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Review

Chiral separations performed by supercritical fluid chromatography

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ABSTRACT

Supercritical fluid chromatography is an attractive alternative for chiral separations of compounds which are not readily analyzed by gas chromatography. It offers fast separations, high-efficiency, low-temperature analysis and access to a wide range of detectors including the "universal" flame ionization detector. In this article the state of the art is reviewed. The technique is compared with other chromatographic techniques and the applicability of different chiral stationary phases as well as the choice of experimental conditions is discussed.

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1. INTRODUCTION

Supercritical fluid chromatography (SFC) is defined as a chromatographic technique in which the mobile phase is subjected to pressures and temperatures above or just below the critical point, the latter case often being referred to as subcritical fluid chromatography (SubFC). Further, the mobile phase should possess solvating

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power under these conditions and thereby contribute to the selectivity of the chromatographic system. By varying the temperature or pressure it is possible to change the nature of the mobile phase from gas-like to liquid-like conditions within a single chromatographic run and thereby SFC will provide a continuum between gas (GC) and liquid chromatography (LC). SFC is usually divided into two categories, open-tubular column and packed column, representing complementary ways of using the SFC technique. The former offers high efficiencies, mild conditions and the possibility of using a large number of different detectors, whereas the latter offers high selectivity, fast elution and the possibility of analysing highly polar solutes.

SFC is a technique that possesses important advantages for the separation of enantiomers in comparison with LC and sometimes also GC. As concluded in previous reviews [1,2], SFC often provides a higher resolution per unit time than LC as the diffusion rates in the mobile phase and consequently also the optimum linear velocities are higher (Fig. 1) [3–7]. The separations are generally performed at temperatures well below

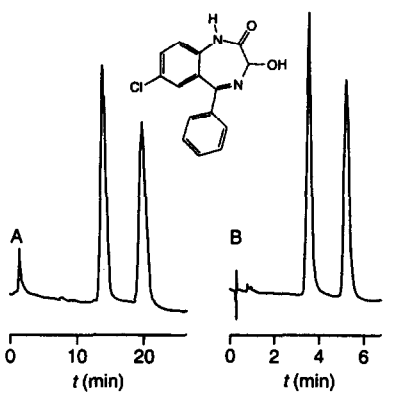

Fig. 1. Separation of (±)-oxazepam at constant resolution under (A) LC and (B) SubFC conditions illustrating the higher resolution per unit time for the latter technique (subFC refers to SFC mobile phases used at conditions just below the critical point). Packed column, 150 mm × 4.6 mm I.D.; CSP derived from (*S*)-N-(3,5-dinitrobenzoyl)tyrosine-*n*-butylamide. (A) LC–UV. Conditions: hexane–ethanol (90:10, v/v), 25°C, flow-rate 2 ml min^{-1}, detection at 230 nm. (B) SubFC–UV. Conditions: carbon dioxide–ethanol (92:8, w/w), 25°C, flow-rate 6 ml min^{-1} at 0°C, average column pressure 200 bar, detection at 229 nm. From ref. 48.

those required for GC analysis, an important consideration since the chiral selectivity decreases with increasing temperature in both techniques [8–10]. Analysis at low temperatures also reduces the probability of racemization and thermal decomposition of chiral stationary phases (CSPs) and analytes.

The mechanism for the separation of different enantiomers is not fully understood. For example, at the same temperature, it has been suggested that the selectivity should be lower in SFC than in GC owing to the solvation of the chiral selector [11–13]. On the other hand, other studies have shown similar or larger chiral selectivities in SFC than in GC even for an analogous combination of stationary phase and analyte [10–14]. In general, however, it should be possible to obtain a higher degree of chiral selectivity in SFC than in GC as the retention is easily controlled by the density and/or the composition of the mobile phase. The temperature can therefore be regarded as a free variable which can be kept at a favourable level. The use of supercritical fluids as mobile phases may also extend the workable temperature range towards lower temperatures by a softening or swelling of the stationary phase which results in a higher efficiency in SFC than in GC at the same low temperature [14,15], although the efficiency in general is higher in GC.

