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Super/subcritical fluid chromatography chiral separations with macrocyclic glycopeptide stationary phases

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Abstract

The chiral recognition capabilities of three macrocyclic glycopeptide chiral selectors, namely teicoplanin (Chirobiotic T), its aglycone (Chirobiotic TAG) and ristocetin (Chirobiotic R), were evaluated with supercritical and subcritical fluid mobile phases. A set of 111 chiral compounds including heterocycles, analgesics (nonsteroidal antiinflamatory compounds), β-blockers, sulfoxides, *N*-protected amino acids and native amino acids was separated on the three chiral stationary phases (CSPs). All separations were done with an outlet pressure regulated at 100 bar, 31 °C and at 4 ml/min. Various amounts of methanol ranging from 7 to 67% (v/v) were added to the carbon dioxide along with small amounts (0.1 to 0.5%, v/v) of triethylamine and/or trifluoroacetic acid. The Chirobiotic TAG CSP was the most effective closely followed by the Chirobiotic T column. Both columns were able to separate, partially or fully, 92% of the enantiomers of the compound set. The ristocetin chiral selector could partially or baseline resolve only 60% of the enantiomers tested. All separations were done in less than 15 min and 70% were done in less than 4 min. The speed of the separations is the main advantage of the use of SFC compared to normal-phase HPLC. In addition, SFC is advantageous for preparative separations with easy solute recovery and solvent disposal.

Keywords: Enantiomer separation; Supercritical fluid chromatography; Subcritical mobile phase; Stationary phases, SFC; Amino acids; Sulfoxides, chiral; Macrocyclic glycopeptide

1. Introduction

The use of supercritical fluids as eluents for chromatographic separations was first proposed by Klesper et al. in 1962 [1]. The advantages of supercritical fluids are numerous: reduced viscosity giving low pressure drop and allowing high flow-rates or long columns, high solute diffusion coefficients giving fast mass transfer and high efficiency, ease of disposal and solute recovery in preparative modes, and the ability to use gas chromatography (GC)-type detectors. Unfortunately in the 1980s, studies greatly overestimated the solvent strength of supercritical CO_2 and led to some disappointment in applicability of supercritical fluid

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chromatography (SFC) [2]. It was rapidly realized that the polarity of supercritical CO_2 was similar to pentane. It was necessary to add significant amounts of polar solvents to the CO_2 eluent to obtain a useful solvent strength. These solvent additions greatly limited the advantages of pure CO_2, and the use of capillary SFC never became a mainstream method.

With the increased environmental concerns, SFC with packed columns recently saw a rebirth as a potential replacement for normal-phase liquid chromatography [3]. The use of packed-column SFC grew relative to capillary SFC, which also had limited sample capacity and lacked preparative capabilities. Instrumentation for packed-column SFC was made more reliable using many of the same components as traditional liquid chromatography (LC) [2]. A LC pump with a chilled head is used to measure CO_2 in the liquid state and a second LC pump dispenses the organic modifier. The composition of the eluent is controlled by varying the flows delivered by each pump as in any high pressure LC gradient system [3]. In the column oven, the density may change, but some software is able to compensate for this. A backpressure regulator is required at the system outlet to control the pressure and prevent expansion of the eluent into a gas in the detector cell. This implies that the detector cell, which is similar to an LC–UV detector, must be capable of withstanding elevated pressure.

Chiral SFC with packed columns was first proposed for the separation of chiral phosphorous-containing derivatives by Mourier et al. in 1985 [4]. The properties of supercritical fluids are especially useful in chiral separations that use almost exclusively subcritical mobile phases containing large amounts of modifier and mild conditions [5–7]. Using SFC for chiral separations, it is expected that the increased diffusivity will lead to sharper peaks and increased resolution. The low viscosity of SFC eluents should allow faster separations and rapid method development. The last point is essential. With rapid column equilibration, simple mobile phase composition and a reduced number of columns to evaluate, SFC is selected as the first try for chiral separations in some industrial cases [8]. Many different chiral stationary phases can be used [9].

The macrocyclic glycopeptide chiral stationary phases (CSPs) have been found to be extremely useful in the chiral separation of native amino acids [10–12], food flavors [13], reagents and catalysts advertized as being enantiomerically pure [14,15], and a wide variety of compounds of various polarities [16–18]. Macrocyclic antibiotic columns were used with SFC mobile phases. A vancomycin based CSP was able to separate the enantiomers of β-adrenergic blocking agents and other pharmaceuticals [19]. Cyclic ketones and dioxalene derivatives were separated by chiral SFC using teicoplanin and vancomycin based columns [20]. A ristocetin CSP was tested with SFC mobile phases to resolve the enantiomers of acidic drugs [21]. Forty-four racemates were evaluated for separation on six different CSPs, including teicoplanin and vancomycin, with SFC mobile phases [22].

An in depth evaluation of the capabilities of macrocyclic glycopeptide-based CSPs used with SFC mobile phases has not been reported to our knowledge. In this work a set of 111 chiral compounds with widely differing functionalities, acids, bases, heterocyclic compounds, β-blockers, chiral sulfoxides, derivatized and native amino acids, was tested with three commercially available macrocyclic glycopeptide based CSPs: teicoplanin (T), ristocetin (R) and the recently introduced teicoplanin aglycone (TAG) [23]. Experimental conditions were deliberately chosen to favor fast (high flow-rates) rather than efficient (high plate number) separations. The results obtained on the three CSPs are compared and discussed in terms of enantiorecognition capabilities.

2. Experimental

2.1. SFC

A Berger Instrument SFC system with a flow control module, an automatic injector (10 μl loop) with a 96-sample tray, a diode array detector and Berger Instruments ChemStation software (Berger Instruments, Newark, DE, USA) was used. The chromatograph had two reciprocating pumps, one with a refrigerated head dispensing the liquid CO_2, the second one controlled the organic modifier. A scale was placed under the CO_2 cylinder as a weight gauge indicating the amount of CO_2 remaining.

2.2. Chiral stationary phases

Three different 25 cm×4.6 mm I.D. columns were obtained from Astec (Whippany, NJ, USA). They were Chirobiotic T, Chirobiotic R and Chirobiotic TAG whose chiral selectors were, respectively, the teicoplanin, $C_{88}H_{97}Cl_2N_9O_{33}$, molecular mass ($M_r$) 1878, and ristocetin, $C_{95}H_{110}N_8O_{44}$, M_r 2066, macrocyclic glycopeptides and the aglycone core of teicoplanin, $C_{58}H_{45}Cl_2N_7O_{18}$, M_r 1197. These CSPs were extensively described in previous articles [11–17].

2.3. The solutes

One hundred and eleven solutes of a wide variety of functionalities were evaluated on the three Chirobiotic CSPs. They were sorted into six classes referred to by letters A to F. Class A contains a variety of heterocyclic compounds that are mainly amides (oxazolidinone or imidazolidinone) and esters (lactone, furanone). Three compounds of this class, hydrobenzoin (A15), *N*,*S*-dimethyl-*S*-phenylsulfoximine (A16) and norgestrel (A18) are not heterocyclic compounds. Norgestrel was not racemic (two chiral centers) but an epimer mixture. Class B is made of chiral acids, especially anti-inflammatory molecules (the ''profen'' family) and other propionic acid derivatives. Class C is the β-blockers compounds. Class D is made of 31 chiral sulfoxides, many of them especially synthesized by the group of Dr. Jenks at Iowa State University. Class E compounds are dinitrophenyl (DNP), dinitropyridyl (DNPyr) or carboxybenzyl (CBZ) *N*-derivatized amino acids. Class F gathers underivatized amino acids.

2.4. Other chemicals

SFC-grade CO_2 (Matheson Gas, Chicago, IL, USA) in 17.7 kg cylinders, supplied with full length eductor tube, was used. Triethylamine (TEA) was purchased from Aldrich (Milwaukee, WI, USA). Methanol, trifluoroacetic acid (TFA) and glycerol were from Fisher Scientific (Fairlawn, NJ, USA). The chiral sulfoxides were synthesized by the Jenks group at Iowa State University (Ames, IA, USA). All the other chiral compounds were obtained from Sigma (St. Louis, MO, USA).

2.5. Protocol

The solutes were dissolved in methanol (concentration between 1 and 5 mg/ml) except native amino acids that were dissolved in water (pH 1 adjusted with HCl). All separations were done under isocratic conditions at 31 °C and regulating the pressure at the detector outlet at 100 kg/cm^2 (100 bar, 10 MPa or 1430 p.s.i.). The organic additive pump was fed by the methanol+TFA and/or TEA mixture. Small amounts of glycerol and/or water were added to elute native amino acids. The columns were equilibrated for at least 30 min any time the organic additive was changed. Three wavelengths, 214, 220 and 254 nm, were continuously monitored. The same columns were used for the six classes of compounds.

