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Chiral packed column subcritical fluid chromatography on polysaccharide and macrocyclic antibiotic chiral stationary phases.

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Abstract

The polysaccharide chiral stationary phases (CSPs) Chiralcel OD and Chiralpak AD, and the macrocyclic antibiotic CSPs Chirobiotic V and Chirobiotic T were evaluated in packed column subcritical fluid chromatography (pSFC) for the separation of different types of racemic compounds (β-blockers, β-agonists, benzodiazipines, non-steroidal anti-inflammatory drugs, barbiturates, free and derivatized amino acids, etc.). The same conditions and program could be applied to check the applicability and enantioselectivity of one of the CSPs for a given racemic mixture. The conditions are: temperature 30°C, pressure 200 bar, flow-rate 2 ml/min, carbon dioxide, modifier methanol containing 0.1% trifluoroacetic acid (TFAA) and 0.1% triethylamine (TEA) with a gradient from 5% (5 min) to 30% at 5%/min. A resolution of 0.4 under those conditions generally indicates that baseline separation can be realized on the CSP by fine-tuning the different parameters of the SFC separation. The best CSP for pSFC proved to be Chiralpak AD which provided separation for 70% of the 44 substances tested, followed by Chiralcel OD (66%), Chirobiotic T (50%) and Chirobiotic V (48%). For comparison, the enantioselectivity in pSFC of the brush type CSPs Chirex 3022 with π-donor characteristics and Chirex 3005 with π-acceptor characteristics was also evaluated. As expected, these phases perform poorly under SFC conditions, on Chirex 3022 (34%) and on Chirex 3005 (20%). © 1997 Elsevier Science B.V.

Keywords: Chiral stationary phases, SFC; Enantiomer separation; Subcritical fluid chromatography; Polysaccharide chiral stationary phases; Macrocyclic antibiotic chiral stationary phases

1. Introduction

In recent years the importance of chirality in pharmaceutical and agricultural chemicals has been recognized. As illustration, a racemic drug can no longer be registered by the US Food and Drug Administration without characterization of the biological activity of the individual enantiomers. Hence the need for methods to separate racemates. Chiral chromatography at present plays a vital role in the separation of enantiomers. Liquid chromatography is by far the most versatile technique for chiral separation, on the one hand because of the large number of chiral stationary phases (CSPs) and on the other hand because of its broad applicability. Moreover analytical LC often constitutes the first step in the search for an appropriate semi- or preparative separation system.

Packed column supercritical fluid chromatography (pSFC) has recently witnessed a remarkable breakthrough. pSFC is more and more considered as an improved alternative to normal phase LC. pSFC

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therefore also represents a versatile tool for enantiomeric separations usually performed by LC on CSPs operated in the normal phase mode. The low viscosity of the mobile phase combined with increased diffusivity in pSFC often translates in improved resolution and shorter analysis times compared to LC. Other reasons for using pSFC instead of LC, include faster column equilibration, faster method development, lower pressure drop across the column, ease of solvent removal and possibilities for semi- and preparative sample collection.

The selected temperature in pSFC for the separation of enantiomers is often below the critical temperature and subcritical conditions are applied. Because there is no discontinuity of the fluid properties when the temperature is below the critical value, this hardly influences the separation capabilities. Subcritical operation should in fact be defined as LC. A back pressure regulator or restrictor is, however, still required to prevent the fluid from expanding in the column into gaseous carbon dioxide and therefore we prefer to continue the use of the abbreviations SFC and pSFC.

Chiral pSFC was pioneered by Mourier et al. [1]. After a short dormant period, pSFC has been rediscovered for enantiomeric separation mainly because of the introduction of reliable and rugged instrumentation. Consequently, a large number of chiral separations on different CSPs by pSFC have been described in the last ten years.

Polysaccharide CSPs based on cellulose and amylose derivatives have proven to be widely applicable in pSFC. Most of the published work has been performed on cellulose tri (3,5-dimethylphenylcarbamate) — Chiralcel OD [2–9] and cellulose tri(benzoate) — Chiralcel OB [5,10,11]. Cellulose tri(4-methylbenzoate) — Chiralcel OJ [5,12,13] and cellulose tri(phenylcarbamate) — non-commercial [14] has been used sporadically. The performance of amylose CSPs in pSFC and more especially of amylose (3,5-dimethylphenylcarbamate) — Chiralpak AD have been reported [7,15] and compared to its cellulose analogue — Chiralcel OD [16–18]. Cyclodextrins have been used in capillary column SFC (cSFC) [19,20] and pSFC [11,21]. We found, however, that for a series of pharmaceuticals, resolution on CD CSPs in general is poor [7]. Very popular phases in LC are Pirkle or brush CSPs and they were among the first stationary phases evaluated in pSFC [1]. Their applicability in pSFC seems rather limited although some excellent separations have been reported [22,23]. A special brush type SFC phase, 3,5-dinitrobenzoyltyrosine (ChyRoSine-A) has been synthesized by Siret et al. [24] and its use in LC and pSFC has been described [25,26]. The column was evaluated by us but showed enantioselectivity for only a very small number of racemates. More recently new CSPs with π-acid and π-base characteristics have been introduced by Blum et al. [27] and Terfloth el al. [28]. The Whelk-O 1 and polyWhelk-O columns seems very promising in pSFC [29,30] and moreover this approach opens the way to rationally design CSPs applicable in pSFC [31]. Another group of CSPs, recently introduced by Armstrong et al. [32] are the macrocyclic antibiotic CSPs Chirobiotic V (vancomycin) and Chirobiotic T (teicoplanin) and evaluation of those phases in pSFC seems straightforward because of their multimodal character and their complementary nature e.g. Chirobiotic V offers advantages over protein and cellulose CSPs and Chirobiotic T is an excellent alternative to crown ether and ligand exchange based CSPs [33]. To the best of our knowledge the performances of Chirobiotic V and T in pSFC have not been reported so far in the literature. The only application we are aware of was performed by scientists at the Roche Products Laboratories (Hertfordshire, UK) who succeeded in separating a drug racemate on Chirobiotic V which was not separated on Chiralcel OD and Chiralpak AD (J.A. Whatley, Roche Products, Hertfordshire, UK, personal communication).

In this contribution, our experiences in pSFC with the polysaccharide phases Chiralcel OD and Chiralpak AD, with the brush type phases Chirex 3022 and Chirex 3005 and with the macrocyclic antibiotic phases Chirobiotic V and Chirobiotic T are summarized. Forty-four racemates were analysed on the six CSP columns. Different amino acid derivatives have been analyzed on Chiralpak AD and some free amino acids on Chirobiotic T.

