanismus separace v SFC, zabývá se dostupnými chirálními stacionárními fázemi a shrnuje publikované chirální analýzy v letech 2000-2013.

Metoda SFC je založena na použití superkritické kapaliny jako hlavní složky MP. Superkritické kapaliny mají unikátní vlastnosti, jejich hustota a rozpouštěcí schopnost je obdobná jako u kapalin, nízkou viskozitou a difuzními vlastnostmi se podobá plynům [54]. Výsledkem těchto vlastností je nižší tlak v chromatografickém systému, a tedy možnost používat několikanásobně vyšší průtoky MP v porovnání s běžnou HPLC bez ztráty separační účinnosti [55]. Nejčastěji se používá oxid uhličitý, který je snadno dostupný, netoxický, nehořlavý a jeho kritických hodnot se dosahuje snadno (T_K = 31 °C, p_K = 74 bar). Jako organické modifikátory se obvykle používají MeOH, ACN, ethanol nebo izopropanol, dále se používají kyselá a zásaditá aditiva, např. kyselina trifluoroctová a triethylamin. Separační systém lze k získání vyšších hodnot rozlišení a lepšího tvaru píků modifikovat přídavkem aditiv, ať už organických látek nebo kyselin/bází. Vlivem přídavku organických modifikátorů probíhá většina SFC separací v subkritické oblasti. Pro přípravu CSP lze použít širokou škálu chirálních selektorů. V dnešní době se uplatňují především polysacharidové CSP na bázi derivatizované celulózy a amylózy. Dále se používají CSP na bázi derivatizovaných CD a CF, teikoplaninu, syntetických polymerů, Pirklovy nebo iontově výměnné chininové a chinidinové CSP.

Publikace VI

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

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

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Review

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

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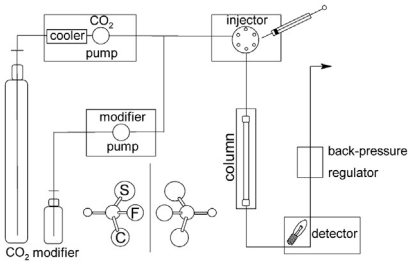
HIGHLIGHTS

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

GRAPHICAL ABSTRACT



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ABSTRACT

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

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

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Contents

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1. Introduction

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

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

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

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

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

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

2. Chiral stationary phases and chiral SFC separation process

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

2.1. Polysaccharide-based CSPs

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

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

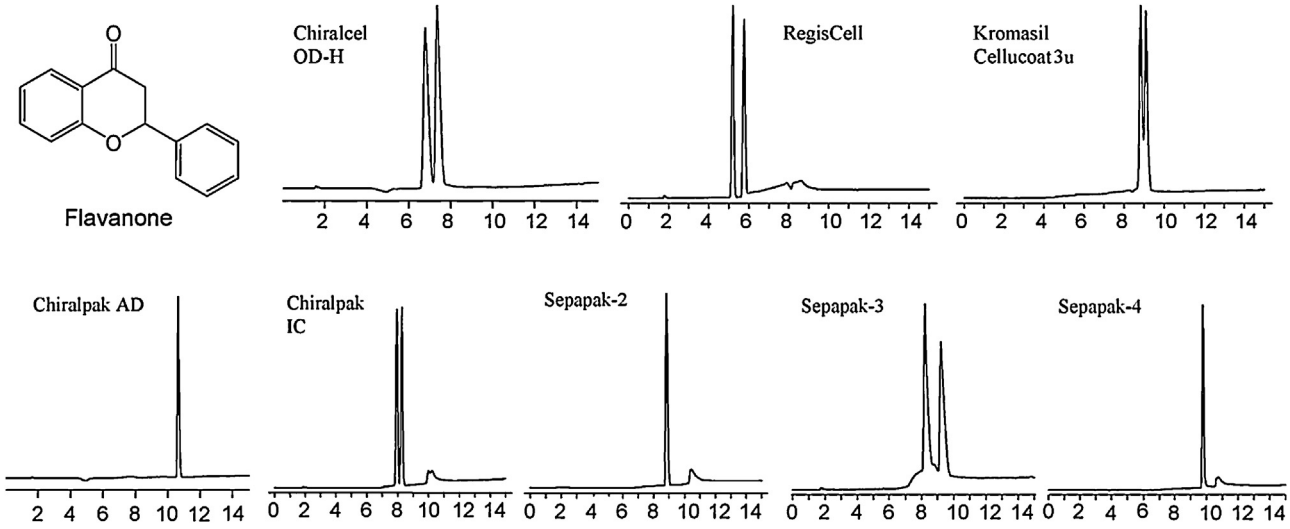

Fig. 1. Comparison of enantioseparation of flavanone on the generic OD-H columns and the Chiralpak AD-H and five other polysaccharide-based CSPs. Separation conditions: gradient elution, CO_2 and 4% (MeOH with 25 mM IBA) for 4 min, then ramp at 4% min^{-1} to 40%, hold for 2 min at 40%, 200 bar, 2 mL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

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

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

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

2.2. Cyclic oligosaccharides

2.2.1. Cyclodextrins

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

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

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

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

2.2.2. Cyclofructans

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

2.3. Ion exchange CSPs

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

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

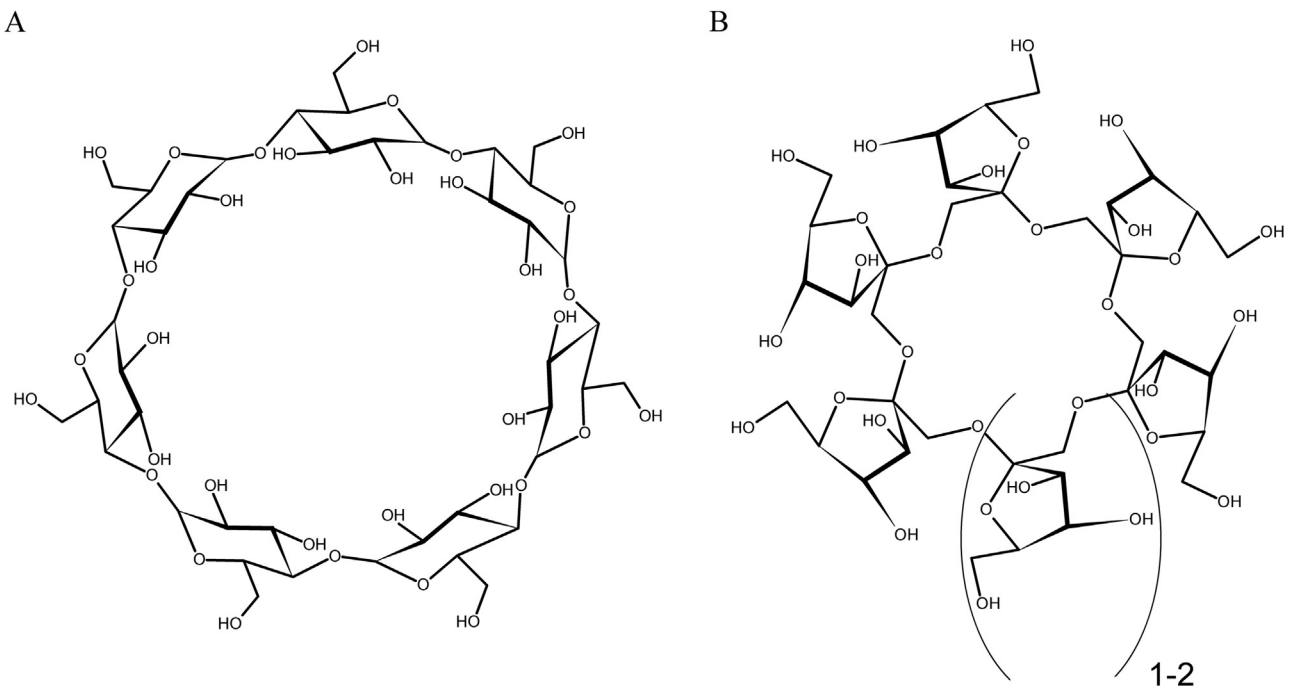

Fig. 2. Structure of cyclic oligosaccharides. (A) β-Cyclodextrin and (B) cyclofructan.

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

2.4. Macrocyclic glycopeptide CSPs

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

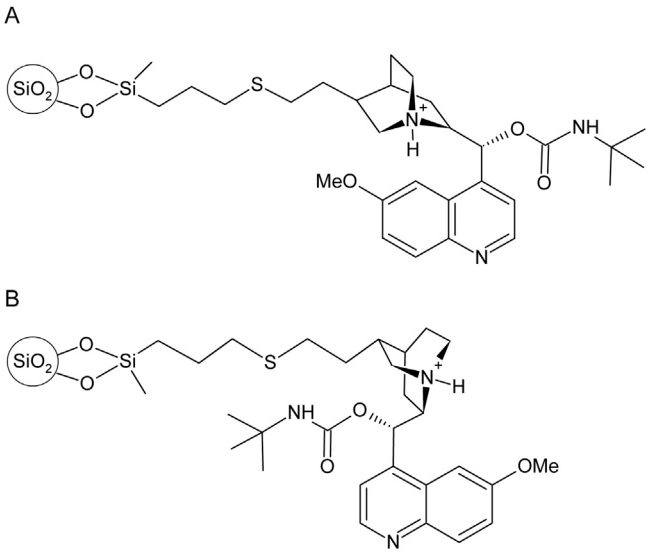

Fig. 3. Structures of weak anion exchangers. (A) QN-AX (quinine-based) and (B) QD-AX (quinidine-based).