Another important aspect when considering the choice of separation technique is the range of detectors available. With selected mobile phases there are more detection principles compatible with SFC than for any other chromatographic technique. Most detection methods developed for LC and GC, including flame ionization detection (FID) and mass spectrometry (MS), are therefore available for SFC. Fig. 2 shows one example of how MS can be a valuable tool for the verification of the peak identity in a chiral separation. The total ion current of a suitable mass range is monitored during the elution of the sample (Fig. 2A) and the peak identity is conveniently verified by matching of the mass spectra from each peak (Fig. 2B). After obtaining the mass spectra of the peaks of interest, it is possible, as shown in Fig. 2C, to improve the signal-to-noise ratio considerably by employing

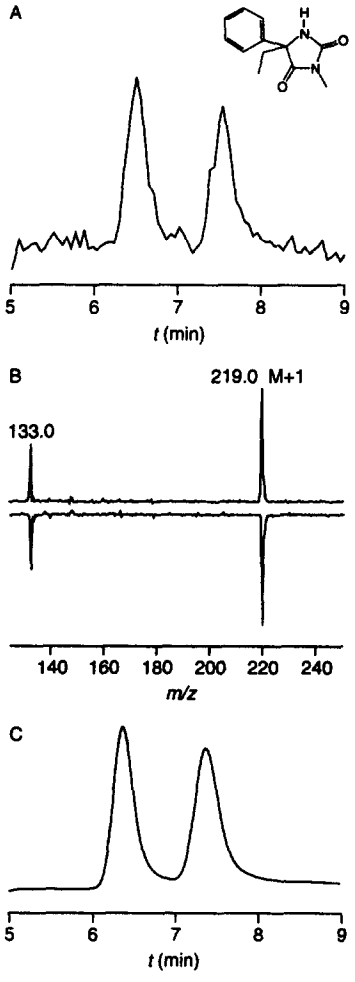

Fig. 2. SFC–MS quantification and verification of peak identity. (A) Total ion current for m/z between 125 and 250 monitored during the elution of the sample, (±)-mephenytoin. (B) Verification of peak identity by matching of mass spectra. (C) Selected ion monitoring, $m/z = 133$ and 219. Open-tubular column: 2.5 m × 50 μm I.D., copolymeric poly[(1*R*)-*trans* - N,N'-1,2 - cyclohexylenebisbenzamide]-oligoalkylsiloxane, $d_f \approx 0.25$ μm. Conditions: carbon dioxide, 60°C, density programmed from 0.20 to 0.58 g ml^{-1} at 0.50 g ml^{-1} min^{-1} after a 1-min isopycnic period. From ref. 17.

selected ion monitoring. This improves the determination of enantiomeric purity not only by increasing the signal-to-noise ratio but also by eliminating the signals from other compounds that may co-elute.

For the reasons described above, SFC should be well suited for the separation of non-ionic chiral compounds with low to medium volatility. This is reflected in the increased interest in chiral separations performed by SFC. During the period 1985–90 one to five papers were published each year on this topic, whereas during 1991 and 1992 the number increased to over ten per year. When chiral columns that are especially made for SFC are commercially available, this increase should be even more pronounced.

2. CHIRAL STATIONARY PHASES

Most types of chiral stationary phases developed for LC and GC have been used in SFC with or without modification with good results. There is, however, one important exception in the protein-based CSPs frequently used in LC, which have not yet been evaluated for the use with supercritical fluids.

Usually CSPs are classified on the basis of the type of chiral selector used, *e.g.*, CSPs based on cyclodextrins or amides. The chromatographic properties of the CSP are influenced not only by the type of chiral selector used but also by the amount of the chiral selector and the type of the achiral part of the CSP.

For CSPs based on cyclodextrins, it has been found, for example, that the chiral selectivity increases asymptotically with increasing amount of chiral selectors in the CSP [16,17] up to a certain limit, *i.e.*, 20–30% (w/w) cyclodextrin, above which little or no chiral selectivity is gained, which has been explained by theoretical models for the relationship between the chiral selectivity and the amount of cyclodextrin in the CSP [18]. For CSPs based on amides it has been found that the chiral selectivity may even decrease at high substitution ratios owing to stacking interactions between the chiral selectors hampering the chiral selectivity [19]. A high substitution ratio should also result in lower efficiencies as the CSP becomes more rigid. One advantage with the use of high-percentage chiral substitution should, however, be larger sample capacity.

The achiral part of the CSP is often as important for the performance of the CSP as the chiral part. It should provide a high diffusion rate within the stationary phase to ensure good

efficiency and also impart chemical and thermal stability. Further, it has to be compatible with the chiral part to ensure homogeneity in the preparation and should not add additional sites that act in a competitive way with the chiral recognition or give increased retention of the analytes [19,20]. To fulfil these requirements, different siloxane polymers are usually employed as the achiral part of the CSP.