2. Experimental

2.1. Instrumentation

Two SFC systems were used for the analyses. The

first system was an HP G 1205A SFC (Hewlett–Packard, Little Falls, DE, USA) coupled to an HP 1050 diode array detector. Data were processed on an HP Windows CHEMSTATION. The second system was a Gilson modular SFC system Series SF3 (Gilson Medical, Villiers-le-Bel, France) composed of a 308 high pressure pump for carbon dioxide delivery and a 306 high pressure pump for the delivery of modifier, a 811 C mixer, a 821 pressure controlling unit, a 831 temperature regulator, a Rheodyne 5-μl injection valve, a 119 dual wavelength UV–VID detector and a 506 C interface. Data were collected and processed by a Gilson 715 software package running under Windows. Both systems perform equally well for the separations described.

2.2. Columns

Columns 25 cm×0.46 cm I.D. packed with the 3,5-dimethylphenylcarbamate derivative of cellulose coated on 10 μm silica-gel (Chiralcel OD) and the 3,5-dimethylphenylcarbamate derivative of amylose coated on 10-μm silica-gel (Chiralpak AD) manufactured by Diacel (Tokyo, Japan) were purchased from J.T. Baker (Deventer, The Netherlands). The brush type columns (25 cm×0.32 cm I.D., 5-μm particles) Chirex 3005 (π-acceptor) and Chirex 3022 (π-donor) were gifts from Phenomenex (Torrance, CA, USA). In Chirex 3005 *R*-1-naphthylglycine and 3,5 dinitrobenzoic acid are linked via an amide bond to aminopropyl silica-gel, whereas in Chirex 3022, *S*-indoline-2-carboxylic acid and *R*-1-(a naphthyl)ethyl amine are linked to aminopropyl silica-gel via an urea bond. Chirobiotic V and Chirobiotic T columns of 25 cm×0.46 cm I.D., 5-μm particles were manufactured by Astec (Whippany, NJ, USA) and purchased from ICT (Bad Homburg, Germany). Solutions of the solutes were made in hexane–isopropanol (90:10, v/v), methanol or acetone at the 0.1% level. The injected volume was 5 μl.

2.3. Chemicals

SFC/SFE grade carbon dioxide from Air Products (Sambreffe, Belgium) was used. All solvents and modifiers were HPLC grade and obtained from Labscan (Dublin, Ireland). Triethylamine (TEA), trifluoroacetic acid (TFAA) and acetic acid (AA) were purchased from Janssen Chimica (Beerse, Belgium). Benzoyl chloride, 3-nitrobenzoyl chloride, 4-nitrobenzoyl chloride, 3,5-dinitrobenzoyl chloride were from Aldrich (Bornem, Belgium) and the aqueous solution of 10% picryl sulphonic acid was from Sigma (St. Louis, MO, USA).

The racemates were collected over the years and obtained from different sources: Sigma, Janssen, AKZO (Arnhem, The Netherlands), Pharmaceutical Institute (University of Gent, Belgium). The amino acids were purchased from Aldrich.

2.4. Derivatisation

The trinitrophenyl derivatives were prepared in the following way. To 50–100 mmol of the amino acids dissolved in 5 ml water, 200 mg sodium bicarbonate was added followed by 2 ml of the picryl sulphonic acid solution. The sample was mixed and placed in the darknesss for 2 h at room temperature. Hydrochloric acid was added to reach pH 2 and the sample was placed in a refrigerator at 4°C for 1 h. The precipitated amino acid derivatives were filtered, washed with a 0.1 *M* HCl solution and redissolved in acetone.

The benzoyl derivatives were prepared by dissolving 1 mmol amino acid with 2 mmol reagent in 5 ml tetrahydrofuran. The sample was then heated at 75°C until the amino acids were completely dissolved. The solvent was removed under a stream of nitrogen and the residue was redissolved in acetone. Methylation of the benzoylated amino acids was performed by adding diazomethane to the acetone solution.

3. Results and discussion

3.1. Chiralpak AD and Chiralcel OD

Based on our previous studies [7,34], a simple strategy was devised for the separation of racemates by pSFC on the polysaccharide phases. Initial pSFC experiments were always performed with the following conditions: column temperature 30°C, pressure 200 bar, flow rate 2 ml/min, methanol as modifier containing 0.1% triethylamine (TEA) and 0.1% trifluoroacetic acid (TFAA) and programmed from 5% (5 min) to 30% at 5%/min.

The composition of the mobile phase has the

largest effect on retention, peak shape and enantioselectivity of the CSPs. Methanol has proven to be the most versatile modifier. A basic or an acidic additive is usually required to improve peak shapes for basic or acidic compounds analysed on Chiralcel OD or Chiralpak AD. We have observed that addition of both additives had, at least for the racemates studied, no negative influence on the enantioselectivity of the CSPs and on the peak shape. The selection of the modifier gradient program is based on the fact that most solutes exhibit sufficient retention under those conditions. Temperature is the second most important parameter. The highest enantioselectiviy is usually observed at low temperatures. This is the reason why a subcritical temperature has been applied. Pressure seems to have little influence on enantioselectivity. At too low inlet pressure e.g less than 150 bar, bad peak shapes have been noted for some racemates. A flow of 2 ml/min can be applied because, on the one hand, the Van Deemter curves are quite flat [7] and, on the other hand, the separations are mainly controlled by the CSP selectivity.

The apparent retention factors k^G for the first eluting enantiomer and the resolution R_s^G for 44 racemates, in alphabetical order, on Chiralpak AD and Chiralcel OD are listed in Table 1. Because it is not very common to calculate resolution under gradient conditions, the intrinsic value of the R_s^G numbers is illustrated in Fig. 1 for separations on different CSPs. The same SFC conditions were indeed applied for the macrocyclic antibiotics Chirobiotic T and Chirobiotic V and for the brush type phases Chirex 3022 and 3005 as will be discussed further.