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

2.5. Pirkle or brush type CSPs

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

2.6. Synthetic polymeric columns

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

2.7. Molecularly imprinted polymers CSPs

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

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

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

Table 1 (*Continued*)

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

Table 1 (*Continued*)

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

Table 1 (*Continued*)

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

Table 1 (*Continued*)

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

Table 1 (*Continued*)

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

Table 1 (*Continued*)

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

Table 1 (*Continued*)

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

Table 1 (*Continued*)

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

Table 1 (*Continued*)

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

Table 1 (*Continued*)

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

Table 1 (*Continued*)

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

Table 1 (*Continued*)

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

Table 1 (*Continued*)

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

Table 1 (*Continued*)

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

Table 1 (*Continued*)

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

Table 1 (*Continued*)

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

Table 1 (*Continued*)

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

Table 1 (*Continued*)

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

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

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

2.8. Miscellaneous CSPs

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

2.9. Packed capillary CSPs

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

2.10. Tandem-column coupling and two-dimensional SFC

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

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

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

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

2.11. Preparative SFC

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

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

2.12. Supercritical fluid simulated moving bed chromatography

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

2.13. Mobile phases in SFC

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

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

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

Table 2 (*Continued*)

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

See Table 1 footnotes for abbreviations.

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

2.14. Temperature and pressure

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

3. Applications

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

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

4. Conclusions

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

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

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References

[1] G. Guiochon, A. Tarafder, Fundamental challenges and opportunities for preparative supercritical fluid chromatography, J. Chromatogr. A 1218 (2011) 1037–1114.
[2] E. Lesellier, Retention mechanisms in super/subcritical fluid chromatography on packed columns, J. Chromatogr. A 1216 (2009) 1881–1890.
[3] K. De Klerck, D. Mangelings, Y.V. Heyden, Supercritical fluid chromatography for the enantioseparation of pharmaceuticals, J. Pharm. Biomed. Anal. 69 (2012) 77–92.
[4] E. Klesper, A.H. Corwin, D.A. Turner, High pressure gas chromatography above critical temperatures, J. Org. Chem. 27 (1962) 700–701.
[5] N.M. Karayannis, A.H. Corwin, E.W. Baker, E. Klesper, J.A. Walter, Apparatus and materials for hyperpressure gas chromatography of nonvolatile compounds, Anal. Chem. 40 (1968) 1736–1739.
[6] R.E. Jentoft, T.H. Gouw, Pressure-programmed supercritical fluid chromatography of wide molecular weight range mixtures, J. Chromatogr. Sci. 8 (1970) 138–142.
[7] R.E. Jentoft, T.H. Gouw, Apparatus for supercritical fluid chromatography with carbon dioxide as the mobile phase, Anal. Chem. 44 (1972) 681–686.
[8] M. Saito, History of supercritical fluid chromatography: instrumental development, J. Biosci. Bioeng. 115 (2013) 590–599.
[9] L.T. Taylor, Supercritical fluid chromatography for the 21st century, J. Supercrit. Fluids 47 (2009) 566–573.
[10] B. Bolaños, M. Greig, M. Ventura, W. Farrell, C.M. Aurigemma, H. Li, T.L. Quenzer, K. Tivel, J.M.R. Bylund, P. Tran, C. Pham, D. Philipson, SFC/MS in drug discovery at Pfizer, La Jolla, Int. J. Mass Spectrom. 238 (2004) 85–97.
[11] F.B. Li, Y.S. Hsieh, Supercritical fluid chromatography–mass spectrometry for chemical analysis, J. Sep. Sci. 31 (2008) 1231–1237.
[12] T.A. Berger, Demonstration of high speeds with low pressure drops using 1.8 μm particles in SFC, Chromatographia 72 (2010) 597–602.
[13] A.G.G. Perrenoud, J.L. Veuthey, D. Guillarme, Comparison of ultra-high performance supercritical fluid chromatography and ultra-high performance liquid chromatography for the analysis of pharmaceutical compounds, J. Chromatogr. A 1266 (2012) 158–167.