Owing to the solvating power of the supercritical mobile phase it is necessary, in contrast to GC, to immobilize the stationary phase. In packed-column SFC this is generally no problem as the chiral selector is covalently bonded to the packing material via a spacer, in analogy with the preparation of LC columns. Alternatively, the CSP consists of a rigid, high-molecular-mass polymer, insoluble in the mobile phase, which has been coated on the packing material. In open-tubular column SFC, the CSP is immobilized by thermal or radical-initiated cross-linking, as has been done successfully for achiral columns [21]. However, in the case of CSPs, the presence of the chiral selector often disturbs the cross-linking and it becomes necessary to incorporate in the non-chiral part of the CSP groups that facilitate the cross-lining, *e.g.*, octyl, vinyl or tolyl groups [21,22].

2.1. Open-tubular columns

The open-tubular columns used for chiral separations in SFC generally have an inside diameter (I.D.) between 50 and 100 μm and a length ranging from 2.5 to 20 m. The question has arisen of whether it is possible to fulfil the requirement of detecting enantiomeric impurities at levels less than 1% using 50 μm I.D. columns as the sample capacity is limited to *ca.* 50–250 ng of each analyte. Using SFC–FID this is in fact possible, provided that baseline resolution is obtained. Fig. 3 illustrates such an application in which the separation of the enantiomers of the sedative dihydrodiazepam was performed with a 1.0% impurity of the (−)-isomer.

The film thickness of the stationary phase (d_f) is usually between 0.15 and 0.25 μm, but it has been reported [23] that it is possible to use film thicknesses of up to 1.0 μm in order to increase

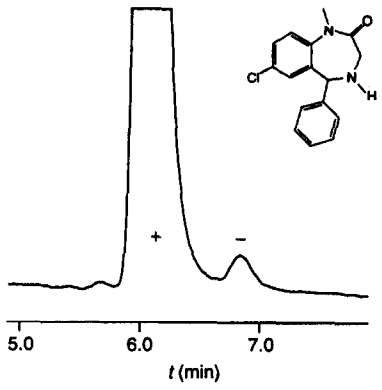

Fig. 3. SFC–FID of the (+)-form of the sedative dihydrodiazepam with a 1% impurity of the (−)-form. Open-tubular column: 5 m × 50 μm I.D., side-arm-substituted methyloctylsiloxane having permethylated β-cyclodextrin as the chiral selector, $d_f \approx 0.25$ μm. Conditions: carbon dioxide, 85°C, density programmed from 0.20 to 0.52 g ml^{-1} at 0.50 g ml^{-1} min^{-1} after a 2 min-isopycnic period. From ref. 17.

the sample capacity without a significant loss of efficiency.

Today carbon dioxide is the most commonly used mobile phase for open-tubular column SFC owing to its low critical temperature and pressure, compatibility with most detectors, low toxicity and low cost. To our knowledge, pure carbon dioxide without modifiers is the only mobile phase that has been used for chiral separations carried out with open-tubular column SFC. The drawback associated with the use of carbon dioxide is that very polar compounds such as ions and primary amines are difficult or impossible to elute.

Among the different types of CSPs used in open-tubular column SFC, four approaches can be identified: (i) side-arm-substituted siloxanes [24], *i.e.*, the chiral selector is attached to a siloxane polymer via a spacer in analogy with classical CSPs developed for GC, *e.g.*, **1** (Fig. 4); (ii) copolymeric siloxanes [25], *i.e.*, CSPs consisting of alternating chiral and achiral blocks, a strategy which may result in a chiral secondary structure and thereby improved chiral selectivity, *e.g.*, **6**; (iii) mixed phases [26], *i.e.*, mixtures where one chiral part provides selectivity and one achiral part facilitates the formation of a uniform film that it is possible to cross-link; and (iv) immobilizable chiral selectors [27], *i.e.*,