The injector tray was loaded with a compound family and, for each compound, two injections were done successively. The tray was reloaded another day and a third injection was done to check for reproducibility and column stability. The solvent UV signal was used as the dead volume marker. Redoing selected experiments after 5 months of intensive use of the columns in a variety of experimental conditions, the columns showed less than 4% change in retention times and between 15 and 20% decrease in efficiency.

3. Results and discussion

3.1. Selecting the experimental conditions

All previous studies using SFC and macrocyclic glycopeptide CSPs have shown that the enantioselectivity factors decreased as the temperature increased [20–22]. So a constant and low temperature, 31 °C (the critical temperature of pure CO_2 is 31.3 °C), was selected for all separations. Similarly, it was found that raising the pressure decreased the enantioresolution factors [20]. A constant outlet pressure of 100 bar (10 MPa or 1430 p.s.i.) was used in all cases. The SFC instrument controls the mobile phase pressure at the column outlet, after the detector cell (see Experimental). This means that the actual

inlet pressure is not the same for all experiments although it is constant during a given isocratic separation. The inlet pressure depends on the column permeability and on the mobile phase composition. Since the mobile phase viscosity increases when the methanol percentage increases, the inlet column pressure is higher, at constant flow-rate, when a high percentage of methanol is added to CO_2.

It can be argued that the mobile phases used are not all supercritical fluids. However, it was demonstrated that the changes in viscosity and solute diffusion coefficients between supercritical and subcritical or liquid mobile phases were continuous [2,24]. The mobile phase compositions used in this work contained between 4 and 60% (v/v) methanol as organic modifier. When 60% methanol is ''added'' to CO_2, it can clearly be considered that it is actually 40% CO_2 that is ''added'' to liquid methanol. At 31 °C and more than 100 bar of pressure, the physico–chemical properties of the methanol–CO_2 mixtures change gradually from a pure supercritical state (no methanol) to a pure liquid (100% methanol) through the subcritical state. No phase separation occurs [3,24]. So, all CO_2–methanol mixtures will be called SFC mobile phases, the S standing for ''supercritical'' in CO_2 rich mobile phases and for ''subcritical'' in methanol-rich mobile phase.

TFA and/or TEA were also added to the SFC mobile phases. Obviously, addition of TFA will protonate the solute and/or stationary phase basic sites and TEA additions will neutralize analyte and/or acidic stationary phase sites. These ionization changes greatly affect the solute retention behavior and enantioselectivity. A 1-ml volume of TFA and TEA corresponds, respectively, to 13.5 and 7.2 mmol. Then, when equal volumes of TFA and TEA are added to a mobile phase, it remains acidic. The amount of TFA and TEA added to the SFC mobile phases depends on the solutes studied.

3.2. Overall CSP effectiveness

Compound D27, methyl hexyl sulfoxide, is the only compound whose enantiomers could not be separated at all ($R_s=0$). The enantiomers of all other 110 compounds could be fully separated ($R_s>1.5$) by at least one of the macrocyclic glycopeptide CSPs. Table 1 lists the results. By chance, the two teicoplanin based chiral selectors were able to resolve fully ($R_s=1.5$ or greater) or partially ($0.4<R_s<1.5$) the enantiomers of exactly the same number of compounds: 63 compounds (57%) were baseline separated by the two Chirobiotic T and TAG columns and 39 (35%) were partially separated. The enantiomers of nine compounds only (8%) could not be resolved. These identical numbers do not correspond to the same compounds. The Chirobiotic TAG column was the only one able to separate the enantiomers of compounds A8, A16, D2 and D16 (Table 1). Similarly, the enantiomers of compounds C1, C2, C7 and F11 were separated only with the Chirobiotic T column.

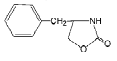
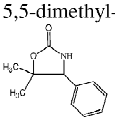
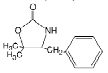
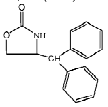
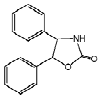
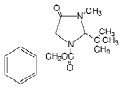
The Chirobiotic R column was significantly less successful. It could separate, with baseline return between peaks, the enantiomers of 25 compounds (22%, Table 1). Forty-two more compounds (38%) were partially resolved and there was no separation for the 44 remaining compounds (40%). However, the enantiomers of D17 and D20 could be separated only by the ristocetin CSP.

Table 1 shows that the resolution factors obtained for the same compound with the three different columns may differ widely. Fig. 1 shows the number of best enantioseparations obtained for each class of compounds and each CSP. Clearly, the Chirobiotic TAG column shows a better effectiveness except in the separation of β-blocker enantiomers (class C) where the Chirobiotic T column is superior. Of the whole set of enantiomers, 55% (61 compounds) are best separated by the Chirobiotic TAG column, 35% (38 compounds) by the Chirobiotic T column and 10% (11 compounds) by the Chirobiotic R column (Fig. 1).

3.3. Class A, heterocyclic compounds

Most of the compounds in this class have a stereogenic center that is part of a heterocyclic ring. This structural feature introduces some rigidity in and around the stereogenic center and renders the two enantiomers easier to differentiate compared to stereogenic centers with four freely rotating substituents [25]. This may be the reason why the three highest resolution factors obtained with Chirobiotic T and TAG columns corresponds to the class A compounds. For Chirobiotic T, the R_s values for

Table 1
Enantiomeric separations on three chirobiotic CSPs by subcritical fluid chromatography

Code	Compound name and formula	CSP[a]	t_1 min	α	Rs	MeOH[b] % v/v	Other additive % v/v in MeOH
	Class A: heterocyclic compounds						
A1	R,S-(+/-)-4-benzyl-2-oxazolidinone	T	3.73	1.39	2.3	40	
		TAG	3.76	1.55	3.9	50	
		R	1.64	1.02	0.4	40	
A2	5,5-dimethyl-4-phenyl-2-oxazolidinone	T	1.87	1.56	4.0	40	
		TAG	2.30	2.94	8.0	40	
		R	1.19	1.08	0.8	40	
A3	R,S-(+/-)-4-benzyl-5,5-dimethyl-2-oxazolidinone	T	2.01	2.96	6.2	40	
		TAG	2.52	1.87	4.1	40	
		R	1.23	1.36	1.6	40	
A4	R,S-(+/-)-4-diphenylmethyl-2-oxazolidinone	T	2.61	1.82	4.1	40	
		TAG	2.83	1.52	3.0	40	
		R	1.49	1.23	1.4	40	
A5	(4R5S/4S5R)-(+/-)-cis-4,5-diphenyl-2-oxazolidinone	T	2.11	1.74	4.3	40	
		TAG	4.09	1.23	1.6	40	
		R	1.38	1.25	1.8	40	
A6	(4R,5S/4S,5R)-(+/-)-4-methyl-5-phenyl-2-oxazolidinone	T	1.96	3.17	12.2	40	
		TAG	3.62	2.53	6.9	40	
		R	1.28	3.26	8.0	40	
A7	(4R,5S/4S,5R)-(+/-)-1,5-dimethyl-4-phenyl-2-imidazolidinone	T	1.27	1.14	1.3	40	
		TAG	1.65	1.42	3.3	40	
		R	6.25	1.04	0.9	7	
A8	1-benzyloxycarbonyl-2-tert-butyl-3-methyl-4-imidazolidinone	T	1.73	1	0.0	7	
		TAG	3.69	1.05	0.8	10	
		R	2.80	1	0.0	5	
A9	4-hydroxy-2-pyrrolidinone	T	2.47	1.05	0.5	40	
		TAG	3.53	1.16	1.7	40	
		R	2.42	1.08	0.9	40	
A10	RS(+/-)-5-(hydroxymethyl)-2-pyrrolidinone	T	2.47	1.20	1.5	40	0.1 TEA TFA
		TAG	3.61	1.22	1.6	60	
		R	2.32	1.05	0.7	40	

compounds A6 and A3 were 12.2 and 6.2, respectively (Table 1). For Chirobiotic TAG, the R_s values were 9.1, 8.0 and 6.9 obtained for compounds A11, A2 and A6, respectively. A R_s value of 8.0 was also obtained on the Chirobiotic R column for the A6 compound. Compounds A15 and A16 are not heterocyclic compounds. They were included with the class A compounds to compare the enantioresolution obtained with two chiral compounds with a free stereogenic center and compounds with ring-blocked