[14] W. Majewski, E. Valery, O. Ludemann-Hombourger, Principle and applications of supercritical fluid chromatography, J. Liq. Chromatogr. Relat. Technol. 28 (2005) 1233–1252.
[15] A. Rajendran, Design of preparative-supercritical fluid chromatography, J. Chromatogr. A 1250 (2012) 227–249.
[16] K.W. Phinney, Enantioselective separations by packed column subcritical and supercritical fluid chromatography, Anal. Bioanal. Chem. 382 (2005) 639–645.
[17] T. Berger, B. Berger, R.E. Majors, A review of column developments for supercritical fluid chromatography, LC GC N. Am. 28 (2010) 344–357.
[18] N.J. Wu, Increasing speed of enantiomeric separations using supercritical fluid chromatography, Adv. Chromatogr. 46 (2008) 213–234.
[19] D. Speybrouck, D. Corens, J.M. Argoullon, Screening strategy for chiral and achiral separations in supercritical fluid chromatography mode, Curr. Top. Med. Chem. 12 (2012) 1250–1263.
[20] G. Felix, A. Berthod, P. Piras, C. Roussel, Part III: Supercritical fluid chromatographic separations, Sep. Purif. Rev. 37 (2008) 229–301.
[21] B. Chankvetadze, Recent developments on polysaccharide-based chiral stationary phases for liquid-phase separation of enantiomers, J. Chromatogr. A 1269 (2012) 26–51.
[22] G. Gübitz, M.G. Schmid, Chiral separation principles in chromatographic and electromigration techniques, Mol. Biotechnol. 32 (2006) 159–179.
[23] T. Ikai, Y. Okamoto, Structure control of polysaccharide derivatives for efficient separation of enantiomers by chromatography, Chem. Rev. 109 (2009) 6077–6101.
[24] H. Steinmeier, P. Zugenmaier, Homogeneous and heterogeneous cellulose triesters and a cellulose triurethane: synthesis and structural investigations of the crystalline state, Carbohydr. Res. 164 (1987) 97–105.
[25] U. Vogt, P. Zugenmaier, Structural models for some liquid-crystalline cellulose derivatives, Phys. Chem. Chem. Phys. 89 (1985) 1217–1224.
[26] Y. Okamoto, E. Yashima, Polysaccharide derivatives for chromatographic separation of enantiomers, Angew. Chem. Int. Ed. 37 (1998) 1020–1043.
[27] L. Chen, R.A. Thompson, B.D. Johnson, J.M. Wyvratt, Enantiomeric separation of a thiazolbenzenesulfonamide compound using packed-column subcritical fluid chromatography, Chirality 14 (2002) 393–399.
[28] S. Svensson, A. Karlsson, O. Gyllenhaal, J. Vessman, Chiral separations of metoprolol and some analogs with carbon dioxide on Chiralcel OD and Chiralpak AD stationary phases. Use of chemometrics, Chromatographia 51 (2000) 283–293.
[29] A. Rajendran, M. Mazzotti, M. Morbidelli, Enantioseparation of 1-phenyl-1-propanol on Chiralcel OD by supercritical fluid chromatography: I. Linear isotherm, J. Chromatogr. A 1076 (2005) 183–188.
[30] S. Ottiger, J. Kluge, A. Rajendran, M. Mazzotti, Enantioseparation of 1-phenyl-1-propanol on cellulose-derived chiral stationary phase by supercritical fluid chromatography: II. Non-linear isotherm, J. Chromatogr. A 1162 (2007) 74–82.
[31] Z.B. Bao, B.G. Su, H.B. Xing, Y.W. Yang, Q.L. Ren, Enantioseparation of racemic paroxol on an amylose-based chiral stationary phase by supercritical fluid chromatography, J. Sep. Sci. 33 (2010) 3256–3262.
[32] C. Wenda, A. Rajendran, Enantioseparation of flurbiprofen on amylose-derived chiral stationary phase by supercritical fluid chromatography, J. Chromatogr. A 1216 (2009) 8750–8758.
[33] A. Rajendran, W.D. Chen, Binary retention time method for rapid determination of competitive Langmuir isotherm parameters, Sep. Purif. Technol. 67 (2009) 344–354.
[34] D.E. Cherrak, S. Khattabi, G. Guiochon, Adsorption behavior and prediction of the band profiles of the enantiomers of 3-chloro-1-phenyl-1-propanol: influence of the mass transfer kinetics, J. Chromatogr. A 877 (2000) 109–122.
[35] C. Wenda, R. Haghpanah, A. Rajendran, M. Amanullah, Optimization of isocratic supercritical fluid chromatography for enantiomer separation, J. Chromatogr. A 1218 (2011) 162–170.
[36] C. West, Y.R. Zhang, L. Morin-Allory, Insights into chiral recognition mechanisms in supercritical fluid chromatography. I. Non-enantiospecific interactions contributing to the retention on tris-(3,5-dimethylphenylcarbamate) of amylose and cellulose stationary phases, J. Chromatogr. A 1218 (2011) 2019–2032.
[37] C. West, G. Guenegou, Y.R. Zhang, L. Morin-Allory, Insights into chiral recognition mechanisms in supercritical fluid chromatography. II. Factors contributing to enantiomer separation on tris-(3,5-dimethylphenylcarbamate) amylose and cellulose stationary phases, J. Chromatogr. A 1218 (2011) 2033–2057.
[38] 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.
[39] K. De Klerck, G. Parewyck, D. Mangelings, Y.V. Heyden, Enantioselectivity of polysaccharide-based chiral stationary phases in supercritical fluid chromatography using methanol-containing carbon dioxide mobile phases, J. Chromatogr. A 1269 (2012) 336–345.
[40] L.X. Jin, W.L. Gao, H.Y. Yang, C.M. Lin, W.P. Liu, Enantiomeric resolution of five chiral pesticides on Chiralpak IB-H column by SFC, J. Chromatogr. Sci. 49 (2011) 739–743.
[41] L. Toribio, M.J. del Nozal, J.L. Bernal, J. Bernal, M.T. Martín, Study of the enantiomeric separation of an acetamide intermediate by using supercritical fluid chromatography and several polysaccharide based chiral stationary phases, J. Chromatogr. A 1218 (2011) 4886–4891.
[42] B.G. Su, Z.B. Bao, H.B. Xing, Y.W. Yang, Q.L. Ren, Enantioseparation of paroxetine intermediate on an amylose-derived chiral stationary phase by supercritical fluid chromatography, J. Chromatogr. A 1216 (2009) 5140–5146.
[43] L. Toribio, M.J. del Nozal, J.L. Bernal, C. Alonso, J.J. Jiménez, Enantiomeric resolution of bifonazole by supercritical fluid chromatography, J. Sep. Sci. 29 (2006) 1373–1378.
[44] L. Toribio, C. Alonso, M.J. del Nozal, J.L. Bernal, J.J. Jiménez, Enantiomeric separation of chiral sulfoxides by supercritical fluid chromatography, J. Sep. Sci. 29 (2006) 1363–1372.
[45] C. West, A. Bouet, I. Gillaizeau, G. Coudert, M. Lafosse, E. Lesellier, Chiral separation of phospine-containing α-amino acid derivatives using two complementary cellulosic stationary phases in supercritical fluid chromatography, Chirality 22 (2010) 242–251.
[46] C. West, M. Cieslikiewicz-Bouet, K. Lewinski, I. Gillaizeau, Enantiomeric separation of original heterocyclic organophosphorus compounds in supercritical fluid chromatography, Chirality 25 (2013) 230–237.
[47] C. Zhang, L.X. Jin, S.S. Zhou, Y.F. Zhang, S.L. Feng, Q.Y. Zhou, Chiral separation of neonicotinoid insecticides by polysaccharide-type stationary phases using high-performance liquid chromatography and supercritical fluid chromatography, Chirality 23 (2011) 215–221.
[48] Y.Q. Xiang, J.R. Dunetz, M. Lovdahl, Chiral separation of amides using supercritical fluid chromatography, J. Chromatogr. A 1293 (2013) 150–158.
[49] A.P. Zhang, W.L. Gao, B.B. Ma, L.X. Jin, C.M. Lin, Enantiomeric separations of chiral polychlorinated biphenyls on three polysaccharide-type chiral stationary phases by supercritical fluid chromatography, Anal. Bioanal. Chem. 403 (2012) 2665–2672.
[50] Y.Q. Zhao, W.A. Pritts, S.H. Zhang, Chiral separation of selected proline derivatives using a polysaccharide-type stationary phase by supercritical fluid chromatography and comparison with high-performance liquid chromatography, J. Chromatogr. A 1189 (2008) 245–253.
[51] P. Franco, T. Zhang, Common approaches for efficient method development with immobilised polysaccharide-derived chiral stationary phases, J. Chromatogr. B 875 (2008) 48–56.
[52] D.B. Laskar, L. Zeng, R.D. Xu, D.B. Kassel, Parallel SFC/MS-MUX screening to assess enantiomeric purity, Chirality 20 (2008) 885–895.
[53] L. Zeng, R.D. Xu, D.B. Laskar, D.B. Kassel, Parallel supercritical fluid chromatography/mass spectrometry system for high-throughput enantioselective optimization and separation, J. Chromatogr. A 1169 (2007) 193–204.
[54] C. Hamman, M.L. Wong, M. Hayes, P. Gibbons, A high throughput approach to purifying chiral molecules using 3 μm analytical chiral stationary phases via supercritical fluid chromatography, J. Chromatogr. A 1218 (2011) 3529–3536.
[55] M.M. Wong, W.B. Holzheuer, G.K. Webster, A comparison of HPLC and SFC chiral method development screening approaches for compounds of pharmaceutical interest, Curr. Pharm. Anal. 4 (2008) 101–105.
[56] A. Akin, F.J. Antosz, J.L. Ausec, K.F. Greve, R.L. Johnson, L.E. Magnusson, T. Ramstad, S.L. Secreast, D.S. Seibert, G.K. Webster, An orthogonal approach to chiral method development screening, Curr. Pharm. Anal. 3 (2007) 53–70.
[57] M. Maftouh, C. Granier-Loyaux, E. Chavana, J. Marini, A. Pradines, Y.V. Heyden, C. Picard, Screening approach for chiral separation of pharmaceuticals. Part III. Supercritical fluid chromatography for analysis and purification in drug discovery, J. Chromatogr. A 1088 (2005) 67–81.
[58] C. White, Integration of supercritical fluid chromatography into drug discovery as a routine support tool: Part I. Fast chiral screening and purification, J. Chromatogr. A 1074 (2005) 163–173.
[59] P. Borman, B. Boughtflower, K. Cattanach, K. Crane, K. Freebairn, G. Jonas, I. Mutton, A. Patel, M. Sanders, D. Thompson, Comparative performances of selected chiral HPLC, SFC, and CE systems with a chemically diverse sample set, Chirality 15 (2003) S1–S12.
[60] E. Schneiderman, A.M. Stalcup, Cyclodextrins: a versatile tool in separation science, J. Chromatogr. B 745 (2000) 83–102.
[61] Y. Xiao, S.C. Ng, T.T.Y. Tan, Y. Wang, Recent development of cyclodextrin chiral stationary phases and their applications in chromatography, J. Chromatogr. A 1269 (2012) 52–68.
[62] W.H. Tang, S.C. Ng, Monosubstituted positively charged cyclodextrins: synthesis and applications in chiral separation, J. Sep. Sci. 31 (2008) 3246–3256.
[63] R. Duval, H. Léveque, Y. Prigent, H.Y. Aboul-Enein, Enantioseparation of aminoglutethimide and thalidomide by high performance liquid chromatography or supercritical fluid chromatography on mono-2 and mono-6-O-pentenyl-β-cyclodextrin-based chiral stationary phases, Biomed. Chromatogr. 15 (2001) 202–206.
[64] H.F. Kasai, M. Tsubuki, S. Matsuo, T. Honda, Sub- and supercritical chiral separation of racemic compounds on columns with stationary phases having different functional groups, Chem. Pharm. Bull. 53 (2005) 1270–1276.
[65] T.T. Ong, R.Q. Wang, I.W. Muderawan, S.C. Ng, Synthesis and application of mono-6-(3-methylimidazolium)-6-deoxyperphenylcarbamoyl-β-cyclodextrin chloride as chiral stationary phases for high-performance liquid chromatography and supercritical fluid chromatogaphy, J. Chromatogr. A 1182 (2008) 136–140.
[66] R.Q. Wang, T.T. Ong, S.C. Ng, Synthesis of cationic β-cyclodextrin derivatives and their applications as chiral stationary phases for high-performance liquid chromatography and supercritical fluid chromatography, J. Chromatogr. A 1203 (2008) 185–192.
[67] R.Q. Wang, T.T. Ong, S.C. Ng, Chemically bonded cationic β-cyclodextrin derivatives as chiral stationary phases for enantioseparation applications, Tetrahedron Lett. 53 (2012) 2312–2315.