On Chiralpak AD 31 and on Chiralcel OD 29 out of the 44 compounds exhibited resolutions equal to or greater than 0.4. On the two phases, 39 of the 44 compounds are separated with resolutions of at least 1.0; three solutes, clenbuterol (7), felodipine (12) and promethazine (34) exhibited resolution between 0.4 and 0.9, while the racemates of amlodipine (4) and salmeterol (36) were not separated at all. Chiralpak AD and Chiralcel OD are complementary in nature and should be the first chiral phases to evaluate in pSFC for a given enantiomeric pair. Forty-two of the forty-four racemates (95%) indeed show resolution equal to or larger than 0.4. The amylose derivative is slightly more versatile than the cellulose analogue. This is an important observation because most of the applications of pSFC until now were performed on cellulose based derivatives. In addition to the mechanisms of hydrogen bonding, dipole–dipole interaction and $\pi-\pi$ interactions, the helical nature of amylose better allows inclusion compared to the planar cellulose surface. In the past we have stated that Chiralpak AD and Chiralcel OD are best suited for acidic and basic drugs, respectively [7,34]. This is, however not a general rule as illustrated in Fig. 2 for the separation of mandelic acid on Chiralpak AD and Chiralcel OD using the SFC conditions described. The homologue tropic acid, on the other hand, is not separated at all on Chiralcel OD while the resolution is 3.2 on Chiralpak AD. Nevertheless, non-steroidal anti-inflammatory drugs, benzodiazipines and barbiturates show the highest enantioselectivity on Chiralpak AD and β-blockers on Chiralcel OD.

From the data in Table 1, one can deduce whether a given enantiomeric pair can be separated to the baseline or not on Chiralcel OD or Chiralpak AD. If the enantiomers show no sign of separation under the initial gradient conditions e.g. amlodipine (4) and salmeterol (36), optimisation will be difficult and it is more fruitful to select a different CSP (see further). If the initial conditions provide R_s^G values of at least 0.4, baseline separation can in general be achieved by fine-tuning one of the initially applied parameters of the SFC conditions. In this optimisation, the concentration and the nature of the modifier are of utmost importance. This is illustrated in Fig. 3 for the separation of the felodipine enantiomers on Chiralpak AD showing a R_s^G value of 0.6 under the programmed conditions.

The first peak elutes at 9 min ($k^G=4.7$) where the methanol concentration is 25% (Fig. 3A). This is definitely too high to provide baseline separation. Fig. 3B and C show the analyses at 5% with a program of 1%/min and at 5% isocratic, respectively. In cases where the enantiomers are still inadequately resolved, a different modifier should be selected. The order of preference in pSFC, based on our experiences over the last three years is ethanol, isopropanol and propanol as protic solvents [17]. The aprotic solvent acetonitrile can eventually be added in combination with a protic solvent to avoid on-

Table 1
Apparent retention factors k^G and resolution values R_s^G for 44 racemates

No.	Compound	Column											
		Chiralpak AD		Chiralcel OD		Chirobiotic T		Chirobiotic V		Chirex 3022		Chirex 3005	
		k^G	R_s^G	k^G	R_s^G	k^G	R_s^G	k^G	R_s^G	k^G	R_s^G	k^G	R_s^G
1	Acebutolol	5.44	–	5.33	1.0	11.3	0.9[a]	13.8	2.0[a]	14.68	0.8	11.83	–
2	Alprenolol	2.04	0.2	3.07	5.1	9.11	1.5	7.95	0.4	8.71	0.6	6.92	–
3	Althiazide	17.55	–	6.84	2.6	15.70	–	14.20	–	13.18	–	14.60	–
4	Amlodipine	4.50	–	5.55	–	20.53	–	13.62	1.0	9.70	3.1	17.65	–
5	Atropine	5.46	–	5.06	1.6	21.30	–	17.50	–	11.06	–	10.28	–
6	Bendrotlumethiazide	693	1.4	6.60	0.3	11.60	0.8	11.20	1.3	16.88	–	13.63	–
7	Clenbuterol	4.18	0.9	4.09	0.4	12.30	1.7	9.43	1.5	14.66	0.7	9.38	–
8	Cyclopenthiazide	8.33	1.5	6.69	2.6	17.00	0.9	14.60	1.5	19.74	1.4	14.96	–
9	Cyclothiazide	10.66	3.2	6.77	2.9	14.70	–	10.10	–	19.01	0.4	14.55	–
10	Dysopyramide	5.27	1.2	5.28	–	30.40	–	20.00	1.4	11.80	–	11.50	–
11	Ephedrine	2.51	0.2	3.19	1.4	10.90	–	10.20	–	9.00	–	7.86	–
12	Felodipine	4.69	0.6	8.91	–	5.78	–	5.56	–	529	–	3.63	–
13	Fenoprofen	4.35	3.3	4.13	–	4.00	0.8	3.20	–	3.77	–	3.31	1.0
14	Fenoterol	5.92	3.2	6.28	–	12.90	1.6[a]	23.00	1.8[a]	14.70	0.7	12.90	–
15	Flurbiprofen	4.87	11.7	3.85	–	4.15	0.6	3.30	–	3.73	–	3.39	0.5
16	Guaifenesine	5.30	1.6	4.61	3.6	5.99	–	5.80	–	3.68	–	5.57	0.7
17	Hexobarbital	3.45	21.2	3.77	0.7	3.40	–	3.25	0.9	1.65	–	1.63	–
18	Ibuprofen	2.02	3.1	1.41	–	2.26	0.9	1.60	–	1.70	–	1.33	–
19	Indapamide	7.95	0.4	8.02	2.0	11.42	–	10.27	0.9	13.65	–	12.71	–
20	Ketamine	2.97	1.4	3.57	0.2	12.86	1.0	10.41	–	9.07	0.9	7.22	–
21	Ketoprofen	5.21	1.2	4.22	–	5.38	1.1	5.03	–	5.78	–	5.24	0.4
22	Lormethazepam	8.95	11.2	6.04	1.3	6.86	2.2	6.20	1.1	9.07	1.6	9.94	–
23	Mandelic acid	2.00	1.2	1.06	4.8	6.89	–	5.80	–	0.93	–	0.94	–
24	Medetomindine	4.20	–	4.43	3.3	11.54	–	9.55	3.4	11.85	3.3	9.94	–
25	Mephobarbital	4.52	25.6	3.82	1.2	3.40	–	3.13	1.0	1.78	–	1.72	0.6
26	Metroprolol	4.55	2.9	4.15	10.0	11.32	1.0	9.30	0.4[a]	9.40	–	8.71	–
27	Nadolol	5.92	0.4	5.80	1.8	27.40	1.1[a]	19.62	0.2	12.05	–	2.03	–
28	Naproxen	6.21	2.6	4.78	1.4	4.90	1.6	4.60	–	4.96	–	7.56	1.4
29	Oxazepam	8.81	4.7	6.87	3.0	11.02	–	8.01	–	12.43	0.8	11.93	0.4
30	Oxprenolol	3.26	1.2	4.30	6.8	10.46	1.7	8.24	0.4	9.06	0.2	8.49	–
31	2-Phenyl cyclohexanone	2.37	–	1.50	1.3	1.08	–	0.87	–	0.92	–	0.88	0.4
32	Pindolol	5.58	1.2	6.27	11.7	19.81	1.0	24.00	0.6	12.78	–	13.09	–
33	Polythiazide	7.00	3.5	6.22	–	10.92	0.4	10.32	–	16.17	–	13.19	–
34	Promethazine	5.12	0.8	5.03	–	13.01	–	6.28	1.4	10.85	0.8	9.33	–
35	Propranolol	4.64	1.4	5.59	6.0	12.83	2.0	9.91	1.3	10.71	0.3	10.72	0.2
36	Salmeterol	5.98	–	6.24	–	24.31	0.6	19.73	1.3	13.68	1.0	12.13	–
37	Spironolactone	11.9	–	6.62	2.6	7.30	–	11.81	–	9.90	–	9.15	–
38	Tetramisole	5.53	0.2	5.65	1.2	14.5[a]	–	15.5[a]	–	14.48	–	18.11	–
39	Tiaprofenic acid	6.90	1.5	5.03	–	6.05	–	5.68	–	8.79	–	8.54	–
40	*trans*-Stilbene oxide	3.53	13.0	2.10	4.3	0.40	–	0.28	–	0.29	–	0.56	–
41	Trifluoro-anthranylethanol	6.31	–	5.83	8.8	5.24	0.3	4.88	–	8.11	–	5.74	3.4
42	Tropic acid	5.59	2.5	4.33	–	6.27	0.6	5.99	–	7.13	–	7.92	–
43	Verapamil	4.61	0.2	5.35	1.5	10.56	–	10.89	0.5	10.85	0.9	10.46	–
44	Warfarin	6.75	8.1	5.64	6.0	6.83	1.3	6.12	3.9	10.69	1.0	10.12	–