Table 1. Continued

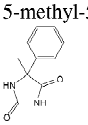
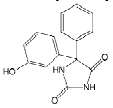
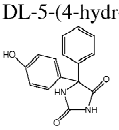
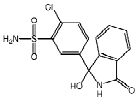
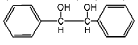
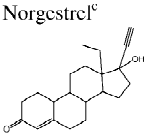
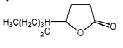
Code	Compound name and formula	CSP[a]	t_1 min	α	Rs	MeOH[b] % v/v	Other additive % v/v in MeOH
A11	5-methyl-5-phenylhydantoin	T	2.32	1.05	0.7	40	
		TAG	3.20	3.87	9.1	40	
		R	2.00	1.39	2.2	40	
A12	5-(3-hydroxyphenyl)-5-phenyl-hydantoin	T	6.97	1.16	1.4	40	0.1 TEA TFA
		TAG	4.58	1.38	2.0	60	0.1 TEA TFA
		R	4.14	1.18	0.9	40	
A13	DL-5-(4-hydroxyphenyl)-5-phenylhydantoin	T	8.27	1.16	1.3	40	
		TAG	6.14	1.08	0.6	60	
		R	4.35	1.12	0.7	40	
A14	Chlorthalidon	T	3.61	1.37	2.9	40	0.1 TEA TFA
		TAG	4.72	1.82	3.6	45	
		R	8.65	1.08	1.0	30	
A15	(R,R/S,S)-(+/-)-Hydrobenzoin	T	6.70	1.04	0.6	7	
		TAG	6.59	1.04	0.7	10	
		R	4.75	1	0.0	10	
A16	(R,S)-(+/-)-*N*,*S*-dimethyl-*S*-phenylsulfoximine	T	4.41	1	0.0	5	
		TAG	4.80	1.06	0.6	7	
		R	3.98	1	0.0	5	
A17	α-methyl-α-phenyl-succinimide	T	1.60	1.26	3.0	2	
		TAG	2.83	1.47	4.5	15	
		R	4.58	1.03	0.6	7	
A18	Norgestrel[c]	T	11.92	1.05	1.0	7	
		TAG	8.08	1.08	0.9	15	
		R	10.49	1.02	0.6	7	
A19	(1R,5S/1S,5R)-(+/-)-2-Oxabicyclo[3,3,0]-oct-6-en-3-one	T	1.11	1.51	2.9	40	
		TAG	1.63	1.53	2.7	30	
		R	0.90	1.20	1.0	40	
A20	(R,S)-(+/-)-Dihydro-5-(hydroxymethyl)-2(3H)-furanone	T	1.31	1.11	0.8	40	
		TAG	1.63	1.12	0.7	20	
		R	1.21	1	0.0	40	
A21	(R,S)-(+/-)-5-hydroxymethyl-)-2-(5H)-furanone	T	5.25	1.13	1.3	40	
		TAG	4.93	1.14	1.2	15	
		R	4.72	1	0.0	10	
A22	γ-nonanoic lactone	T	1.53	1.14	1.1	7	
		TAG	1.35	1.13	0.8	15	
		R	2.79	1.11	0.8	5	

Table 1. Continued

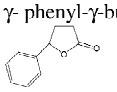
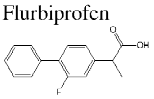
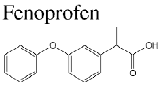
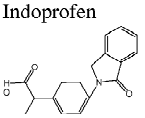
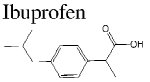
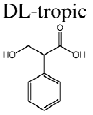
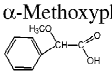
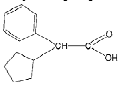
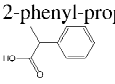
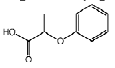
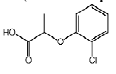
Code	Compound name and formula	CSP[a]	t_1 min	α	Rs	MeOH[b] % v/v	Other additive % v/v in MeOH
A23	γ- phenyl-γ-butyrolactone 	T	2.35	1.29	1.8	7	
		TAG	1.75	1.54	2.4	25	
		R	5.16	1.03	0.6	5	
A24	DL-pantolactone 	T	1.67	1.11	1.5	15	
		TAG	2.13	1.23	2.3	15	
		R	7.85	1	0.0	5	
A25	(+/-) β-butyrolactone 	T	2.65	1.12	0.8	7	
		TAG	3.04	1.18	1.4	5	
		R	2.48	1	0.0	5	
	class B, chiral acids						
B1	Flurbiprofen 	T	4.05	1.07	1.2	5	0.5 TFA
		TAG	6.44	1.06	1.2	5	0.5 TFA
		R	2.93	1	0.0	7	0.5 TFA
B2	Fenoprofen 	T	3.95	1.07	1.2	5	0.5 TFA
		TAG	5.73	1.05	1.1	5	0.5 TFA
		R	2.90	1	0.0	7	0.5 TFA
B3	Indoprofen 	T	6.99	1.11	1.9	15	0.5 TFA
		TAG	8.60	1.14	1.6	20	0.5 TFA
		R	6.63	1	0.0	15	0.5 TFA
B4	Ibuprofen 	T	1.89	1.13	1.5	7	0.5 TFA
		TAG	2.25	1.14	2.2	7	0.5 TFA
		R	2.25	1.14	2.2	7	0.5 TFA
B5	DL-tropic acid 	T	8.45	1.07	1.3	7	0.5 TFA
		TAG	3.95	1.07	1.3	15	0.5 TFA
		R	5.85	1	0.0	7	0.5 TFA
B6	α-Methoxyphenyl acetic acid 	T	3.90	1.04	0.5	7	0.5 TFA
		TAG	8.47	1.09	1.2	5	0.5 TFA
		R	3.47	1.24	1.8	7	0.5 TFA
B7	α-phenylcyclopentaneacetic acid 	T	6.84	1.04	0.9	5	0.2 TFA
		TAG	4.28	1.06	1.1	5	0.5 TFA
		R	3.17	1.04	0.8	5	0.2 TFA
B8	2-phenyl-propionic acid 	T	2.09	1.10	1.5	7	0.5 TFA
		TAG	2.64	1.12	1.5	7	0.5 TFA
		R	2.11	1	0.0	7	0.5 TFA
B9	2-phenoxy-propionic acid 	T	3.34	1.09	0.6	7	0.1 TFA
		TAG	1.50	1.72	2.6	60	0.1 TEA
		R	2.43	1	0.0	7	0.5 TFA
B10	2-(2-chlorophenoxy) propionic acid 	T	2.01	1.51	1.7	40	0.1 TEA
		TAG	2.05	1.61	1.6	40	0.1 TEA
		R	2.74	1	0.0	7	0.5 TFA

Table 1. Continued

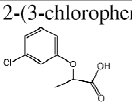
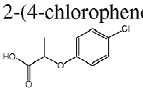
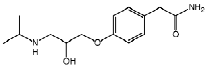
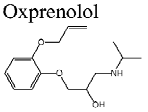
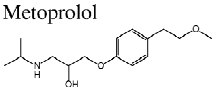
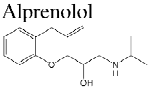
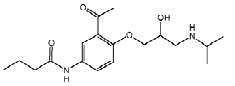
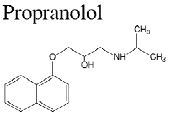
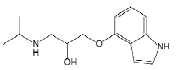
Code	Compound name and formula	CSP[a]	t_1 min	α	Rs	MeOH[b] % v/v	Other additive % v/v in MeOH
B11	2-(3-chlorophenoxy) propionic acid 	T	2.02	1.53	0.9	40	0.1 TEA
		TAG	2.88	1.37	1.7	40	0.1 TEA
		R	2.42	1	0.0	7	0.5 TFA
B12	2-(4-chlorophenoxy) propionic acid 	T	2.07	1.59	1.8	40	0.1 TEA
		TAG	2.45	1.74	2.3	40	0.1 TEA
		R	2.44	1	0.0	7	0.5 TFA
	Class C, β-blockers						
C1	Atenolol 	T	10.63	1.15	1.5	70	0.1 TFA TEA
		TAG	4.93	1	0.0	60	0.1 TFA TEA
		R	10.62	1	0.0	40	0.1 TFA TEA
C2	Oxprenolol 	T	4.75	1.14	1.8	60	0.1 TEA
		TAG	3.75	1	0.0	40	0.1 TFA TEA
		R	6.61	1	0.0	20	0.1 TFA TEA
C3	Metoprolol 	T	2.67	1.14	1.5	40	0.1 TFA TEA
		TAG	3.97	1.07	0.8	60	0.1 TFA TEA
		R	7.99	1	0.0	20	0.1 TFA TEA
C4	Alprenolol 	T	2.06	1.15	1.5	40	0.1 TFA TEA
		TAG	5.19	1.08	1.0	30	0.1 TFA TEA
		R	4.89	1	0.0	20	0.1 TFA TEA
C5	Acebutolol 	T	8.90	1.10	1.1	60	0.1 TEA
		TAG	12.49	1.11	1.0	40	0.1 TFA TEA
		R	4.14	1	0.0	40	0.1 TFA TEA
C6	Propranolol 	T	3.08	1.16	1.8	40	0.1 TFA TEA
		TAG	2.89	1.38	2.0	50	0.1 TFA TEA
		R	9.35	1	0.0	20	0.1 TFA TEA
C7	Pindolol 	T	4.41	1.12	1.4	40	0.1 TFA TEA
		TAG	8.11	1	0.0	40	0.1 TFA TEA
		R	8.71	1	0.0	30	0.1 TFA TEA
	class D, chiral sulfoxides						
D1	 methyl phenyl sulfoxide	T	5.14	1.03	0.4	7	
		TAG	8.52	1.14	0.9	7	
		R	4.17	1.06	0.9	7	
D2	 methyl-4-toluyl sulfoxide	T	5.37	1	0.0	7	
		TAG	9.54	1.12	1.2	7	
		R	3.97	1	0.0	7	
D3	 methyl 3-toluyl sulfoxide	T	4.37	1.22	3.1	7	
		TAG	3.18	1.29	3.2	15	
		R	3.56	1.03	0.5	7	
D4		T	4.90	1	0.0	7	
		TAG	3.77	1.10	1.1	15	