[68] R.Q. Wang, T.T. Ong, S.C. Ng, Chemically bonded cationic β-cyclodextrin derivatives and their application in supercritical fluid chromatography, J. Chromatogr. A 1224 (2012) 97–103.
[69] P. Sun, C. Wang, Z.S. Breitbach, Y. Zhang, D.W. Armstrong, Development of new HPLC chiral stationary phases based on native or derivatized cyclofructans, Anal. Chem. 81 (2009) 10215–10226.
[70] J. Vozka, K. Kalíková, C. Roussel, D.W. Armstrong, E. Tesařová, An insight into the use of dimethylphenyl carbamate cyclofructan 7 chiral stationary phase in supercritical fluid chromatography: the basic comparison with HPLC, J. Sep. Sci. 36 (2013) 1711–1719.
[71] C.L. Wang, Y.R. Zhang, Effects of column backpressure on supercritical fluid chromatography separations of enantiomers using binary mobile phases on 10 chiral stationary phases, J. Chromatogr. A 1281 (2013) 127–134.
[72] P. Franco, T. Zhang, A. Gargano, M. Mahut, M. Lämmerhofer, W. Lindner, Enantiomer and topoisomer separation of acidic compounds on anion-exchanger chiral stationary phases by HPLC and SFC, LC GC Eur. 25 (2012) 600–610.
[73] N.M. Maier, L. Nicoletti, M. Lämmerhofer, W. Lindner, Enantioselective anion exchangers based on cinchona alkaloid-derived carbamates: influence of C_8/C_9 stereochemistry on chiral recognition, Chirality 11 (1999) 522–528.
[74] R. Pell, W. Lindner, Potential of chiral anion-exchangers operated in various subcritical fluid chromatography modes for resolution of chiral acids, J. Chromatogr. A 1245 (2012) 175–182.
[75] 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.
[76] Y. Liu, A.W. Lantz, D.W. Armstong, High efficiency liquid and super-/subcritical fluid-based enantiomeric separations: an overview, J. Liq. Chromatogr. Relat. Technol. 27 (2004) 1121–1178.
[77] M. Johannsen, S. Peper, A. Depta, Simulated moving bed chromatography with supercritical fluids for the resolution on bi-naphthol enantiomers and phytol isomers, J. Biochem. Biophys. Methods 54 (2002) 85–102.
[78] Y. Liu, A. Berthod, C.R. Mitchell, T.L. Xiao, B. Zhang, D.W. Armstrong, Super/subcritical fluid chromatography chiral separations with macrocyclic glycopeptide stationary phases, J. Chromatogr. A 978 (2002) 185–204.
[79] Y. Liu, R.V. Rozhkov, R.C. Larock, T.L. Xiao, D.W. Armstrong, Fast super/subcritical fluid chromatography enantiomeric separations of dihydrofurocoumarin derivatives with macrocyclic glycopeptide stationary phases, Chromatographia 58 (2003) 775–779.
[80] G. Lavison, D. Thiébaut, Evaluation of a ristocetin bonded stationary phase for subcritical fluid chromatography of enantiomers, Chirality 15 (2003) 630–636.
[81] K.W. Phinney, L.C. Sander, Additive concentration effects on enantioselective separations in supercritical fluid chromatography, Chirality 15 (2003) 287–294.
[82] C.J. Welch, M. Biba, J.R. Gouker, G. Kath, P. Augustine, P. Hosek, Solving multicomponent chiral separation challenges using a new SFC tandem column screening tool, Chirality 19 (2007) 184–189.
[83] W.B. Holzheuer, M.M. Wong, G.K. Webster, Evaluation of Pirkle stationary phases in chiral method development screening for compounds of pharmaceutical interest, Curr. Pharm. Anal. 5 (2009) 10–20.
[84] S.H. Hoke, J.D. Pinkston, R.E. Bailey, S.L. Tanguay, T.H. Eichhold, Comparison of packed column supercritical fluid chromatography-tandem mass spectrometry with liquid chromatography-tandem mass spectrometry for bioanalytical determination of (R)- and (S)-ketoprofen in human plasma following automated 96 well solid-phase extraction, Anal. Chem. 72 (2000) 4235–4241.
[85] P. Oswald, K. Desmet, P. Sandra, J. Krupcík, P. Májek, D.W. Armstrong, Determination of the enantiomerization energy barrier of some 3-hydroxy-1,4-benzodiazepine drugs by supercritical fluid chromatography, J. Chromatogr. B 779 (2002) 283–295.
[86] P. Oswald, K. Desmet, P. Sandra, J. Krupcík, D.W. Armstrong, Evaluation of reversible and irreversible models for the determination of the enantiomerization energy barrier for N-(p-methoxybenzyl)-1,3,2-benzodithiazol-1-oxide by supercritical fluid chromatography, Chirality 14 (2002) 334–339.
[87] Y. Zhang, J. Dai, D.B. Wang-Iverson, A.A. Tymiak, Simulated moving columns technique for enantioselective supercritical fluid chromatography, Chirality 19 (2007) 683–692.
[88] N. Byrne, E. Hayes-Larson, W.W. Liao, C.M. Kraml, Analysis and purification of alcohol-sensitive chiral compounds using 2,2,2-trifluoroethanol as a modifier in supercritical fluid chromatography, J. Chromatogr. B 875 (2008) 237–242.
[89] T. Szczerba, P. Wrezel, Effect of varying co-solvents in SFC method development on a Whelk-O1® chiral stationary phase, LC GC N. Am. (2008) 21.
[90] M. Lämmerhofer, Chiral recognition by enantioselective liquid chromatography: mechanisms and modern chiral stationary phases, J. Chromatogr. A 1217 (2010) 814–856.
[91] F. Antelo, C.C. Santana, T.L.M. Alves, A.G. Barreto, Thermodynamic study of enantioseparation of mitotane by supercritical fluid chromatography, Sep. Sci. Technol. 47 (2012) 636–640.
[92] S.K. Han, K.H. Row, Chiral separation of ibuprofen by supercritical fluid chromatography, Chin. J. Chem. Eng. 13 (2005) 741–746.
[93] Y.W. Yang, B.G. Su, Q.H. Yan, Q.L. Ren, Separation of naproxen enantiomers by supercritical/subcritical fluid chromatography, J. Pharm. Biomed. Anal. 39 (2005) 815–818.
[94] S. Peper, M. Lübbert, M. Johannsen, G. Brunner, Separation of ibuprofen enantiomers by supercritical fluid simulated moving bed chromatography, Sep. Sci. Technol. 37 (2002) 2545–2566.
[95] S.K. Han, Y.Z. Jin, K.H. Row, Correlation equation for retention factor and resolution of ibuprofen in SFC, Bull. Korean Chem. Soc. 25 (2004) 1807–1811.
[96] X. Han, J.W. Remsburg, L.F. He, T.E. Beesly, D.W. Armstrong, Effect of the orientation of amide linkage groups on the enantioselectivity of two related synthetic polymeric chiral stationary phases, Chromatographia 67 (2008) 199–210.
[97] W.W. Barnhart, K.H. Gahm, Z. Hua, W. Goetzinger, Supercritical fluid chromatography comparison of the poly (trans-1,2-cyclohexanediyl-bis acrylamide) (P-CAP) column with several derivatized polysaccharide-based stationary phases, J. Chromatogr. B 875 (2008) 217–229.
[98] X. Han, A. Berthod, C. Wang, K. Huang, D.W. Armstrong, Super/subcritical fluid chromatography separations with four synthetic polymeric chiral stationary phases, Chromatographia 65 (2007) 381–400.
[99] T. Payagala, E. Wanigasekara, D.W. Armstrong, Synthesis and chromatographic evaluation of new polymeric chiral stationary phases based on three (1S,2S)-(−)-1,2-diphenylethylenediamine derivatives in HPLC and SFC, Anal. Bioanal. Chem. 399 (2011) 2445–2461.
[100] T.E. Beesley, Updates in technology and application of chiral stationary phases, LC GC N. Am. 24 (2006) 28–31.
[101] R.J. Ansell, Molecularly imprinted polymers for the enantioseparation of chiral drugs, Adv. Drug Deliv. Rev. 57 (2005) 1809–1835.
[102] J. Lin, S. Liu, J. Lin, X. Lin, Z. Xie, Novel highly hydrophilic methacrylate-based monolithic column with mixed-mode of hydrophilic and strong cation-exchange interactions for pressurized capillary electrochromatography, J. Chromatogr. A 1218 (2011) 4671–4677.
[103] R.J. Ansell, J.K.L. Kuah, D. Wang, C.E. Jackson, K.D. Bartle, A.A. Clifford, Imprinted polymers for chiral resolution of (±)-ephedrine, 4: packed column supercritical fluid chromatography using molecularly imprinted chiral stationary phases, J. Chromatogr. A 1264 (2012) 117–123.
[104] A. Ellwanger, P.K. Owens, L. Karlsson, S. Bayoudh, P. Cormack, D. Sherrington, B. Sellergren, Application of molecularly imprinted polymers in supercritical fluid chromatography, J. Chromatogr. A 897 (2000) 317–327.
[105] C.L. Wang, D.W. Armstrong, D.S. Risley, Empirical observations and mechanistic insights on the first boron-containing chiral selector for LC and supercritical fluid chromatography, Anal. Chem. 79 (2007) 8125–8135.
[106] V. Schurig, M. Fluck, Enantiomer separation by complexation SFC on immobilized Chirasil-nickel and Chirasil-zinc, J. Biochem. Biophys. Methods 43 (2000) 223–240.
[107] L.A. Svensson, P.K. Owens, Enantioselective supercritical fluid chromatography using Ristocetin A chiral stationary phases, Analyst 125 (2000) 1037–1039.
[108] N.J. Wu, Z. Chen, J.C. Medina, J.S. Bradshaw, M.L. Lee, Fast chiral separations using packed capillary columns and near-critical fluid carbon dioxide mobile phase, J. Microcolumn Sep. 12 (2000) 454–461.
[109] Y. Shen, N.J. Wu, Q.L. Tang, Z. Chen, J.S. Bradshaw, M.L. Lee, Effect of pore size on speed of chiral separations using packed capillary column SFC, J. Microcolumn Sep. 12 (2000) 475–481.
[110] K. De Klerck, D. Mangelings, Y. Vander Heyden, Advances in supercritical fluid chromatography for the analysis of chiral and achiral pharmaceuticals, LC GC Eur. (2013) 24–30.
[111] O. Gyllenhaal, Packed column supercritical fluid chromatography of a peroxysome proliferator-activating receptor agonist drug – achiral and chiral purity of substance, formulation assay and its enantiomeric purity, J. Chromatogr. A 1042 (2004) 173–180.
[112] W.W. Barnhart, K.H. Gahm, S. Thomas, S. Notari, D. Semin, J. Cheetham, Supercritical fluid chromatography tandem-column method development in pharmaceutical sciences for a mixture of four stereoisomers, J. Sep. Sci. 28 (2005) 619–626.
[113] A.J. Alexander, A. Staab, Use of achiral/chiral SFC/MS for the profiling of isomeric cinnamonitrile/hydrocinnamonitrile products in chiral drug synthesis, Anal. Chem. 78 (2006) 3835–3838.
[114] L. Zeng, R.D. Xu, Y.N. Zhang, D.B. Kassel, Two-dimensional supercritical fluid chromatography/mass spectrometry for the enantiomeric analysis and purification of pharmaceutical samples, J. Chromatogr. A 1218 (2011) 3080–3088.
[115] L. Miller, Preparative enantioseparations using supercritical fluid chromatography, J. Chromatogr. A 1250 (2012) 250–255.
[116] T.E. Beesley, Review of chiral stationary phase development and chiral applications, LC GC Eur. 24 (2011) 270–276.
[117] C.J. Welch, W.R. Leonard, J.O. DaSilva, M. Biba, J. Albaneze-Walker, D.W. Henderson, B. Laing, D.J. Mathre, Preparative chiral SFC as a green technology for rapid access to enantiopurity in pharmaceutical process research, LC GC Eur. 18 (2005) 264–272.
[118] R.Q. Wang, T.T. Ong, S.C. Ng, W.H. Tang, Recent advances in pharmaceutical separations with supercritical fluid chromatography using chiral stationary phases, Trends Anal. Chem. 37 (2012) 83–100.
[119] F. Geiser, R. Shah, Enantioseparation of hydrochloride salts using carbon dioxide-based mobile phases with on-line polarimetric detection, Chirality 16 (2004) 263–266.
[120] L. Miller, I. Sebastian, Evaluation of injection conditions for preparative supercritical fluid chromatography, J. Chromatogr. A 1250 (2012) 256–263.
[121] J.C. Xie, H.L. Han, L. Sun, H.Y. Zeng, B.G. Sun, Resolution of racemic 3-hydroxy-4,5-dimethyl-2(5H)-furanone (sotolon) by packed column supercritical fluid chromatography, Flavour Fragr. J. 27 (2012) 244–249.
[122] R. Gaggeri, D. Rossi, S. Collina, B. Mannucci, M. Baierl, M. Juza, Quick development of an analytical enantioselective high performance liquid