[a] Gradient up to 40%.

column racemization as in the case of oxazepam [7]. Contrary to the observations made in LC [35], the addition of aprotic solvents (acetonitrile, methyl-*tert.*-butyl-ether) alone is not successful in pSFC and often completely destroys enantioselectivity. When modifier optimisation is not leading to improved

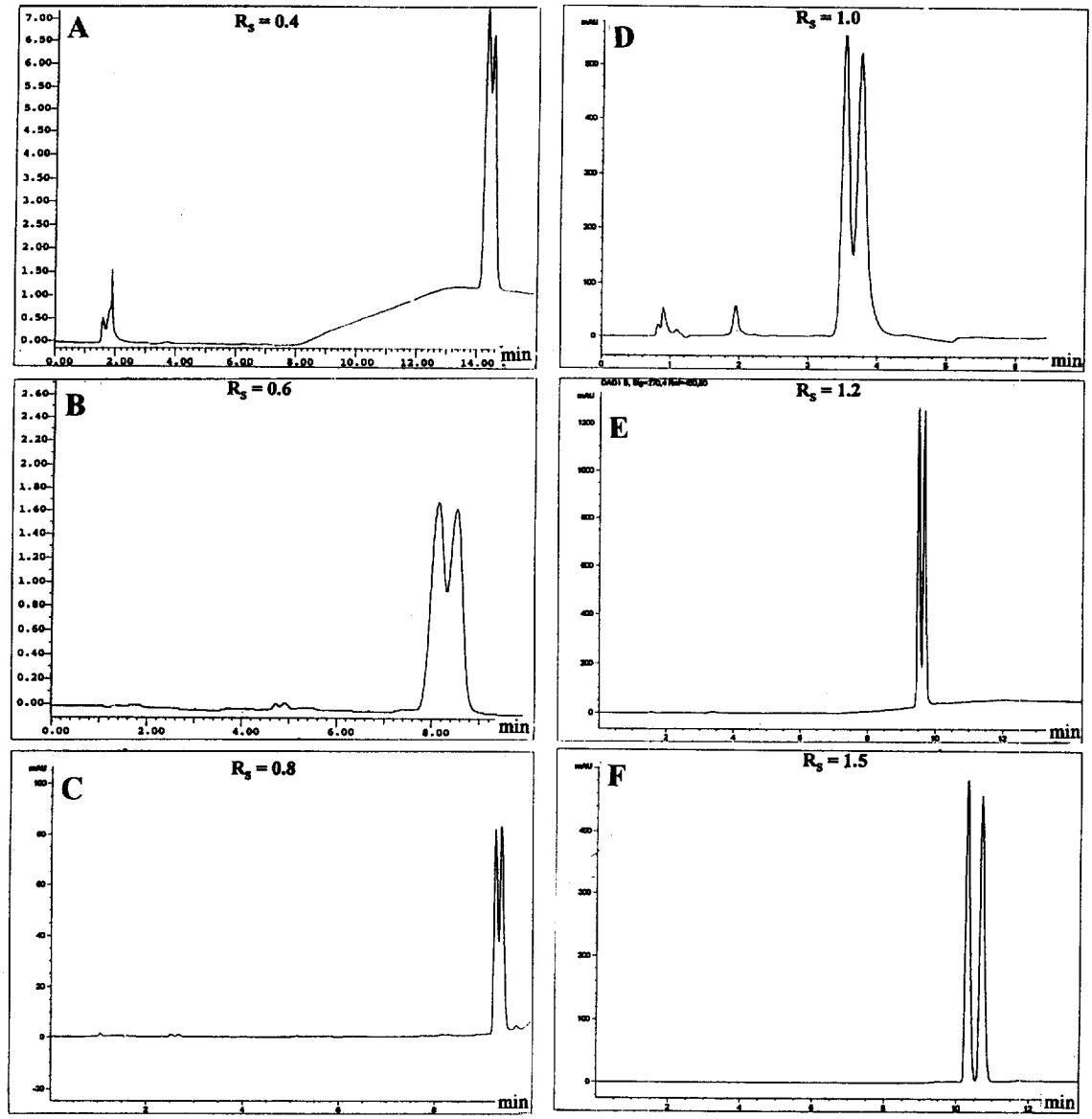


Fig. 1. Illustration of R_s^G values in the gradient run. (A) Alprenolol on Chirobiotic V (B) flurbiprofen on Chirobiotic T (C) promethazine on Chirex 3022 (D) fenoprofen on Chirex 3005 (E) ketoprofen on Chiralpak AD (F) verapamil on Chiralcel OD.

resolution, it is worthwhile to investigate the influence of temperature by varying the column temperature to 20°C and 40°C. This will indicate whether or not temperature is an important tool for resolution improvement for a specific racemate. In general we have observed that enantioselectivity increases at lower temperature but efficiency decreases, while the opposite is true for higher temperatures.