Table 1. Continued

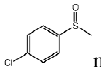
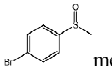
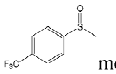
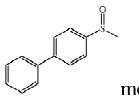
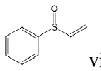
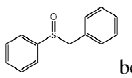
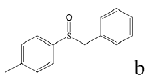
Code	Compound name and formula	CSP[a]	t_1 min	α	Rs	MeOH[b] % v/v	Other additive % v/v in MeOH
	 methyl-2-toluyl sulfoxide	R	3.88	1.06	0.9	15	
D5	 methyl-4-fluorophenyl sulfoxide	T	4.3	1.15	2.4	7	
		TAG	7.28	1.32	3.6	7	
		R	3.4	1	0.0	7	
D6	 methyl-4-chlorophenyl sulfoxide	T	4.81	1.19	1.9	7	
		TAG	8.86	1.28	2.3	7	
		R	4.0	1	0.0	7	
D7	 methyl-3-chlorophenyl sulfoxide	T	4.31	1.18	2.8	7	
		TAG	3.43	1.31	3.4	15	
		R	3.93	1	0.0	7	
D8	 methyl-2-chlorophenyl sulfoxide	T	3.37	1.12	1.8	7	
		TAG	3.06	1.20	1.8	15	
		R	2.86	1	0.0	7	
D9	 methyl-4-bromophenyl sulfoxide	T	5.75	1.18	2.6	7	
		TAG	11.21	1.30	3.5	7	
		R	4.62	1.03	0.8	7	
D10	 methyl-3-bromophenyl sulfoxide	T	4.98	1.22	3.0	7	
		TAG	4.04	1.37	4.0	15	
		R	4.52	1	0.0	7	
D11	 methyl-2-bromophenyl sulfoxide	T	4.09	1.16	1.8	7	
		TAG	3.91	1.22	2.3	15	
		R	3.41	1	0.0	7	
D12	 methyl-(4-trifluoromethyl phenyl) sulfoxide	T	2.65	1.04	0.8	7	
		TAG	3.65	1.10	1.3	7	
		R	2.2	1	0.0	7	
D13	 methyl-(4-phenyl-phenyl) sulfoxide	T	9.3	1.12	1.7	7	
		TAG	8.09	1.06	0.8	15	
		R	7.19	1	0.0	7	
D14	 *t*-butyl phenyl sulfoxide	T	2.73	1.07	1.0	7	
		TAG	1.95	1.19	1.8	15	
		R	2.66	1.11	1.4	7	
D15	 vinyl phenyl sulfoxide	T	3.32	1.11	2.0	7	
		TAG	2.68	1.18	2.7	15	
		R	3.21	1	0.0	7	
D16	 benzyl phenyl sulfoxide	T	5.25	1	0.0	7	
		TAG	10.62	1.02	0.4	7	
		R	5.25	1	0.0	7	
D17	 benzyl toluyl sulfoxide	T	5.63	1	0.0	7	
		TAG	4.22	1	0.0	15	
		R	4.89	1.11	1.7	7	

Table 1. Continued

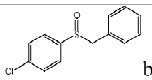
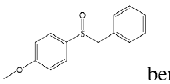
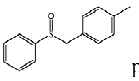
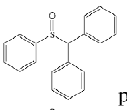
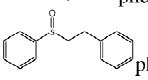
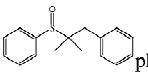
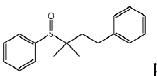
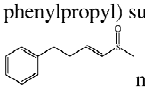
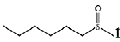
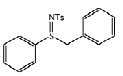
Code	Compound name and formula	CSP[a]	t_1 min	α	Rs	MeOH[b] % v/v	Other additive % v/v in MeOH
D18	 benzyl-(4-chlorophenyl) sulfoxide	T	4.79	1.05	0.8	7	
		TAG	4.15	1.04	0.7	15	
		R	4.70	1.24	2.6	7	
D19	 benzyl-(4-methoxyphenyl) sulfoxide	T	8.55	1.03	0.6	7	
		TAG	5.89	1.07	1.0	15	
		R	7.1	1.21	1.8	7	
D20	 phenyl-(4-methylbenzyl) sulfoxide	T	5.07	1	0.0	7	
		TAG	10.01	1	0.0	7	
		R	4.87	1.01	0.4	7	
D21	 phenyl-diphenylmethyl sulfoxide	T	5.98	1.19	3.1	7	
		TAG	11.73	1.19	2.4	7	
		R	2.39	1.20	2.1	15	
D22	 phenyl-(2-phenylethyl) sulfoxide	T	4.8	1.05	0.6	7	
		TAG	3.39	1.10	1.1	15	
		R	4.14	1.05	1.0	7	
D23	 phenyl-(1,1-dimethyl-2-phenylethyl) sulfoxide	T	3.63	1.17	2.1	7	
		TAG	5.94	1.22	1.9	7	
		R	3.59	1.23	3.7	7	
D24	 phenyl-(1,1-dimethyl-3-phenylpropyl) sulfoxide	T	3.69	1.07	1.2	7	
		TAG	6.03	1.15	1.5	7	
		R	3.67	1.05	0.9	7	
D25	 methyl-(4-phenyl-1-butene) sulfoxide	T	7	1.29	4.5	7	
		TAG	5.21	1.35	3.8	15	
		R	4.98	1	0.0	7	
D26	 methyl-1-hexene sulfoxide	T	3.55	1.37	5.7	7	
		TAG	2.53	1.52	4.7	15	
		R	2.61	1	0.0	7	
D27	 methyl hexyl sulfoxide	T	3.59	1	0.0	7	
		TAG	2.37	1	0.0	15	
		R	2.79	1	0.0	7	
D28	 Ts = tosyl, $-SO_2-C_6H_4-CH_3$	T	8.29	1.03	0.7	15	
		TAG	6.55	1.05	0.7	25	
		R	7.12	1.02	0.5	15	
D29	 Ts = tosyl, $-SO_2-C_6H_4-CH_3$	T	6.81	1.06	1.0	15	
		TAG	5.74	1.05	0.7	25	
		R	5.96	1	0.0	15	
D30	 methyl-1-naphthyl sulfoxide	T	8.06	1.24	2.8	7	
		TAG	6.62	1.36	2.9	15	
		R	5.88	1.08	0.9	7	
D31		T	9.18	1.05	0.9	7	
		TAG	7.23	1.11	1.4	15	

Table 1. Continued

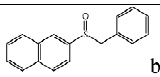
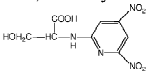
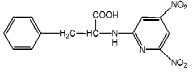
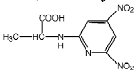
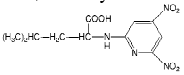
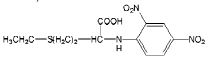
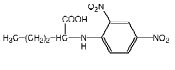
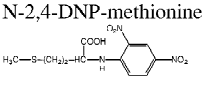
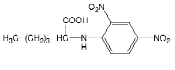
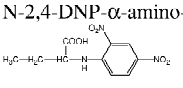
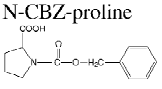
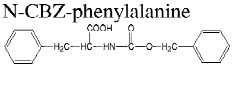
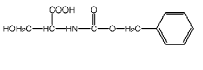
Code	Compound name and formula	CSP[a]	t_1 min	α	Rs	MeOH[b] % v/v	Other additive % v/v in MeOH
	 benzyl-2-naphthyl sulfoxide	R	8.54	1.11	1.5	7	
	class E, *N*-blocked amino-acids						
E1	N-3,5-DNPyr-serine 	T	4.09	1.74	3.1	25	
		TAG	4.36	1.52	1.5	40	
		R	5.83	1.30	1.5	40	
E2	N-3,5-DNPyr-phenylalanine 	T	1.44	1.50	1.5	40	
		TAG	2.51	1.29	0.9	40	
		R	3.00	2.95	6.9	40	
E3	N-3,5-DNPyr-alanine 	T	1.80	2.07	4.2	25	
		TAG	3.49	1.80	2.6	25	
		R	2.36	1.61	3.3	40	
E4	N-3,5-DNPyr-leucine 	T	1.82	2.25	5.4	40	0.1 TEA
		TAG	2.86	1.47	1.9	15	
		R	2.87	1.52	2.9	25	
E5	N-2,4-DNP-ethionine 	T	1.91	1.62	2.0	40	
		TAG	4.33	1.72	2.0	40	
		R	2.28	1.05	0.5	40	0.1 TEA
E6	N-2,4-DNP-norvaline 	T	2.22	1.57	2.4	25	
		TAG	4.76	1.46	1.6	25	
		R	4.66	1	0.0	40	0.1 TEA
E7	N-2,4-DNP-methionine 	T	1.94	1.89	2.4	40	
		TAG	4.96	2.06	2.5	40	
		R	6.53	1.06	0.8	40	0.1 TEA
E8	N-2,4-DNP-norleucine 	T	2.14	1.41	2.2	25	
		TAG	4.28	1.29	1.0	25	
		R	3.79	1	0.0	40	0.1 TEA
E9	N-2,4-DNP- α-amino-n-caprylic acid 	T	3.03	1.48	2.8	40	0.1 TEA
		TAG	5.13	1.21	1.0	40	0.1 TEA
		R	3.08	1	0.0	40	0.1 TEA
E10	N-2,4-DNP-α-amino-n-butyric acid 	T	5.78	1.28	1.8	40	0.1 TEA
		TAG	4.61	1.12	0.7	60	0.1 TEA
		R	5.85	1.31	2.4	40	0.1 TEA
E11	N-CBZ-proline 	T	6.56	1.06	0.8	7	
		TAG	3.96	1.10	1.0	15	
		R	1.82	1	0.0	40	0.1 TEA
E12	N-CBZ-phenylalanine 	T	2.41	1.25	1.0	40	0.1 TEA
		TAG	3.06	1.27	1.2	40	0.1 TEA
		R	3.16	1.53	2.2	40	0.1 TEA
E13	N-CBZ-serine 	T	4.05	1.39	1.0	40	0.1 TEA
		TAG	1.87	1.66	1.8	60	0.1 TEA
		R	5.69	1.25	1.7	40	0.1 TEA