chromatography separation and preparative scale-up for the flavonoid Naringenin, J. Chromatogr. A 1218 (2011) 5414–5422.
[123] L. Miller, M. Potter, Preparative chromatographic resolution of racemates using HPLC and SFC in pharmaceutical discovery environment, J. Chromatogr. B 875 (2008) 230–236.
[124] F. Denet, W. Hauck, R.M. Nicoud, O. Di Giovanni, M. Mazzotti, J.N. Jaubert, M. Morbidelli, Enantioseparation through supercritical fluid simulated moving bed (SF-SMB) chromatography, Ind. Eng. Chem. Res. 40 (2001) 4603–4609.
[125] M. Mazzotti, G. Storti, M. Morbidelli, Optimal operation of simulated moving bed units for nonlinear chromatographic separations, J. Chromatogr. A 769 (1997) 3–24.
[126] M. Mazotti, G. Storti, M. Morbidelli, Supercritical fluid simulated moving bed chromatography, J. Chromatogr. A 786 (1997) 309–320.
[127] Y. Zhang, D.R. Wu, D.B. Wang-Iverson, A.A. Tymiak, Enantioselective chromatography in drug discovery, Drug Discovery Today 10 (2005) 571–577.
[128] T.Q. Yan, C. Orihuela, D. Swanson, The application of preparative batch HPLC, supercritical fluid chromatography, steady-state recycling and simulated moving bed for the resolution of a racemic pharmaceutical intermediate, Chirality 20 (2008) 139–146.
[129] A. Rajendran, S. Peper, M. Johannsen, M. Mazzotti, M. Morbidelli, G. Brunner, Enantioseparation of 1-phenyl-1-propanol by supercritical fluid-simulated moving bed chromatography, J. Chromatogr. A 1092 (2005) 55–64.
[130] D. Mangelings, Y. Vander Heyden, Chiral separations in sub- and supercritical fluid chromatography, J. Sep. Sci. 31 (2008) 1252–1273.
[131] 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.
[132] 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.
[133] L. Miller, Evaluation of non-traditional modifiers for analytical and preparative enantioseparation using supercritical fluid chromatography, J. Chromatogr. A 1256 (2012) 261–266.
[134] K. De Klerck, D. Mangelings, D. Clicq, F. De Boever, Y. Vander Heyden, Combined use of isopropylamine and trifluoroacetic acid in methanol-containing mobile phases for chiral supercritical fluid chromatography, J. Chromatogr. A 1234 (2012) 72–79.
[135] 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.
[136] C. Hamman, D.E. Schmidt, M.L. 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.
[137] O. Gyllenhaal, M. Stefansson, Reversal of elution order for profen acid enantiomers in packed-column SFC on Chiralpak AD, Chirality 17 (2005) 257–265.
[138] O. Gyllenhaal, A. Karlsson, Enantioresolution of dihydropyridine substituted acid by supercritical fluid chromatography on Hypercarb® with Z-(L)-arginine as chiral counter ion, Chromatographia 52 (2000) 351–355.
[139] A. Salvador, B. Herbreteau, M. Dreux, Preliminary studies of supercritical-fluid chromatography on porous graphitic carbon with methylated cyclodextrin as chiral selector, Chromatographia 53 (2001) 207–209.
[140] A. Salvador, B. Herbreteau, M. Dreux, A. Karlsson, O. Gyllenhaal, Chiral supercritical fluid chromatography on porous graphitic carbon using commercial dimethyl β-cyclodextrins as mobile phase additive, J. Chromatogr. A 929 (2001) 101–112.
[141] O. Gyllenhaal, A. Karlsson, Enantiomeric separations of amino alcohols by packed-column SFC on Hypercarb with L-(+)-tartaric acid as chiral selector, J. Biochem. Biophys. Methods 54 (2002) 169–185.
[142] P.J. Schoenmakers, P.E. Rothfusz, F.C.C.J.G. Verhoeven, Calculation of pressure, density and temperature profiles in packed-column supercritical fluid chromatography, J. Chromatogr. A 395 (1987) 91–110.
[143] R.W. Stringham, J.A. Blackwell, Factors that control successful entropically driven chiral separations in SFC and HPLC, Anal. Chem. 69 (1997) 1414–1420.
[144] J.L. Bernal, L. Toribio, M.J. del Nozal, E.M. Nieto, M.I. Montequi, Separation of antifungal chiral drugs by SFC and HPLC: a comparative study, J. Biochem. Biophys. Methods 54 (2002) 245–254.
[145] K. Desmet, P. Sandra, K. Vízvárdi, G.J. Hoornaert, E. Van der Eycken, Enantioselective packed column SFC for the separation of 2-oxatetracyclo[5.4.0.0^{1,8}.0^{5,11}] undec-9-ene derivatives, J. Microcolumn Sep. 13 (2001) 163–168.
[146] M.J. del Nozal, L. Toribio, J.L. Bernal, E.M. Nieto, J.J. Jiménez, Separation of albendazole sulfoxide enantiomers by chiral supercritical-fluid chromatography, J. Biochem. Biophys. Methods 54 (2002) 339–345.
[147] L. Toribio, M.J. del Nozal, J.L. Bernal, C. Cristofol, C. Alonso, Study of the enantiomeric separation of oxfendazole and cetirizine using subcritical fluid chromatography on an amylose-based column, J. Chromatogr. A 1121 (2006) 268–273.
[148] R. Geryk, J. Vozka, K. Kalíková, E. Tesařová, HPLC method for chiral separation and quantification of antidepressant citalopram and its precursor citadiol, Chromatographia 76 (2013) 483–489.
[149] B. Tienpont, F. David, A. Pereira, P. Sandra, In-vial pyrolysis (PyroVial) with pre- and post-sample treatment combined with different chromatographic techniques, J. Chromatogr. A 1218 (2011) 8303–8312.
[150] L. Toribio, M.J. del Nozal, J.L. Bernal, C. Alonso, J.J. Jiménez, Enantiomeric separation of several antimycotic azole drugs using supercritical fluid chromatography, J. Chromatogr. A 1144 (2007) 255–261.
[151] J.L. Bernal, M.J. del Nozal, L. Toribio, M.I. Montequi, E.M. Nieto, Separation of ketoconazole enantiomers by chiral subcritical-fluid chromatography, J. Biochem. Biophys. Methods 43 (2000) 241–250.
[152] L. Toribio, J.L. Bernal, M.J. del Nozal, J.J. Jiménez, E.M. Nieto, Applications of the Chiralpak AD and Chiralcel OD chiral columns in the enantiomeric separation of several dioxolane compounds by supercritical fluid chromatography, J. Chromatogr. A 921 (2001) 305–313.
[153] J.L. Bernal, L. Toribio, M.J. del Nozal, E.M. Nieto, J.J. Jiménez, Chiral separation of four 1,3-dioxolane derivatives by supercritical fluid chromatography on an amylose-based column, J. Chromatogr. A 871 (2000) 127–137.
[154] M.J. del Nozal, L. Toribio, J.L. Bernal, N. Castaño, Separation of triadimefon and triadimenol enantiomers and diastereoisomers by supercritical fluid chromatography, J. Chromatogr. A 986 (2003) 135–141.
[155] M.J. del Nozal, L. Toribio, J.L. Bernal, C. Alonso, J.J. Jiménez, Chiral separation of omeprazole and several related benzimidazoles using supercritical fluid chromatography, J. Sep. Sci. 27 (2004) 1023–1029.
[156] S. Andersson, H. Nelander, K. Öhlén, Preparative chiral chromatography and chiroptical characterization of enantiomers of omeprazole and related benzimidazoles, Chirality 19 (2007) 706–715.
[157] L. Toribio, M.J. del Nozal, J.L. Bernal, C. Alonso, J.J. Jiménez, Comparative study of the enantioselective separation of several antiulcer drugs by high-performance liquid chromatography and supercritical fluid chromatography, J. Chromatogr. A 1091 (2005) 118–123.
[158] M. Johannsen, Separation of enantiomers of ibuprofen on chiral stationary phases by packed column supercritical fluid chromatography, J. Chromatogr. A 937 (2001) 135–138.
[159] K.W. Phinney, L.C. Sander, Preliminary evaluation of a standard reference material for chiral stationary phases used in liquid and supercritical fluid chromatography, Anal. Bioanal. Chem. 372 (2002) 101–108.
[160] N. Matthijs, M. Maftouh, Y.V. Heyden, Chiral separation strategy in polar organic solvent chromatography and performance comparison with normal-phase liquid and supercritical-fluid chromatography, J. Sep. Sci. 29 (2006) 1353–1362.
[161] R.A. Coe, J.O. Rathe, J.W. Lee, Supercritical fluid chromatography-tandem mass spectrometry for fast bioanalysis of R/S-warfarin in human plasma, J. Pharm. Biomed. Anal. 42 (2006) 573–580.
[162] L. Toribio, M.J. del Nozal, J.L. Bernal, J.J. Jiménez, C. Alonso, Chiral separation of some triazole pesticides by supercritical fluid chromatography, J. Chromatogr. A 1046 (2004) 249–253.
[163] M. Garzotti, M. Hamdan, Supercritical fluid chromatography coupled to electrospray mass spectrometry: a powerful tool for the analysis of chiral mixtures, J. Chromatogr. B 770 (2002) 53–61.
[164] L.M. Nogle, C.W. Mann, W.L. Watts Jr., Y. Zhang, Preparative separation and identification of derivatized β-methylphenylalanine enantiomers by chiral SFC, HPLC and NMR for development of new peptide ligand mimetics in drug discovery, J. Pharm. Biomed. Anal. 40 (2006) 901–909.
[165] G.V.N. Rao, G. Gnanadev, B. Ravi, D. Dhananjaya, P. Manoj, B. Indu, R.V. Nadh, Supercritical fluid (carbon dioxide) based ultra performance convergence chromatography for the separation and determination of fulvestrant diastereomers, Anal. Methods 5 (2013) 4832–4837.
[166] I. Brondz, D. Ekeberg, D.S. Bell, A.R. Annino, J.A. Hustad, R. Svendsen, V. Vlachos, P. Oakley, G.J. Langley, T. Mohini, C.G. Amaury, F. Mikhalitsyn, Nature of the main contaminant in the drug primaquine diphosphate: SFC and SFC–MS methods of analysis, J. Pharm. Biomed. Anal. 43 (2007) 937–944.
[167] J. Qian-Cutrone, B. Dasgupta, E.S. Kozlowski, R. Dalterio, D. Wang-Iverson, V.M. Vrudhula, Separation of maxi-K channel opening 3-substitued-4-arylquinolinone atropisomers by enantioselective supercritical fluid chromatography, J. Pharm. Biomed. Anal. 48 (2008) 1120–1126.
[168] J. Xie, J. Cheng, H. Han, B. Sun, G.W. Yanik, Resolution of racemic γ-lactone flavors on Chiralpak AD by packed column supercritical fluid chromatography, Food Chem. 124 (2011) 1107–1112.
[169] J. Qian-Cutrone, R. Hartz, V.T. Ahuja, V.M. Vrudhula, D.R. Wu, R.A. Dalterio, D. Wang-Iverson, J.J. Bronson, Chiral separation of potent corticotropin-releasing factor-1 receptor antagonists by supercritical fluid chromatography, J. Pharm. Biomed. Anal. 54 (2011) 602–606.
[170] C.M. Kraml, D. Zhou, N. Byrne, O. McConnell, Enhanced chromatographic resolution of amine enantiomers as carbobenzyloxy derivatives in high-performance liquid chromatography and supercritical fluid chromatography, J. Chromatogr. A 1100 (2005) 108–115.
[171] Z. Pirzada, M. Personick, M. Biba, X. Gong, L. Zhou, W. Schafer, C. Roussel, C.J. Welch, Systematic evaluation of new chiral stationary phases for supercritical fluid chromatography using a standard racemate library, J. Chromatogr. A 1217 (2010) 1134–1138.
[172] K. De Klerck, C. Tistaert, D. Mangelings, Y.V. Heyden, Updating a generic screening approach in sub- or supercritical fluid chromatography for the enantioresolution of pharmaceuticals, J. Supercrit. Fluids 80 (2013) 50–59.
[173] C. Hamman, M. Wong, I. Aliagas, D.F. Ortwine, J. Pease, D.E. Schmidt Jr., J. Victorino, The evaluation of 25 chiral stationary phases and the utilization of sub-2.0 μm coated polysaccharide chiral stationary phases via supercritical fluid chromatography, J. Chromatogr. A 1305 (2013) 310–319.
[174] T. Szczerba, T. Berger, Going faster, going greener with high efficiency Regis chiral stationary phases in supercritical fluid chiral chromatography (SFC), LC GC N. Am. (2011) 18.