For racemates with high retention factors k^G and resolution values R_s^G in Table 1, i.e. $k^G>6$, $R_s^G>2.5$, or a combination of both e.g. k^G–R_s^G: 7–2, 6–2.5, 5–3, 4–3.5, 3–4; the modifier can be kept constant at 30% providing baseline resolution in short analysis

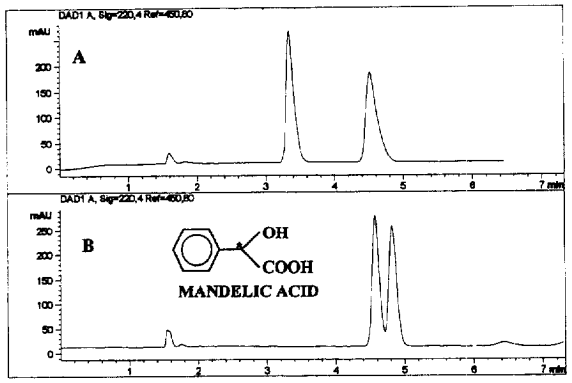


Fig. 2. pSFC analysis of mandelic acid on Chiralcel OD (A) and Chiralpak AD (B). Gradient SFC conditions see text.

times. This is the case for cyclothiazide, fenoterol, flurbiprofen, hexobarbital, lormethazepam, mephobarbital, naproxen, oxazepam, polythiazide, tiaprofenic acid, *trans*-stilbene oxide, tropic acid and warfarin on Chiralpak AD and for althiazide, cyclopenthiazide, cyclothiazide, guaifenesine, indapamide, lormethazepam, mandelic acid, metoprolol, nadolol, oxazepam, oxprenolol, pindolol, propanolol, *trans*-stilbene oxide, trifluoro-anthranylethanol and warfarin on Chiralcel OD.

3.2. Chirobiotic T and chirobiotic V

The macrocyclic antibiotic phases are multimodal and can be used in the normal phase, the reversed phase and the polar organic phase mode, hence the interest in evaluating these CSPs in pSFC. Enantioseparation on Chirobiotic V and T may be possible via several mechanisms like $\pi-\pi$ complexation, inclusion, hydrogen bonding, dipole interaction, steric interaction, etc. This multichiral character can be advantageous i.e. more versatility, but also disadvantegeous i.e. different mechanisms are counteracting each other. The combination of different chiral selectors in one chromatographic column can also be realised by mixing stationary phases [36] or by coupling chiral columns [7,37]. The pros and cons have been discussed [36–38]. Without going into detail, the column tandem Chiralpak AD–Chiralcel OD has been evaluated for the analysis of the 44 racemates. On the tandem 37 of the 44 compounds (84%) were resolved which is more than on one of

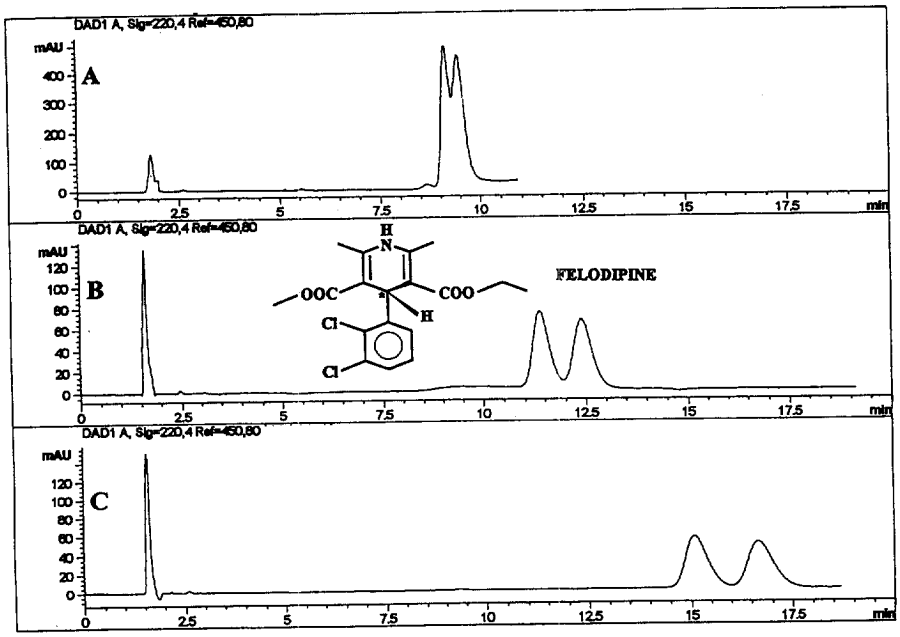


Fig. 3. pSFC optimisation for felodipine. (A) Gradient SFC conditions see text (B) gradient SFC conditions at 5% (5 min) with 1%/min modifier (C) isocratic at 5% modifier.

the individual columns, illustrating the versatility of this approach, but less than the 87% on the individual columns together. Some solutes e.g. ketamine exhibited indeed a reversed elution order on the individual columns and therefore coeluted on the tandem.

The same SFC conditions as for Chiralpak AD and Chiralcel OD were applied for the analysis of the 44 enantiomeric pairs on Chirobiotic T and Chirobiotic V and the data are shown in Table 1. To elute some of the racemates, the percentage modifier had to be increased to 40%. Of the 44 compounds on Chirobiotic T (22) and on Chirobiotc V (21) out exhibited resolutions equal to or greater than 0.4 while this number is 30 on the two phases together. Compared to Chiralpak AD and Chiralcel OD, the macrocyclic antibiotics show less applicability in pSFC although salmeterol which was not at all separated on Chiralpak AD and Chiralcel OD is baseline resolved on Chirobiotic V. We have also observed that retention on the macrocyclic antibiotics under the same SFC conditions is in general twice as long as on the polysaccharide phases while the efficiency is about half as high i.e. mean apparent efficiency in the gradient run 11 600 plates and 5500 plates on polysaccharides and antibiotics, respectively. Note that the polysaccharide CSPs had a d_p of 10 μm, while the antibiotics were linked to 5-μm silica particles.

If resolution is equal to or higher than 0.4 the same optimisation procedure as described for Chiralpak AD and Chiralcel OD can be applied. This is illustrated with the analysis of an asymmetric silicon compound, which is not included in the list, on Chirobiotic V (Fig. 4) and with tropic acid (compound 42) exhibiting a R_s^G value of 0.6 on Chirobiotic T (Fig. 5).