Table 1. Continued

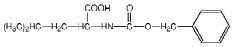
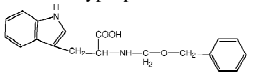
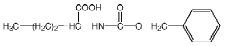
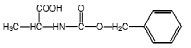
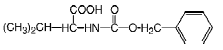
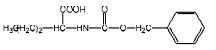
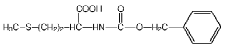
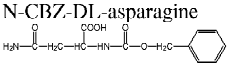
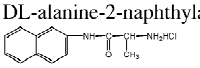
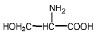
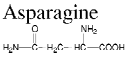
Code	Compound name and formula	CSP[a]	t_1 min	α	Rs	MeOH[b] % v/v	Other additive % v/v in MeOH
E14	N-CBZ-leucine 	T	6.15	1.29	2.2	15	0.1 TEA
		TAG	3.03	1.24	1.7	15	
		R	3.56	1.12	0.9	15	
E15	N-CBZ-tryptophan 	T	4.31	1.15	0.7	40	0.1 TEA
		TAG	7.24	1.25	1.2	40	0.1 TEA
		R	5.39	1.39	2.1	40	0.1 TEA
E16	N-CBZ-Norleucine 	T	1.43	1.45	1.4	40	0.1 TEA
		TAG	1.60	1.76	2.5	40	0.1 TEA
		R	1.55	1.20	1.0	40	0.1 TEA
E17	N-CBZ-alanine 	T	1.89	1.66	2.4	40	0.1 TEA
		TAG	2.43	2.32	3.3	40	0.1 TEA
		R	2.34	1.51	2.8	40	0.1 TEA
E18	N-CBZ-valine 	T	1.42	1.18	0.8	40	0.1 TEA
		TAG	1.61	1.37	1.3	40	0.1 TEA
		R	1.61	1.22	1.1	40	0.1 TEA
E19	N-CBZ-norvaline 	T	1.51	1.54	1.6	40	0.1 TEA
		TAG	1.69	1.90	2.6	40	0.1 TEA
		R	1.68	1.33	1.6	40	0.1 TEA
E20	N-CBZ-methionine 	T	2.21	1.50	1.8	40	0.1 TEA
		TAG	2.70	1.82	2.8	40	0.1 TEA
		R	2.61	1.35	2.1	40	0.1 TEA
E21	N-CBZ-DL-asparagine 	T	2.72	1.19	0.8	60	0.1 TEA
		TAG	4.06	1.16	0.8	60	0.1 TEA
		R	4.07	1.11	0.8	60	0.1 TEA
E22	DL-alanine-2-naphthylamide hydrochloride 	T	4.51	1	0.0	40	0.1 TFA TEA
		TAG	5.09	1.11	0.9	40	0.1 TFA TEA
		R	3.88	1.19	1.8	40	0.1 TFA TEA
	class F, Native amino-acids						
F1	Alanine 	T	2.60	1.94	3.1	48	0.1 TEA TFA 2 H_2O 0.3 glol
		TAG	4.41	2.06	5.0	47.5	0.15 TEA TFA 2.5 H_2O, 0.3 glol
		R	2.80	1.39	1.3	48	0.1 TEA TFA 2 H_2O
F2	Serine 	T	3.20	1.40	1.8	46.5	0.1 TEA TFA 3.5 H_2O
		TAG	---	---	---	Not detected	
		R	4.32	1.17	1.1	48	0.1 TEA TFA 2 H_2O
F3	Asparagine 	T	4.02	1.54	1.6	57.6	0.15 TEA TFA 2.4 H_2O
		TAG	2.18	2.24	3.8	67.2	0.15 TEA TFA 2.8 H_2O
		R	4.06	1.36	1.8	47.5	0.15 TEA TFA 2.5 H_2O 0.3 glol

Table 1. Continued

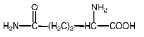
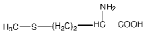
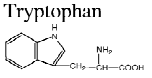
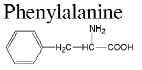
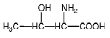
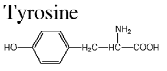
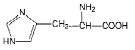
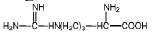
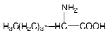
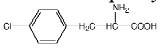
Code	Compound name and formula	CSP[a]	t_1 min	α	Rs	MeOH[b] % v/v	Other additive % v/v in MeOH
F4	Glutamine 	T	4.06	1.36	1.8	57.6	0.15 TEA TFA 2.4 H_2O
		TAG	2.07	1.50	1.5	67.2	0.15 TEA TFA 2.8 H_2O
		R	3.37	1.21	1.2	47.5	0.15 TEA TFA 2.5 H_2O 0.3 glol
F5	Methionine 	T	2.55	2.14	3.0	48	0.1 TEA TFA 2 H_2O 0.3 glol
		TAG	2.25	4.09	3.7	48	0.1 TEA TFA 2 H_2O 0.3 glol
		R	2.59	1.32	1.1	48	0.1 TEA TFA 2 H_2O
F6	Tryptophan 	T	3.61	1.71	1.8	48	0.1 TEA TFA 2 H_2O 0.3 glol
		TAG	3.33	2.02	2.3	48	0.1 TEA TFA 2 H_2O 0.3 glol
		R	4.10	1.20	0.7	48	0.1 TEA TFA 2 H_2O 0.3 glol
F7	Phenylalanine 	T	2.72	1.89	2.2	48	0.1 TEA TFA 2 H_2O 0.3 glol
		TAG	2.24	2.55	2.4	48	0.1 TEA TFA 2 H_2O 0.3 glol
		R	2.90	1.37	1.2	48	0.1 TEA TFA 2 H_2O 0.3 glol
F8	Threonine 	T	2.97	1.52	2.0	48	0.1 TEA TFA 2 H_2O 0.3 glol
		TAG	2.30	2.18	3.8	47.5	0.15 TEA TFA 2.5 H_2O 0.3 glol
		R	3.45	1.27	1.2	48	0.1 TEA TFA 2 H_2O 0.3 glol
F9	Tyrosine 	T	2.87	1.77	1.4	48	0.1 TEA TFA 2 H_2O 0.3 glol
		TAG	2.40	2.63	2.4	48	0.1 TEA TFA 2 H_2O 0.3 glol
		R	3.18	1.31	1.1	48	0.1 TEA TFA 2 H_2O 0.3 glol
F10	Histidine 	T	3.18	1.31	1.1	48	0.1 TEA TFA 2 H_2O 0.3 glol
		TAG	3.51	1.64	1.7	67.2	0.15 TEA TFA 2.8 H_2O
		R	7.95	1.09	0.4	47.5	0.15 TEA TFA 2.5 H_2O 0.3 glol
F11	Lysine 	T	1.85	1.60	2.7	67.2	0.15 TEA TFA 2.8 H_2O
		TAG	---	---	---	Not detected	
		R	---	---	---	Not detected	
F22	Arginine 	T	4.71	1.80	3.4	57.6	0.15 TEA TFA 2.4 H_2O
		TAG	---	---	---	Not detected	

Table 1. Continued

Code	Compound name and formula	CSP[a]	t_1 min	α	Rs	MeOH[b] % v/v	Other additive % v/v in MeOH
		R	4.04	1.50	1.8	47.5	0.15 TEA TFA 2.5 H_2O 0.3 glol
F23	Norvaline 	T	2.62	1.66	1.7	48	0.1 TEA TFA 2 H_2O 0.3 glol
		TAG	2.42	2.10	1.9	48	0.1 TEA TFA 2 H_2O 0.3 glol
		R	2.73	1.38	1.2	48	0.1 TEA TFA 2 H_2O 0.3 glol
F24	DL-4-chlorophenylalanine 	T	1.82	1.80	2.0	60	0.1 TEA TFA
		TAG	1.51	2.17	2.1	60	0.1 TEA TFA
		R	2.22	1.25	0.7	60	0.1 TEA TFA

[a]Chromatographic conditions: pressure 100 bar, temperature 31 °C, flow-rate 4 ml/min; UV detection at 254 nm, 220 nm and 214 nm with a diode array detector.
[b]TEA=Triethylamine, TFA=trifluoroacetic acid, glol=glycerol.
[c]Epimeric separation.

stereogenic centers. A15 an A16 are significantly less enantioresolved by the three CSPs, compared to most other class A compounds.