[175] T. Szczerba, Z. Ali, R. Gaggeri, D. Rossi, S. Collina, B. Mannucci, S. Abel, D. Frühauf, M. Juza, An analytical and scale-up separation by HPLC and SFC of the flavonoid naringenin on a RegisPack® chiral stationary phase, LC GC N. Am. (2010) 84.
[176] T.A. Berger, B.K. Berger, Chromatographic resolution of 7 of 8 stereoisomers of vitamin K_1 on an amylose stationary phase using supercritical fluid chromatography, Chromatographia 76 (2013) 549–552.
[177] T. Szczerba, Improving SFC chiral separations by employing a new chlorinated polysaccharide chiral stationary phase: the RegisPack® CLA-1, LC GC N. Am. (2011) 19.
[178] S. Khater, C. West, Evaluation of the complementarity of three Regis chiral stationary phases in supercritical fluid chromatography, LC GC N. Am. (2012) 70.
[179] Y. Xiang, G.W. Sluggett, Development and validation of a GC method for quantitative determination of enantiomeric purity of a proline derivative, J. Pharm. Biomed. Anal. 53 (2010) 878–883.
[180] S. Khater, Y. Zhang, C. West, In-depth characterization of six cellulose tris-(3,5-dimethylphenylcarbamate) chiral stationary phases in supercritical fluid chromatography, J. Chromatogr. A 1303 (2013) 83–93.
[181] L. Di, O.J. McConnell, E.H. Kerns, A.G. Sutherland, Rapid, automated screening method for enzymatic transformations using a robotic system and supercritical fluid chromatography, J. Chromatogr. B 809 (2004) 231–235.
[182] H.F. Kasai, M. Tsubuki, K. Takahashi, M. Shirao, Y. Matsumoto, T. Honda, Y. Seyama, Separation of stereoisomers of several furan derivatives by capillary gas chromatography-mass spectrometry, supercritical fluid chromatography, and liquid chromatography using chiral stationary phases, J. Chromatogr. A 977 (2002) 125–134.
[183] K. Yaku, F. Morishita, Separation of drugs by packed-column supercritical fluid chromatography, J. Biochem. Biophys. Methods 43 (2000) 59–76.
[184] Z. Wang, M. Jonca, T. Lambros, S. Ferguson, R. Goodnow, Exploration of liquid and supercritical fluid chromatographic chiral separation and purification of Nutlin-3-A small molecule antagonist of MDM2, J. Pharm. Biomed. Anal. 45 (2007) 720–729.
[185] H. Nelander, S. Andersson, K. Öhlén, Evaluation of the chiral recognition properties as well as the column performance of four chiral stationary phases based on cellulose (3,5-dimethylphenylcarbamate) by parallel HPLC and SFC, J. Chromatogr. A 1218 (2011) 9397–9405.
[186] Y.S. Hsieh, L. Favreau, K.C. Cheng, J.W. Chen, Chiral supercritical fluid chromatography/tandem mass spectrometry for the simultaneous determination of pindolol and propranolol in metabolic stability samples, Rapid Commun. Mass Spectrom. 19 (2005) 3037–3041.
[187] J.W. Chen, Y.S. Hsieh, J. Cook, R. Morrison, W.A. Korfmacher, Supercritical fluid chromatography-tandem mass spectrometry for the enantioselective determination of propranolol and pindolol in mouse blood by serial sampling, Anal. Chem. 78 (2006) 1212–1217.
[188] C. Garcia-Martinez, G. Hernandez, M. Biba, C.J. Welch, Determination of enantiomeric composition of (−)-(R)-2-tert-butyltetrahydroimidazolidin-4-one by polarimetry, ^{1}H NMR, and chiral SFC, Chirality 17 (2005) 212–217.
[189] Y. Shen, N. Wu, Q. Tang, Z. Chen, J.S. Bradshaw, M.L. Lee, Effect of pore size on speed of chiral separations using packed capillary column SFC, J. Microcolumn Sep. 12 (2000) 475–481.
[190] R. Pell, G. Schuster, M. Lämmerhofer, W. Lindner, Enantioseparation of chiral sulfonates by liquid chromatography and subcritical fluid chromatography, J. Sep. Sci. 35 (2012) 2521–2528.
[191] L.A. Svensson, J. Dönnecke, K.E. Karlsson, A. Karlsson, J. Vessman, Polar organic phase liquid chromatography with packed capillary columns using a vancomycin chiral stationary phase, Chirality 12 (2000) 606–613.
[192] S. Peper, M. Johannsen, G. Brunner, Preparative chromatography with supercritical fluids: comparison of simulated moving bed and batch processes, J. Chromatogr. A 1176 (2007) 246–253.
[193] G. Brunner, M. Johannsen, New aspects on adsorption from supercritical fluid phases, J. Supercrit. Fluids 38 (2006) 181–200.
[194] L. Toribio, M.J. del Nozal, Y.L. Bernal, C. Alonso, J.J. Jiménez, Semipreparative chiral supercritical fluid chromatography in the fractionation of lansoprazole and two related antiulcer drugs enantiomers, J. Sep. Sci. 31 (2008) 1307–1313.
[195] L. Toribio, M.J. del Nozal, J.L. Bernal, E.M. Nieto, Use of semipreparative supercritical fluid chromatography to obtain small quantities of the albendazole sulfoxide enantiomers, J. Chromatogr. A 1011 (2003) 155–161.
[196] L. Toribio, C. Alonso, M.J. del Nozal, J.L. Bernal, M.T. Martín, Semipreparative enantiomeric separation of omeprazole by supercritical fluid chromatography, J. Chromatogr. A 1137 (2006) 30–35.
[197] G. Cox, R. Stringham, A. Matabe, Resolution of flurbiprofen enantiomers by preparative chiral supercritical fluid chromatography, LC GC N. Am. (2002) 24.
[198] C.J. Welch, F. Fleitz, F. Antia, P. Yehl, R. Waters, N. Ikemoto, J.D. Armstrong, D.J. Mathre, Chromatography as an enabling technology in pharmaceutical process development: expedited multikilogram preparation of a candidate HIV protease inhibitor, Org. Process Res. Dev. 8 (2004) 186–191.
[199] T. Wang, M. Barber, I. Hardt, D.B. Kassel, Mass-directed fractionation and isolation of pharmaceutical compounds by packed-column supercritical fluid chromatography/mass spectrometry, Rapid Commun. Mass Spectrom. 15 (2001) 2067–2075.