The silicon compound was analysed subsequently on Chiralpak AD, Chiralcel OD, Chirobiotic T and Chirobiotic V and only the last provided separation under the standard gradient as shown in Fig. 4A. Reducing the modifier program from 5%/min to 0.5%/min resulted in the baseline separation shown in Fig. 4B. The situation was more complicated in the case of tropic acid (Fig. 5A). Changing the modifier program did not yield improved resolution but replacing methanol by isopropanol gave the separation shown in Fig. 5B.

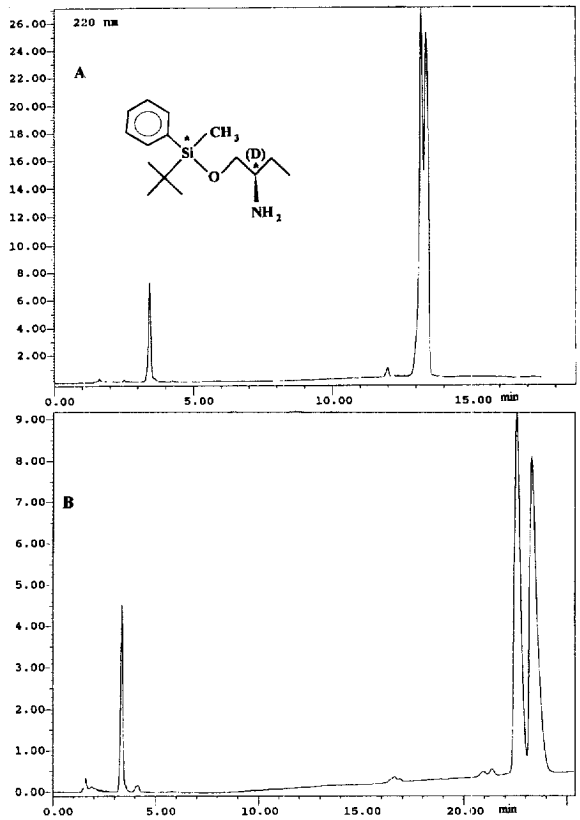


Fig. 4. pSFC optimisation for an asymmetric Si compound on Chirobiotic V. (A) Gradient SFC conditions see text (B) gradient SFC conditions at 5% (5 min) with 0.5%/min modifier.

3.3. Chirex 3022 and Chirex 3005

The brush or Pirkle phases have shown very high selectivity and versatility in LC operation in which, depending on their design, they can be used in the reversed phase, the normal phase or the polar organic phase mode. Chirex 3022 is usually used in the normal phase mode, whereas Chirex 3005 gives the highest enantioselectivity in the polar organic phase mode. Consequently, the best results in pSFC are expected with Chirex 3022. This is evidenced from the data in Table 1. The same SFC program was used as for the other CSPs. On Chirex 3022 15 and on Chirex 3005 9 out of the 44 compounds exhibit resolutions equal to or greater than 0.4 while this number is 23 on the two phases together. For all racemates with the exception of amlodipine (peak 4) better results are obtained on the previously dis-

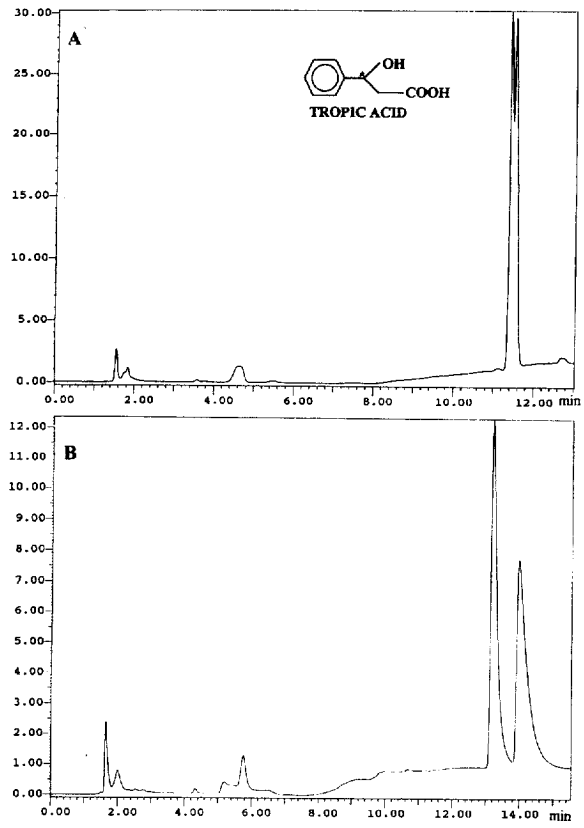


Fig. 5. pSFC optimisation for tropic acid on Chirobiotic T. (A) Gradient SFC conditions see text (B) gradient SFC conditions with isopropanol instead of methanol.

cussed CSPs. pSFC optimisation was therefore not carried out. Retention on the brush type columns is similar to the retention on the macrocyclic antibiotic CSPs but compared to the polysaccharide and antibiotic CSPs the efficiency is substantially higher i.e. mean apparent efficiency in the gradient run 17 500 plates.

3.4. pSFC of amino acids and derivatives

Many LC methods have been developed to separate amino acid enantiomers and without any doubt, pSFC cannot compete with LC for the analysis of those solutes. Nevertheless it is interesting to evaluate the possibilities of pSFC in this respect because this can provide insight in the possibilities of pSFC and at the same time indicate the limits of pSFC for the analysis of very polar solutes. A selection of our most important data is presented.

Camel et al. [39] described the separation of some underivatised amino acids by pSFC on diol phases. High concentrations of a modifier composed of methanol–water–triethylamine and pyridine, ethylene glycol or glycerol (87.95:7:0.05:5, v/v) had to be added to carbon dioxide on the one hand to solubilize the solutes and on the other hand to elute the compounds with good peak shape in a reasonable elution time. Underivatised amino acids are very well separated on Chirobiotic T with methanol or ethanol–water as mobile phase [33]. This brought us to the idea to separate underivatized amino acids by pSFC on Chirobiotic T by applying the mobile phase composition described by Camel et al. [39]. Chirobiotic T is a very retentive stationary phase and the modifier concentration had to be increased to 40% to elute the compounds. The pSFC separation of tyrosine and tryptophan is shown in Fig. 6A and B.

For comparison both solutes were analysed by LC using a water–ethanol (60:40, v/v) mixture. The resolution obtained by pSFC was slightly higher than in LC; for tyrosine 5.6 versus 4.2 and for tryptophan 4.6 versus 3.8, respectively.