The speed of these enantiomeric separations should be noted. With the standard experimental conditions used, 100 bar, 31 °C and 4 ml/min, and different methanol content as listed in Table 1, all heterocyclic compounds were eluted in less than 10 min. Eighteen class A compounds (70%) were eluted in less than 5 min. Several compounds of this class were separated by HPLC with similar resolution factors, but the duration of analysis was commonly three times higher [16].

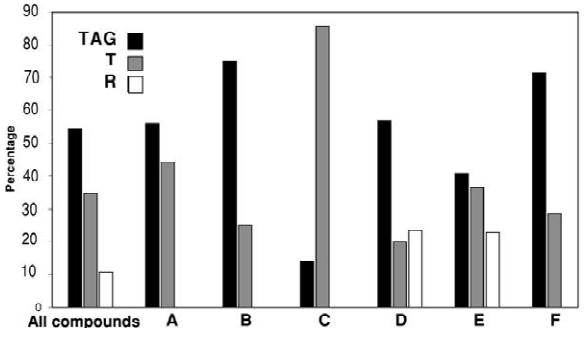


Fig. 1. Overview of successful separation for each class of compound on the three CSPs. A=Heterocyclic compounds, B=chiral acids, C=β-blockers, D=chiral sulfoxides, E=*N*-blocked amino acids, F=native amino acids, T=teicoplanin CSP, TAG=teicoplanin aglycone CSP and R=ristocetin A CSP.

3.4. Class B, chiral acids

The ristocetin chiral selector is not able to separate the enantiomers of the class B chiral acids as well as the teicoplanin and its aglycone analogue do. All 12 acids are resolved, at least partially by the Chirobiotic T and TAG columns. The Chirobiotic R column could separate only three.

From a mechanistic point of view, it should be noted that the enantiomers of the acid compounds are separated with two very different mobile phase compositions. They are either acidic SFC mobile phases containing low amounts of methanol (15%, v/v, or less) or basic SFC mobile phases with a high methanol content (more than 40%, v/v) (Table 1). With TFA containing mobile phases, the class B solutes are in their molecular form and the CSPs are positively charged since their carboxylic acid groups are neutral and their amine groups are protonated. Acidic polar organic mobile phases are used to separate these compounds by HPLC [14,15]. With basic mobile phases, the acid solutes are negatively

charged and so are the CSPs. Table 1 shows that good separation of enantiomeric pairs could be obtained in these conditions with methanol-rich SFC mobile phases.

Fig. 2 illustrates this point with the Chirobiotic T column. Fig. 2A shows the separation of compound B12 with an SFC mobile phase containing 7% added methanol. The enantiomers are partially separated with tailing peaks in less than 5 min. If a small amount of TFA (0.1%, v/v) is added, the enantioseparation is lost (Fig. 2B). With an equal 0.1% amount of TEA and TFA added to the 7% methanol, the SFC mobile phase is still acidic and one tailing peak is still obtained (Fig. 2C). When 0.1% (v/v) TEA is added to the mobile phase, the enantiomers are separated but the retention times increased to over 20 min. Increasing the methanol content to 40%, with the 0.1% (v/v) TEA allows baseline resolution (R_s=1.6) to be obtained in less than 3 min (Fig. 2D).

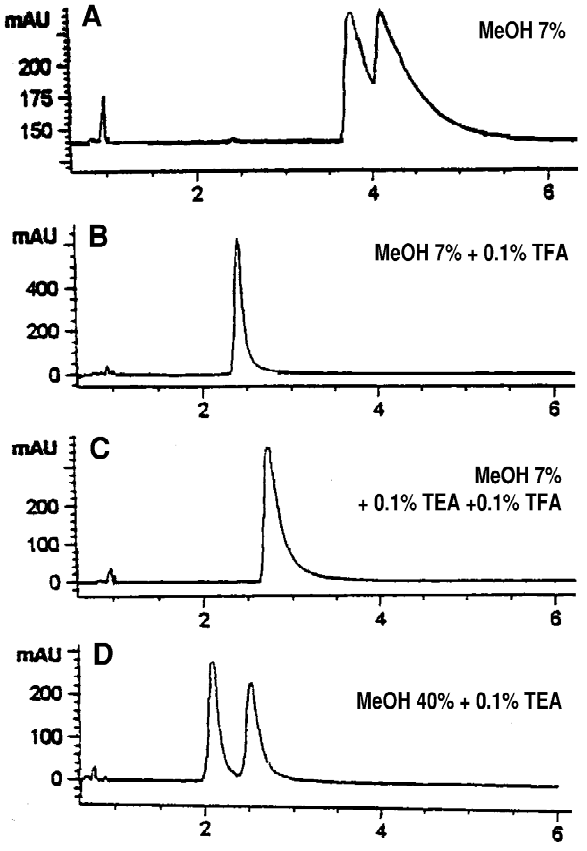


Fig. 2. Effect of additives on the separation of the enantiomers of 2(4-chlorophenoxy) propionic acid, B12. Chromatographic conditions: column Chirobiotic T, 25×0.46 cm I.D., 4 ml/min of indicated SFC mobile phases, 31 °C, 100 bar, UV detection at 254 nm. A 0.5-min integration inhibition was used.

3.5. Class C, β-blockers

The β-adrenergic blockers are all secondary amines with very similar molecular structures [i.e., R–O–CH_2–C*HOH–CH_2–NH–$CH(CH_3)_2$]. The R substituent is always aromatic. It was necessary to use high amounts of methanol (20%, v/v, or more) and to add 0.1% (v/v) of both TEA and TFA to most SFC mobile phases. These mixtures are acidic since 0.1% (v/v) TEA (7.3 m*M*) is completely neutralized by 0.1% (v/v) TFA (13.5 m*M*). It means the β-blockers are in their protonated cationic form when separated by the glycopeptide-based CSP columns.

The ristocetin chiral selector was unable to resolve enantiomers of any class C compounds. Teicoplanin was the best chiral selector for this set of compounds. The teicoplanin aglycone was able to approach the results obtained with teicoplanin for three compounds (C3, C4 and C5) and to match them for C6 (propanolol, Table 1). All successful chiral separations were obtained with 40% (v/v) or more of methanol.

3.6. Class D, chiral sulfoxides

Trivalent sulfur compounds such as sulfoxides have non-planar geometries and, when asymmetrically substituted, can be found as stable enantiomers at room temperature [26]. Traditionally, the sulfoxide group has been represented in illustrations as S=O, implying the existence of a second bond between the two atoms. A more modern understanding is that the S–O bond is more ylide-like, i.e., the molecule bears no overall charge but has a negatively charged oxygen atom bonded to a positively charged sulfur atom [27]. The sulfur stereogenic center is pyramidal, with a lone pair occupying the fourth position of the pseudotetrahedral center. We reviewed in recent work the LC chiral separations of these compounds

and demonstrated that the Chirobiotic T, TAG and R were very effective in separating sulfoxide enantiomers in the normal-phase mode (hexane–ethanol, 90:10, v/v, mobile phase) [28]. It was then logical to try SFC conditions to separate these compounds with the same Chirobiotic CSPs.

No additive other than methanol in moderate amounts (7 or 15%, v/v) was needed to obtain significant enantioselectivity. Fig. 1 shows that the TAG CSP was the most effective stationary phase for this class of compounds. The Chirobiotic TAG column could separate the enantiomers of 28 sulfoxides (90%) of which 17 sulfoxides (55%) were baseline separated. The teicoplanin based CSP could separate 25 compounds (80%) and 15 (48%) with baseline return. The Chirobiotic R column could separate 16 compounds (52%) with only six (20%) at baseline. Compounds D2 and D16 were separated by the TAG CSP only. Similarly, compounds D17 and D20 showed enantioresolution with the R CSP only.