5. Závěr

Předkládaná dizertační práce, tvořená komentovaným souborem šesti publikací, se zabývá problematikou kinetiky trypsinového štěpení peptidů a chirálními separacemi biologicky aktivních látek.

Trypsin je v proteomickém výzkumu používán k popisu aminokyselinových sekvencí proteinů, ale jeho využití je zatíženo několika nevýhodami, jako je autolýza a produkce „miscleavage“ sekvencí. Hlubší pochopení trypsinového štěpení problematických sekvenčních motivů a stanovení odpovídajících rychlostních konstant je přínosem pro identifikační techniky proteinů i pro vývoj nových enzymových modifikací. Zavedení rezistentních sekvencí do programů Peptide Mass Fingerprinting by významně urychlilo a zpřesnilo vyhodnocování výsledků a snížilo by množství falešně pozitivních identifikací.

Provedení enzymové hydrolýzy na trypsinové koloně zapojené přímo v chromatografickém systému představuje další zjednodušení a zpřesnění celého identifikačního procesu. Omezí se tak manipulace se vzorkem, která do procesu analýzy vnáší další nepřesnosti a vyšší množství enzymu zvýší rychlost a opakovatelnost reakce. Byla syntetizována trypsinová kolona 2,1 × 30 mm a jako nosič byly zvoleny BEH částice. Tato kolona vykazovala účinnost srovnatelnou, za některých podmínek i vyšší než kolony komerční.

Chirální separace biologicky aktivních látek jsou důležité nejen pro kontrolu čistoty léčiv, ale také při sledování průběhu některých chorob, stárnutí i pro studie zaměřené na výzkum vzniku života na Zemi. Byly vypracovány tři rozsáhlé rešerše věnované chirálním separacím metodou SFC a enantioseparacím aminokyselin a léčiv chromatografickými metodami. Dále byly vyvinuty metody HPLC a CE vhodné pro purifikační i screeningové analýzy enantiomerů tryptofanu a jeho neproteinogenních derivátů.

Seznam citované literatury

[1] Subramanian, G. Chiral separation techniques: a practical approach, Wiley-VCH 2007, Weinheim, Německo.

[2] Zahradníčková, H.; Hartvich, P.; Holoubek, I. Historie a význam chirálních analýz aminokyselin v biologických matricích a životním prostředí, Chem. Listy 99 (2005) 703.

[3] Cava, F.; Lam, H.; de Pedro, M.A.; Waldor, M.K. Emerging knowledge of regulatory roles of D-amino acids in bacteria, Cell. Mol. Life Sci. 68 (2011) 817.

[4] Brückner, H.; Becker, D.; Lüpke, M. Chirality of amino acids of microorganisms used in food biotechnology, Chirality 5 (1993) 385.

[5] Min, J.Z.; Hatanaka, S.; Yu, H.; Higashi, T.; Inagaki, S.; Toyo'oka, T. Determination of DL-amino acids, derivatized with *R*(−)-4-(3-isothiocyanatopyrrolidin-1-yl)-7-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole, in nail of diabetic patients by UPLC–ESI-TOF-MS, J. Chromatogr. B 879 (2011) 3220.

[6] Hashimoto, K.; Fukushima, T.; Shimizu, E.; Okada, S.; Komatsu, N.; Okamura, N.; Koike, K.; Koizumi, H.; Kumakiri, C.; Imai, K.; Iyo, M. Possible role of D-serine in the pathophysiology of Alzheimer's disease, Prog. Neuro-Psychopharmacol. Biol. Psych. 28 (2004) 385.

[7] He, Y.; Zhong, W.; Yeung, E. S. Multiplexed on-column protein digestion and capillary electrophoresis for high-throughput comprehensive peptide mapping, J. Chromatogr. B 782 (2002) 331.

[8] Yuan, H.M.; Zhang, L.H.; Hou, C.Y.; Zhu, G.J.; Tao, D.Y.; Liang, Z.; Zhang, Y.K. Integrated platform for proteome analysis with combination of protein and peptide separation via online digestion, Anal. Chem. 81 (2009) 8708.

[9] Ren, D.; Pipes, G.D.; Liu, D.; Shih, L.; Nichols, A.C.; Treuheit, M.J.; Brems, D.N.; Bondarenko, P.V. An improved trypsin digestion method minimizes digestion-induced modifications on proteins, Anal. Biochem. 392 (2009) 12.

[10] Rawlings, N.D.; Waller, M.; Barrett, A.J.; Bateman, A. MEROPS: the database of proteolytic enzymes, their substrates and inhibitors, Nucleic Acids Res. 42 (2014) D503.

[11] Monigatti, F.; Berndt, P. Algorithm for accurate similarity measurements of peptide mass fingerprints and its application, J. Am. Soc. Mass Spectrom. 16 (2005) 13.

[12] Siepen, J.A.; Keevil, E.J.; Knight, D.; Hubbard, S.J. Prediction of missed cleavage sites in tryptic peptides aids protein identification in proteomics, J. Proteome Res. 6 (2007) 399.

[13] Calleri, E.; Temporini, C.; Perani, E.; Stella, S.; Rudaz, C.; Lubda, D.; Mellerio, G.; Veuthey, J.-L.; Caccialanza, G.; Massolini, G. Development of a bioreactor based on trypsin immobilized on monolithic support for the on-line digestion and identification of proteins, J. Chromatogr. A 1045 (2004) 99.

[14] Kranz, B.; Burck, J.; Franzreb, M.J.; Ulrich, A.S. Circular dichroism analysis of penicilin G acylase covalently immobilized on silica nanoparticles, J. Colloid Interface Sci. 316 (2007) 413.

[15] Nováková, L.; Douša, M. a kol. Moderní HPLC separace v teorii a praxi II., Lucie Nováková, Michal Douša 2013, Praha, Česká republika.

[16] Nováková, L.; Douša, M. a kol. Moderní HPLC separace v teorii a praxi I., Lucie Nováková, Michal Douša 2013, Praha, Česká republika.

[17] Daugan, A.; Grondin, P.; Ruault, C.; de Gouville, A.C.L.; Coste, H.; Linget, J.M.; Kirilovsky, J.; Hyafil, F.; Labaudiniere, R. 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.

[18] Royer, C.A. Probing protein folding and conformational transitions with fluorescence, Chem. Rev. 106 (2006) 1769.

[19] Kieffer, M.E.; Repka, L.M.; Reisman, S.E. Enantioselective synthesis of tryptophan derivatives by a tandem Friedel-Crafts conjugate addition/asymmetric protonation reaction, J. Am. Chem. Soc. 134 (2012) 5131.

[20] Ilisz, I.; Tourwe, D.; Armstrong, D.W.; Péter, A. High-performance liquid chromatographic enantioseparation of unusual secondary amino acids on a D-penicillamine-based chiral ligand exchange column, Chirality 18 (2006) 539.

[21] Berkecz, R.; Sztojkov-Ivanov, A.; Ilisz, I.; Forró, E.; Fülöp, F.; Hyun, M.H.; Péter, A. High-performance liquid chromatographic enantioseparation of β-amino acid stereoisomers on a (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid-based chiral stationary phase, J. Chromatog A, 1125 (2006) 138.

[22] Perona, J.J.; Craik, C.S. Evolutionary divergence of substrate specificity within the chymotrypsin-like serine protease fold, J. Biol. Chem. 272 (1997) 29987.

[23] Trypsin, general informations, dostupné 30.6.2016 z http://www.sigmaaldrich.com/life-science/metabolomics/enzyme-explorer/analytical-enzymes/trypsin.html.

[24] Introduction to enzymes, Worthington Biochemical Corporation, 2015, dostupné 30.6.2016 z http://www.worthington-biochem.com/introbiochem/Enzymes.pdf.

[25] Schechter, I.; Berger, A. On the size of the active site in proteases. I. Papain, Biochem. Biophys. Res. Co. 27 (1967) 157.

[26] Keil, B. Specificity of Proteolysis, Springer-Verlag 1992, New York.

[27] Guedidi, S.; Portugal, C.A.M.; Innocent, C.; Janot, J.-M.; Deratani, A.; Crespo, J.G. Fluorescence monitoring of trypsin adsorption in layer-by-layer membrane systems, Enzyme Microb. Technol. 51 (2012) 325.

[28] Hinterwirth, H.; Lindner, W.; Lämmerhofer, M. Bioconjugation of trypsin onto gold nanoparticles: Effect of surface chemistry on bioactivity, Anal. Chim. Acta 733 (2012) 90.

[29] Sun, L.; Li, Y.; Yang, P.; Zhu, G.; Dovichi, N.J. High efficiency and quantitatively reproducible protein digestion by trypsin-immobilized magnetic microspheres, J. Chromatogr. A 1220 (2012) 68.

[30] General product information on Poroszyme Immobilized trypsin Cartridge, dostupné 30.6.2016 z https://www.thermofisher.com/order/catalog/product/2312800.

[31] General product information on Perfinity Integrated Digestion Platform, dostupné 30.6.2016 z http://www.ssi.shimadzu.com/products/product.cfm?product=perfinity-2.

[32] Ahuja, S. Chiral separation methods for pharmaceutical and biotechnological research, John Wiley and Sons, Inc. 2011, Hoboken, New Jersey.

[33] Anzenbacher, P.; Jezdinský, J. Léčiva a chiralita, Klin. Farmakol. Farm. 17 (2003) 148.