SFC separation of derivatized amino acids and especially of PITC and FMOC derivatives on bare silica [40] and cyanopropyl silica [41,42] has been described. A high modifier content was needed to dissolve the derivatives. pSFC was unsuccessfully evaluated by us for the separation of tosylated and dansylated amino acid enantiomers because of solubility reasons. The best results in pSFC were obtained on Chiralpak AD with the trinitrophenyl-, benzoyl-, 3-nitrobenzoyl, 4-nitrobenzoyl and 3,5-dinitrobenzoyl derivatives. Methylation of the carboxylic function had an important influence on the enantioselectivity for benzoylated amino acids.

Table 2 presents the data on the separation of a number of N-trinitrophenyl derivatized amino acid racemates using the pSFC programmed conditions. All enantiomeric pairs give a R_s^G value larger than 0.5 which means that baseline separation is possible for all of them by fine-tuning the SFC conditions.

Concerning the benzoylated derivatives, no gener-

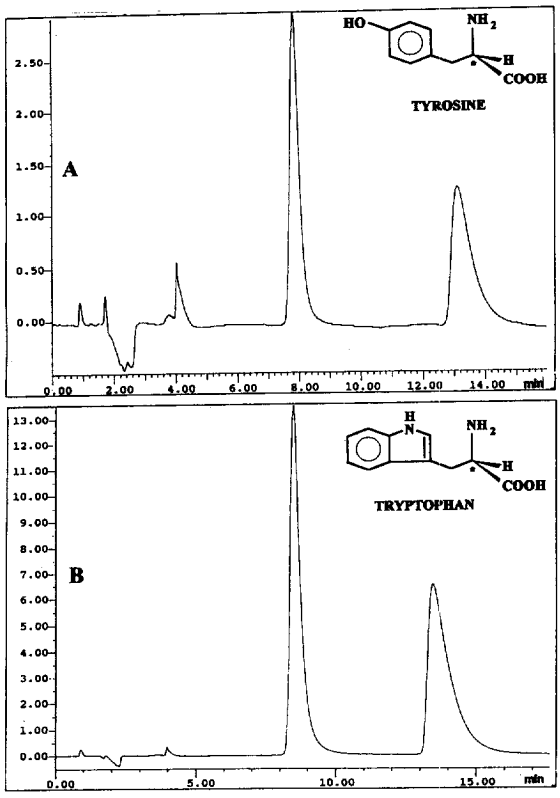


Fig. 6. pSFC separation for underivatized tyrosine (A) and tryptophan (B) on Chirobiotic T. Experimental conditions: pressure 200 bar, column temperature 30°C, flow-rate 2 ml/min, modifier: methanol–water–glycerol (92.8:7.0:0.2, v/v) with 0.1% TEA and 0.1% TFAA, 40% modifier isocratic.

Table 2
Apparent retention factors k^G and resolution values R_s^G for some amino acids as trinitrophenyl derivatives on Chiralpak AD

Amino acid	Derivative (TNF fx1)	
	k^G	R_s^G
Alanine	5.8	1.3
Asparagine[a]	7.4	4.7
Aspartic acid	6.1	0.6
Glutamic acid	7.4	0.5
Histidine	7.4	0.5
Isoleucine	5.7	0.6
Leucine	5.3	2.3
Methionine	6.2	2.6
Norleucine	4.6	1.3
Norvaline	4.7	1.2
Phenylalanine	6.0	2.0
Selenium ethionine	5.9	2.3
Selenium methionine	5.7	3.5
Serine	6.4	0.7
Threonine	5.4	1.6
Tryptophan	7.0	1.7
Tyrosine	6.3	2.2
Valine	4.7	1.6

Gradient SFC conditions see text.
[a] Dibasic amino acids show excessive tailing.

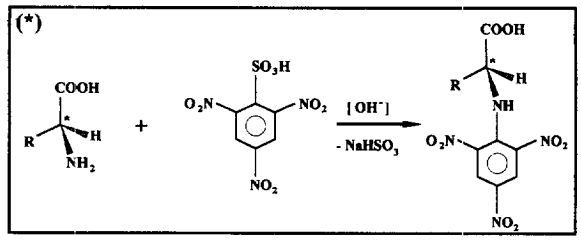
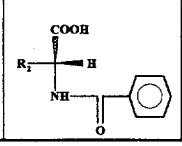
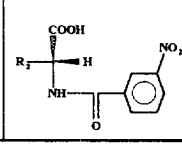
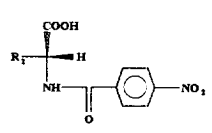
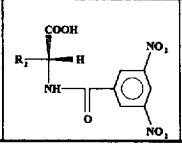
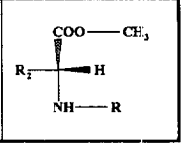


Table 3
R_s^G values for different derivatives of Ala, Aba and Leu on Chiralpak AD

Amino acid	Symbol	Derivative[a]							
		R_s^G							
		Bz	Bz, OMe	3-NBz	3-NBz, OMe	4-NBz	4-NBz, OMe	3,5-DNBz	3,5-DNBBz, OMe
Alanine	Ala	2.2	2.0	1.4	6.6	1.9	8.5	2.8	10.0
Aminobutyric acid	Aba	2.4	1.7	1.0	2.9	2.6	11.1	0.0	6.1
Leucine	Leu	4.7	2.2	4.7	7.8	6.5	8.6	5.2	6.5

Gradient SFC conditions see text.
[a]

Derivative	Bz	3-NBz	4-NBz	3,5-DNBz	OMe
Structure					

al rules can be advanced on which derivative gives the highest enantioselectivity. The differences in function of the selected derivative on the selectivity are remarkable. This is illustrated with alanine (Ala), aminobutyric acid (Aba) and leucine (Leu) as examples (Table 3).

The formation of non-substituted benzoyl derivatives (π-donor) gives good enantioseparation for Ala, Aba and Leu. Methylation slightly decreases resolution. For mono-nitro substituted derivatives, the enantioselectivity is higher for para- than for meta-substitution, while methylation drastically increases resolution. This is illustrated for Ala in Fig. 7. In this particular case, the elution order is even reversed upon methylation.

The formation of dinitrobenzoyl derivatives can destroy enantioselectivity as observed for Aba while high selectivity was obtained for the methyl esters (Fig. 8). The above examples illustrate once more how difficult it is to predict enantioseparations.