These results correspond to those obtained with the same columns and hexane–ethanol (90:10, v/v) or hexane–isopropanol (90:10, v/v) normal mobile phases [28]. Fig. 3 compares the enantioselectivity factors obtained with SFC and HPLC (hexane–ethanol, 90:10, v/v, normal mobile phase) for the *ortho*, *meta* and *para* isomers of the methyl, chloro and bromo methyl-phenyl sulfoxides, compounds D2–D4, D6–D8 and D9–D11, respectively. The similarity of the results is striking. The enantioselectivity factor on the TAG CSP and teicoplanin CSP shows a maximum for all meta isomers, in SFC as well as in HPLC in the normal-phase mode. The enantioselectivity factors obtained with HPLC are slightly (teicoplanin) or significantly (TAG) higher than those obtained with SFC (Fig. 3). D27, the only compound that was not separated in SFC, was also not separated in HPLC with the same three CSPs and normal-phase mobile phases. Though, D27 was baseline separated by the teicoplanin and TAG columns with a methanol–pH 4.1 buffer (20:80, v/v)

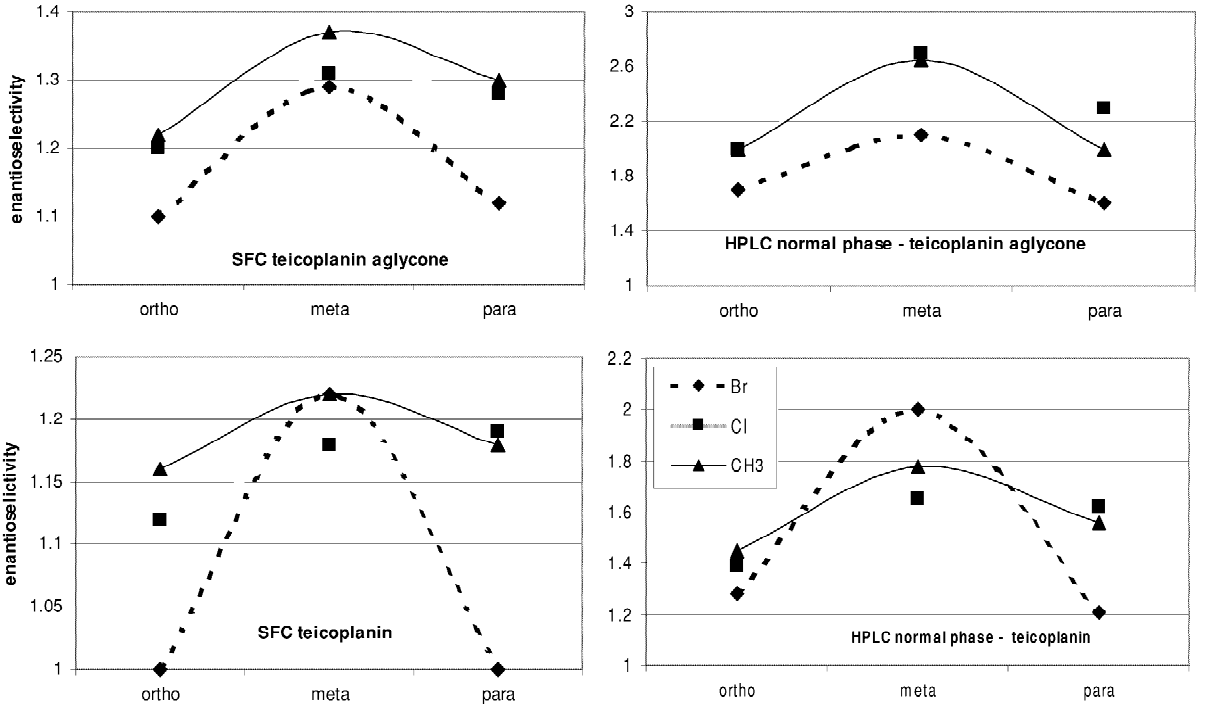


Fig. 3. Comparing SFC and HPLC enantioselectivity for *ortho*, *meta* and *para* substituted phenyl methyl sulfoxides. Top figures: column Chirobiotic TAG. Bottom figures: column Chirobiotic T. Left figures: SFC with 7% (v/v) methanol, 100 bar, 31 °C, 4 ml/min. Right figures: HPLC with hexane–isopropanol (90:10, v/v), 22 °C, 1 ml/min, data from Ref. [25]. The lines are used to show the trend.

reversed mobile phase [28]. It seems that the chiral recognition mechanism for the sulfoxides is very similar with CO_2–methanol mobile phases and normal-phase hexane–alcohol mobile phases.

The similarities between the HPLC findings and the SFC results were not absolute. For example, the ristocetin chiral selector was least effective for sulfoxide enantioresolution; it is, however, the only one that separated the enantiomers of compounds D17 and D20 and the most effective selector for compounds D18 and D19 with SFC mobile phases. All four compounds are derivatives of phenyl benzyl sulfoxides. With HPLC normal-phase mobile phases, compound D19 was the only one that matched the SFC results. It was partially resolved by the ristocetin CSP only [28]. D17, D18 and D20 were better resolved by the Chirobiotic T and TAG CSPs.

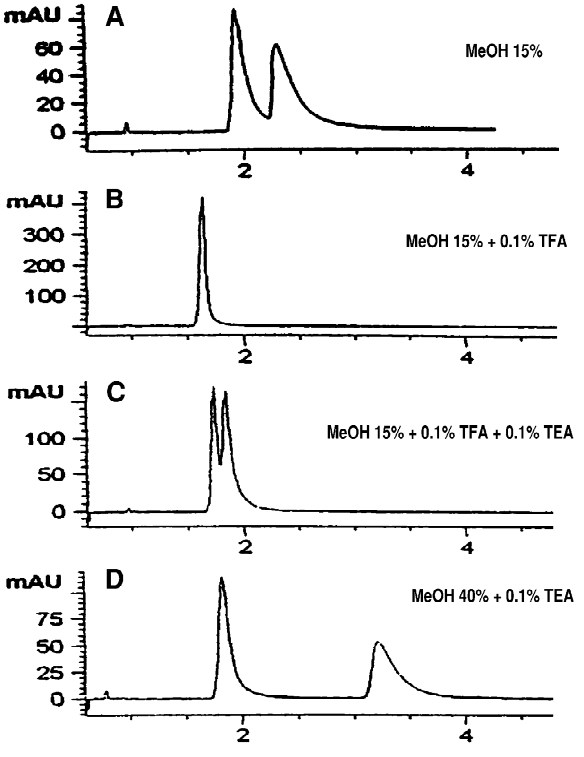


Fig. 4. Effect of additives on the separation of the enantiomers of DNPur-leucine, E4. Chromatographic conditions: column Chirobiotic T, 25×0.46 cm I.D., 4 ml/min, 100 bar outlet pressure, 31 °C, UV detection at 254 nm. A 0.5-min integration inhibition was used.

Compound D17 was partially separated by the TAG CSP only; compounds D18 was partially resolved by the three CSPs, and compound D20 was partially separated by the teicoplanin and TAG CSPs, and not by the ristocetin CSP, an opposite result compared to SFC.

3.7. Class E, N-protected amino acids

N-Protected amino acids are acidic compounds. Therefore, they should be separated using conditions similar to the ones used for class B acidic compounds. It turned out that the 7% (v/v) methanol–0.5% TFA SFC mobile phase always gave a single peak in the analyses of the enantiomers of these compounds. The methanol-rich SFC mobile phases were much more successful. Most baseline separations were obtained with 40 or 60% methanol in the SFC mobile phases. Addition of 0.1% (v/v) TEA was often needed to obtain the enantioseparation, that means the solutes and the stationary phase were in negatively charged forms.

Fig. 4 illustrates the additive effect with the Chirobiotic T column and compound E4 (DNPyr-leucine). Fig. 4A shows that a baseline separation is obtained with 15% methanol and no other additives. The peaks are tailing somewhat. The separation is lost when 0.1% TFA is added (Fig. 4B). Adding both TEA and TFA (0.1%) partially restores the enantioseparation (Fig. 4C). Adding only 0.1% TEA produced an excellent separation but retention times greater than 25 min (not shown). Increasing the methanol content to 40% (v/v) decreased the retention times below 4 min as shown by Fig. 4D with enantioselectivity and resolution factors as high as 2.3 and 5.4, respectively.

The enantiomers of the DNPyr or CBZ derivatives of alanine (E3 and E17, respectively) were extremely well separated on all three CSPs without any optimization. The enantioselectivity factors were higher than 1.6 and the enantioresolution factors were higher than 2.5 (Table 1). This is due to the natural antibiotic property of the three CSPs that bind to the D-Ala–D-Ala terminal group of the terminal dipeptide of the microbial cell wall of Gram+ bacteria [29]. The chiral selectors have a high affinity for the D-Ala amino acid and the L-Ala form is much less

retained. Since the amine group of alanine is derivatized, it shows that the carboxylic acid group is essential in the recognition mechanism [11–15]. Changing the methyl group attached to the stereogenic center of alanine for other groups produces the other amino acids that are also enantiodifferentiated by the CSPs, but somewhat less well than alanine (Table 1).