[34] Agranat, I.; Caner, H.; Caldwell, J. Putting chirality to work: the strategy of chiral switches. Nat. Rev. Drug Discov. 1 (2002) 753.

[35] Rouhi A.M. Chirality at work. Chem. Engn. News 5 (2003) 56.

[36] Ward, T.J.; Ward, K.D. Recent Progress in Chiral Stationary Phase Development and Current Chiral Application, LC GC Europe 32 (2014) 20.

[37] Craik, C.S.; Largman, C.; Fletcher, T.; Roczniak, S.; Barr, P.J.; Fletterick, R.J.; Rutter, W.J. Redesigning trypsin: Alteration of substrate specificity, Science 228 (1985) 291.

[38] Bayramoglu, G.; Celikbicak, O.; Arica, M.Y.; Salih, B. Trypsin immobilized on magnetic beads via click chemistry: Fast proteolysis of proteins in a microbioreactor for MALDI-ToF-MS peptide analysis, Ind. Eng. Chem. Res. 53 (2014) 4554.

[39] Nidhin, M.; Ghosh, D.; Yadav, H.; Yadav, N.; Majumder, S. Structural and functional aspects of trypsin–gold nanoparticle interactions: An experimental investigation, Mat. Sci. Eng. B 202 (2015) 46.

[40] Ahn, J; Jung, M.C.; Wyndham, K.; Yu, Y.Q.; Engen, J.R. Pepsin immobilized on high-strength hybrid particles for continuous flow online digestion at 10 000 psi, Anal. Chem. 84 (2012) 7256.

[41] Gentilucci, L.; Cerisoli, L.; De Marco, R.; Tolomelli, A. A simple route towards peptide analogues containing substituted (S)- or (R)-tryptophans, Tetrahedron Lett. 51 (2010) 2576.

[42] Xiao, Y.; Ng, S.-C.; Tan, T.T.Y.; Wang, W.Y. Recent development of cyclodextrin chiral stationary phases and their applications in chromatography, J. Chromatogr. A 1269 (2012) 52.

[43] 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, Anal. Chem. 81 (2009) 10215.

[44] Iadarola, P.; Ferrari, F.; Fumagalli, M.; Viglio, S. Determination of amino acids by micellar EKC: Recent advances in method development and novel applications to different matrices, Electrophoresis 29 (2008) 224.

[45] Tao, Y.; Julian, R.R. Identification of amino acid epimerization and isomerization in crystallin proteins by tandem LC-MS, Anal. Chem. 86 (2014) 9733.

[46] Fukushima, T.; Lee, J.A.; Korenaga, T.; Ichihara, H.; Kato, M.; Imai, K. Simultaneous detemination of D-lactic acid and 3-hydroxybutyric acid in rat plasma using a column-switching HPLC with fluorescent derivatization with 4-nitro-7-piperazino-2,1,3-benzoxadiazole (NBD-PZ), Biomed. Chromatogr. 15 (2001) 189.

[47] Deakova, Z.; Durackova, Z.; Armstrong, D.W.; Lehotay, J. Two-dimensional high performance liquid chromatography for determination of homocysteine, methionine and cysteine enantiomers in human serum, J. Chromatogr. A 1408 (2015) 118.

[48] Lorenzo, M.P.; Dudzik, D.; Varas, E.; Gibellini, M.; Skotnicki, M.; Zorawski, M.; Zarzycki, W.; Pellati, F.; Garcia, A. Optimization and validation of a chiral GC–MS method for the determination of free D-amino acids ratio in human urine: Application to a Gestational Diabetes Mellitus study, J. Pharm. Biomed. Anal. 107 (2015) 480.

[49] McCudden, C.R.; Kraus, V.B. Biochemistry of amino acid racemization and clinical application to musculoskeletal disease, Clin. Biochem. 39 (2006) 1112.

[50] Engel, M.H.; Goodfriend, G.A.; Qian, Y.; Macko, S.A. Indigeneity of organic matter in fossils: A test using stable isotope analysis of amino acid enantiomers in Quaternary mollusk shells, Proc. Natl. Acad. Sci. USA 91 (1994) 10475.

[51] Amelung, W.; Zhang, X.; Flach, K.W. Amino acids in grassland soils: Climatic effects on concentrations and chirality, Geoderma 130 (2006) 207.

[52] Csapo, J.; Csapo-Kiss, Z.; Csapo Jr., J. Use of amino acids and their racemization for age determination in archaeometry, Trends Anal. Chem. 17 (1998) 140.

[53] Iuliani, P.; Di Federico, L.; Fonteccio, G.; Carlucci, G. RP-HPLC method with fluorescence detection for amino acids D/L ratio determination in fossil bones as a marker of DNA preservation, J. Sep. Sci. 33 (2010) 2411.

[54] Guiochon, G.; Tarafder, A. Fundamental challenges and opportunities for preparative supercritical fluid chromatography, J. Chromatogr. A 1218 (2011) 1037.

[55] De Klerck, K.; Mangelings, D.; Heyden, Y.V. Supercritical fluid chromatography for the enantioseparation of pharmaceuticals, J. Pharm. Biomed. Anal. 69 (2012) 77.

Přílohy:

A. Seznam publikací

Publikace I: Šlechtová, **T.**; Gilar, M; Kalíková, K.; Tesařová, E. Insight into Trypsin Miscleavage: Comparison of Kinetic Constants of Problematic Peptide Sequences, *Analytical Chemistry* 87 (2015) 7636-7643 (IF 5,886).

Publikace II: Šlechtová, **T.;** Gilar, M; Kalíková, K.; Moore, S.S.; Jorgenson, J.W.; Tesařová, E. Comparison of catalytic activity of three trypsin columns used in liquid chromatography, zasláno do periodika *Analytical Chemistry* (IF 5,886).

Publikace III: 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* 158 (2016) 24-30 (IF 3,091).

Publikace IV: Kalíková, K.; **Šlechtová, T.**; Tesařová, E. Cyclic Oligosaccharide-Based Chiral Stationary Phases Applicable to Drug Purity Control; A Review, akceptováno v periodiku *Current Medicinal Chemistry* (IF 3,455).

Publikace V: Kalíková, K.; **Šlechtová, T.**; Tesařová, E. Enantiomeric ratio of amino acids as a tool for determination of aging and disease diagnostics by chromatographic measurements, zasláno do periodika *Separations*.

Publikace VI: Kalíková, K.; **Šlechtová, T.**; Vozka, J.; Tesařová, E. Supercritical Fluid Chromatography as a Tool for Enantioselective Separation; A Review, *Analytica Chimica Acta* 821 (2014) 1-33 (IF 4,712).

Publikace VII: Šlechtová, **T.**; Kalíková, K.; Tesařová, E. Stanovení enantiomerů theaninu pomocí HPLC, porovnání metod detekce, *Chemické Listy* 107 (2013) 228-232 (IF 0,279).

B. Seznam konferenčních příspěvků

Přednášky:

The comparison of kinetic constants of different sequence motifs digested by trypsin
T. Šlechtová, K. Kalíková, E. Tesařová
9th International Student Conference "Modern Analytical Chemistry" Praha, Česká republika, 2013

The study of peptide digestion by trypsin under RP HPLC conditions
T. Šlechtová, K. Kalíková, M. Gilar, E. Tesařová
13th Symposium and Summer School on Bioanalysis, Debrecen, Maďarsko, 2013

The Influence of Protein Structure to Trypsin Digestion Kinetics
T. Šlechtová, K. Kalíková, M. Gilar, E. Tesařová
15th Symposium and Summer School on Bioanalysis, Targu Mures, Rumunsko, 2015

Comparison of three immobilized trypsin reactors used in HPLC
T. Šlechtová, M. Gilar, K. Kalíková, E. Tesařová
16th Symposium and Summer School on Bioanalysis, Varšava, Polsko, 2016

Plakátová sdělení:

Comparison of methods for detection and determination of enantiomers of theanine by HPLC **T. Šlechtová**, K. Kalíková, E. Tesařová
29th International Symposium on Chromatography, Toruň, Polsko, 2012

The analysis of tryptic digests of petides with different sequence motifs
T. Šlechtová, K. Kalíková, M. Gilar, E. Tesařová
4. ročník konference Česká chromatografická škola - HPLC 2013, Seč, Česká republika, 2013

The kinetic study of petide digestion by trypsin
T. Šlechtová, K. Kalíková, M. Gilar, E. Tesařová
39th International Symposium on HPLC Separations & Related Techniques, Amsterdam, Nizozemsko, 2013

Structure related trypsin cleavage of peptides

E. Tesařová, **T. Šlechtová**, K. Kalíková, M. Gilar, J. Ševčík

63th American Crystallographic Association, Honolulu, Hawaii, 2013

An insight into trypsin miscleavage: Comparison of kinetic constants of problematic sequences

T. Šlechtová, K. Kalíková, M. Gilar, E. Tesařová

21st International Symposium on Electro and Liquid Phase Separation Techniques, Natal, Brazílie, 2014

Tryptic digestion kinetics of miscleavage sequence motifs

T. Šlechtová, M. Gilar, K. Kalíková, E. Tesařová

5. ročník konference Česká chromatografická škola - HPLC 2015, Rožnov pod Radhoštěm, Česká republika, 2015

Determination of kinetic constants of tryptic miscleavage patterns

T. Šlechtová, M. Gilar, K. Kalíková, E. Tesařová

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

Comparison of catalytic activity of three trypsin columns used in HPLC

T. Šlechtová, M. Gilar, K. Kalíková, E. Tesařová

44th International Symposium on HPLC Separations & Related Techniques, San Francisco, Spojené státy Americké, 2016

Comparison of performance of three trypsin columns compatible with HPLC apparatus

T. Šlechtová, M. Gilar, K. Kalíková, E. Tesařová

31st International Symposium on Chromatography, Cork, Irsko, 2016