3.5. Considerations on stability and lifetime of CSPs in pSFC

An important aspect of pSFC is the stability and

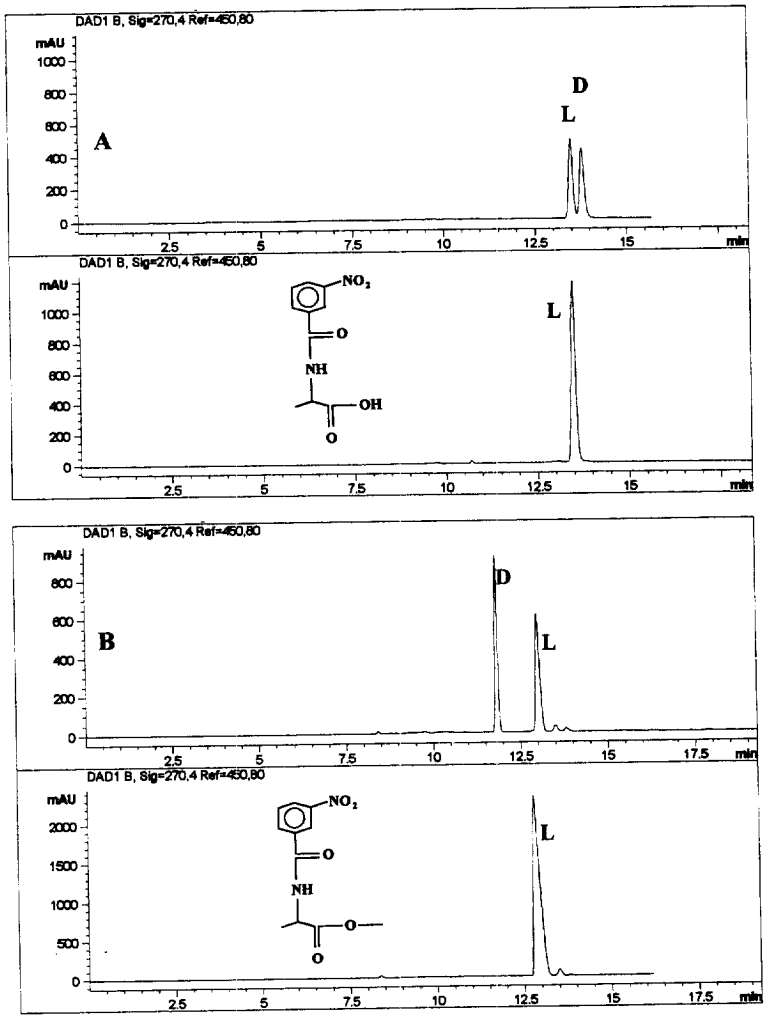


Fig. 7. pSFC separation of N-4-nitrobenzoyl Ala (A) and N-4-nitrobenzoyl methyl ester Ala (B) on Chiralpak AD. Gradient SFC conditions see text.

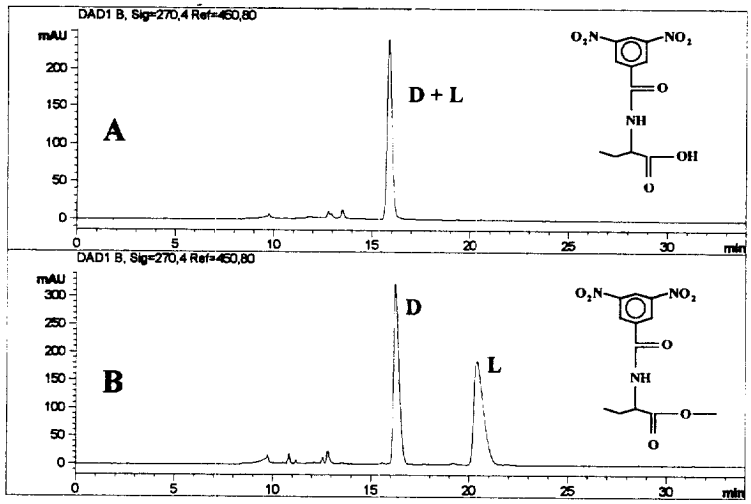


Fig. 8. pSFC separation of N-3,5-dinitrobenzoyl Leu (A) and N-3,5-dinitrobenzoyl methyl ester Leu (B) on Chiralpak AD. Gradient SFC conditions see text.

lifetime of the CSP columns. The functionalised cellulose and amylose coated on silica and the bonded brush type phases tolerate the high flow-rates and outlet pressures extremely well. For all experiments performed in the last three years, only one column of each CSP was purchased. The chromatographic performance of the columns Chiralpak AD, Chirex 3022 and Chirex 3005 is still intact. Four months ago, the Chiralcel OD column exhibited severe tailing after semi-preparative experiments. The column was opened and 0.5 cm of the inlet packing was replaced with home synthesized phase [9], a procedure which restored its initial performance. The reproducubility on the four CSPs was evaluated for several chiral separations over a nine-month period and R.S.D. (%) values on resolution were less than 3. More than 3000 pSFC separations were carried out on the Chiralpak AD and Chiralcel OD columns. Moreover, equilibration times after re-installation are very fast, typically 30 min. Information about the stability of Chiralcel OD in pSFC in an industrial environment is given in [4]. Chirobiotic T and Chirobiotic V have not yet been so intensively used. We have noted, however, that both columns needed an extremely long equilibration time (at least 6 h) in pSFC after use in reversed-phase LC.

4. Conclusion

pSFC is a good alternative to LC for the separation of racemates that dissolve in methanol or a less polar solvent. Based on the results of our studies, a strategy could be devised for optimizing chiral separations by pSFC. Chiralpak AD and Chiralcel OD are recommended for initial experiments. The starting mobile phase is composed of carbon dioxide with 5% methanol containing 0.1% trifluoroacetic acid (TFAA) and 0.1% triethylamine (TEA). A modifier gradient from 5% (5 min) to 30% at 5%/min is applied. A temperature of 30°C and a pressure of 200 bar at a flow-rate 2 ml/min are used. Further optimisation of the initial experiment is done by (a) reducing the modifier gradient or working at 30% modifier, (b) replacing methanol by ethanol, isopropanol, propanol, or eventually mixing with acetonitrile, and (c) varying the temperature by ±10°C. If the separation cannot be performed on Chiralpak AD or Chiralcel OD, the order of preference for the other CSPs is Chirobiotic T, Chirobiotic V, Chirex 3022 and Chirex 3005. Presently we are evaluating the Whelk-O 1 and polyWhelk-O columns [29,30] in pSFC for the same racemates and hope to report soon.

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