Fig. 5 compares the separation of E19 (CBZ-norvaline) in HPLC with a classical methanol–pH 4.1 buffer (20:80, v/v) and the SFC separation with 40% methanol on both the Chirobiotic T and R columns. In all cases, the enantiomers were baseline separated. The peak shape obtained with the classical reversed-phase mobile phases is better than the one obtained with the SFC mobile phases. But the HPLC separations that needed 18 or 13 min on ristocetin or teicoplanin CSPs, respectively, were performed in less than 3 min with SFC mobile phases (Fig. 5).

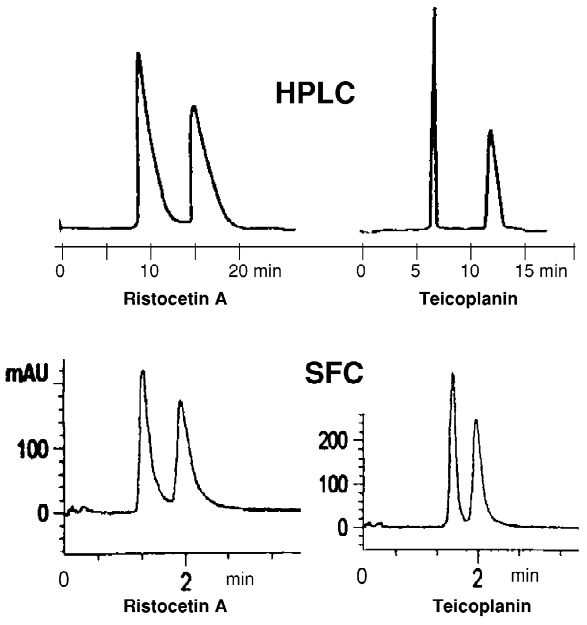


Fig. 5. Comparison of HPLC and SFC enantiomer separations of CBZ-norvaline (E19) on Chirobiotic R (left) and Chirobiotic T (right) columns. HPLC: pH 4 buffer–methanol (80:20, v/v) mobile phase, 1 ml/min, room temperature, UV detection at 254 nm. SFC: CO_2–methanol–TEA (60:39.96:0.04, v/v) mobile phase, 4 ml/min, 31 °C, 100 bar, UV detection at 254 nm.

3.8. Class F, native amino acids

It was mentioned in the Introduction that SFC is a useful substitute to normal-phase chromatography. In this work, polar compounds such as native, underivatized amino acids, that require reversed-phase polar mobile phases in HPLC, were tested to see if they could be enantioresolved by SFC. Two problems were encountered: (1) native amino acids lacking an aromatic substituent poorly absorb UV light making them difficult to detect. Only the 214 nm detector wavelength gave some absorbance. (2) Following the example of Medvedovici et al., small amounts of water and/or glycerol can be added to the mobile phase to enhance the solubility of polar analytes and to improve peak shape [22].

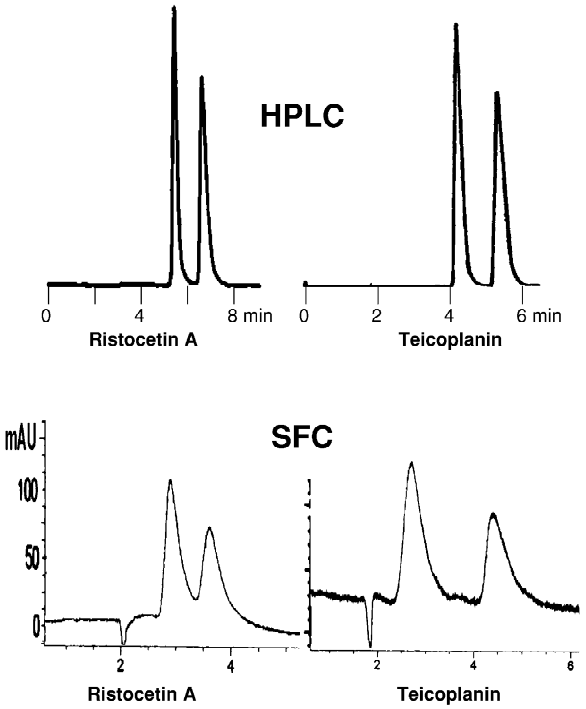


Fig. 6. Comparison of HPLC and SFC enantiomer separations of phenylalanine (F7) on Chirobiotic R (left) and Chirobiotic T (right) columns. HPLC and ristocetin: water–methanol (50:50, v/v) mobile phase, HPLC and teicoplanin: water–ethanol (50:50, v/v) mobile phase, 1 ml/min, room temperature, UV detection at 254 nm. SFC: CO_2–methanol–water–glycerol–TEA–TFA (50:48.75:1:0.15:0.05:0.05, v/v) mobile phase, 4 ml/min, 31 °C, 100 bar, UV detection at 254 nm.

Table 2
Elution order of the compounds eluted by SFC on the three CSPs

Compound[a]	Teicoplanin[c]	TAG[c]	Ristocetin[c]
A1, 4-benzyl-2-ox.	(*R*), (*S*)	(*R*), (*S*)	No separation
A2, 5,5-dimethyl-4-phenyl-2-ox.	(*R*), (*S*)	(*R*), (*S*)	(*R*), (*S*)
A3, 4-benzyl-5,5-dimethyl-2-ox.	(*R*), (*S*)	(*R*), (*S*)	(*R*), (*S*)
A4, 4-diphenylmethyl-2-ox.	(*R*), (*S*)	(*R*), (*S*)	(*R*), (*S*)
A5, *cis*-4,5-diphenyl-2-ox.	(4*S*,5*R*), (4*R*,5*S*)	(4*R*,5*S*), (4*S*,5*R*)	(4*S*,5*R*), (4*R*,5*S*)
A6, 4-methyl-5-phenyl-2-ox.	(4*S*,5*R*), (4*R*,5*S*)	(4*S*,5*R*), (4*R*,5*S*)	(4*S*,5*R*), (4*R*,5*S*)
A7, 1,5-dimethyl-4-phenyl-2-im.	(4*R*,5*S*), (4*S*,5*R*)	(4*R*,5*S*), (4*S*,5*R*)	No separation
A9, 4-hydroxy-2-pyrrolidone	(*S*), (*R*)	(*S*), (*R*)	(*S*), (*R*)
C6, propranolol	(*S*), (*R*)	(*R*), (*S*)	(*S*), (*R*)
D compounds[b]	(*S*), (*R*)	(*S*), (*R*)	(*R*), (*S*)
E compounds[b]	(*S*), (*R*) or (L, D)	(*S*), (*R*) or (L, D)	(*S*), (*R*) or (L, D)
F compounds[b]	(*S*), (*R*) or (L, D)	(*S*), (*R*) or (L, D)	(*S*), (*R*) or (L, D)

[a] ox.=Oxazolidinone, im.=imidazolidinone.
[b] For the separated enantiomers.
[c] Circular dichroism measurements.

All 24 underivatized amino acids were enantioseparated almost always to baseline by the three macrocyclic glycopeptide CSPs with mobile phase containing more than 47.5% (v/v) methanol, 2% or more water, 0.1% or more TEA, 0.1% or more TFA and 0.3% glycerol. Fig. 6 shows the separation of F7 (phenylalanine) on the Chirobiotic T and R columns, comparing the SFC and classical reversed-phase mobile phases. It can be seen that the reversed-phase HPLC separation of phenylalanine is as good or even better that the corresponding separation by SFC. The retention time is slightly lower with SFC but the peak shape is significantly poorer as well.

3.9. Elution order

It is often of great interest to know the enantiomeric elution order of chiral separations. A change in the elution order of some compounds was observed with the same mobile phase when changing the chiral selector [30]. Pure enantiomers are needed to identify the compounds and determine the elution order. It was not possible to have such pure enantiomers for all 111 compounds. Table 2 lists the compounds for which the elution order could be determined.

In most cases, the same elution order was obtained with the three CSPs. For the compounds that were tested for elution order, only A5 and C6 showed inversion of the elution order with the TAG column compared to the T and R columns (Table 2). The sulfoxide compounds (class D) are exceptions. All chiral sulfoxides showed the (*S*)-(+) as the first eluting enantiomer on the teicoplanin and TAG columns. The (*R*)-(−) sulfoxide enantiomer was first eluted with the ristocetin A column. The high affinity of the natural chiral selector for the D-form (*R*) of the amino acids makes this enantiomer always more retained than the L-amino acid (*S* form).

4. Conclusion

The separation time factor is the greatest advantage of the SFC mobile phases. As can be seen in Table 1, all separations done with subcritical mobile phases were performed in less than 15 min, 70% of the separations being done in less than 4 min. The peak shape is not as symmetrical as that obtained in the corresponding HPLC separations. It should also be pointed out that the column equilibrate much faster with CO_2 containing mobile phases than in normal-phase HPLC. This work also showed that the macrocyclic based CSPs are able to separate enantiomers of widely different compounds with various functionalities and polarities. The teicoplanin aglycone and teicoplanin CSPs seem to be the most effective stationary phase with SFC mobile phases.

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