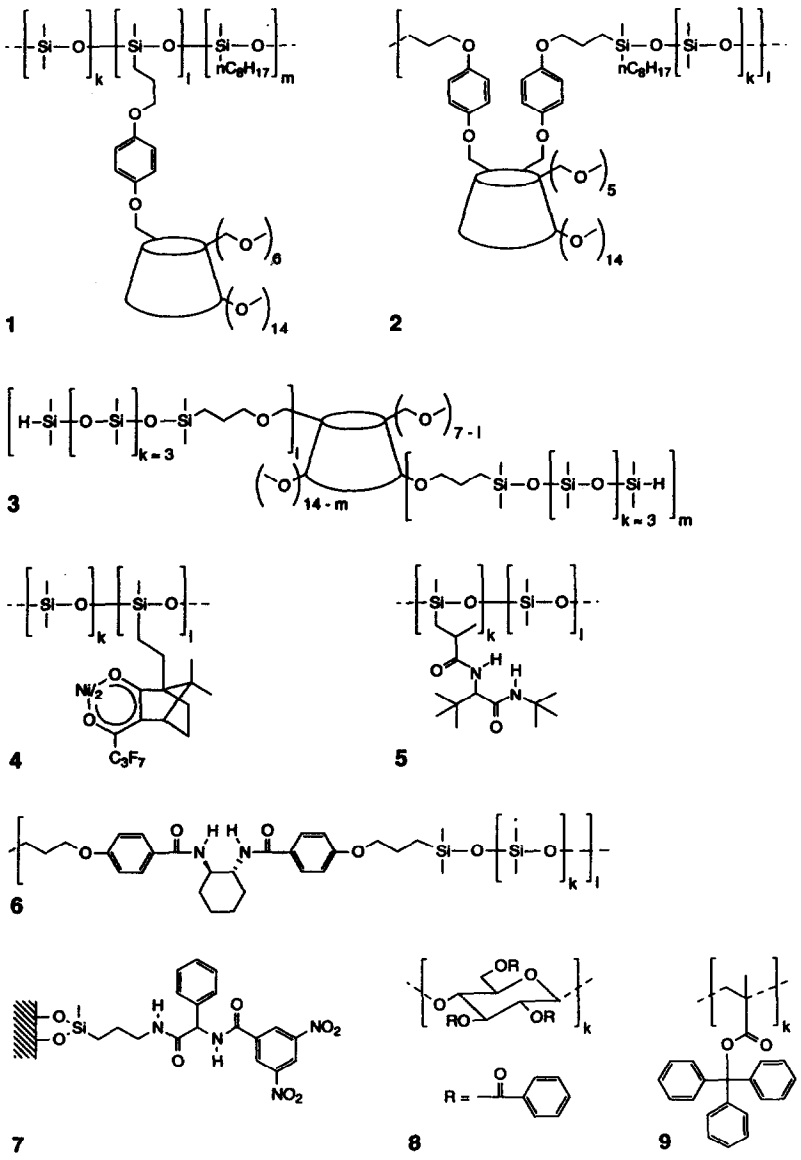

Fig. 4. Structures representing different types of CSPs. 1–3 = CSPs based on cyclodextrins; 4 = CSPs based on metal complexes; 5–7 = CSPs based on amides or amino acids; 8 = CSPs based on polysaccharides; 9 = CSPs based on polymethacrylates of helical conformation.

chiral selectors having one or several short methylsiloxane polymers as substituents that it is possible to coat and use as CSPs after immobilization, *e.g.*, 3.

Röder *et al.* [24] reported the first example of a chiral separation performed by open-tubular column SFC in 1987. The enantiomers of several derivatized amino acids were separated using a CSP based on an amino acid derivative. Since then, the use of a number of different types of CSPs has been reported.

2.1.1. CSPs based on cyclodextrins

The cyclodextrin phases are, to date, the most widely applicable CSPs for chiral separations performed by SFC. Enantiomers of a wide range

of compounds with different functionalities, *e.g.*, mono alcohols, diols, lactones, ketones, amines, carboxylic acids and steroids, have been efficiently separated using either a side-arm-substituted, *e.g.*, **1** [12,13,28–31], or a copolymeric, *e.g.*, **2** [32,33], CSP based on permethylated β-cyclodextrin. Also underivatized enantiomers of relatively large and polar compounds of pharmaceutical and agricultural interest have been separated using these CSPs, *e.g.*, ibuprofen (see Fig. 15), ketoprofen, cicloprofen, flurbiprofen, naproxen, warfarin (Fig. 5), acenocoumarol, hexobarbital, *cis*-permethrinic acid, 10,2-camphorsultam, dihydrodiazepam (Fig. 3) and norgestrel. Recently, Armstrong *et al.* [27] reported the use of a CSP consisting of an immobilizable methylated β-cyclodextrin having short methylsiloxane polymers as substituents (**3**). The applicability of this type of CSP in open-tubular column SFC was illustrated by the separation of the enantiomers of 1-aminoindan and perhydroindole.

2.1.2. CSPs based on metal complexes

Chiral separations based on complexation chromatography, *i.e.*, the coordination of compounds having π- or lone-pair electrons to chiral metal complexes, was extended to SFC by Schurig and co-workers [13,29] through the synthesis of side-arm-substituted siloxane polymers having metal complexes as chiral selectors, *e.g.*, **4**. The combination of CSPs based on metal complexes with SFC may prove to be a fruitful combination as the use of this type of CSP is restricted to temperatures below 140–150°C [34]. In addition, it has been shown that larger selectivities are obtainable in SFC than in GC at the same temperature, probably owing to solvation effects (see below).

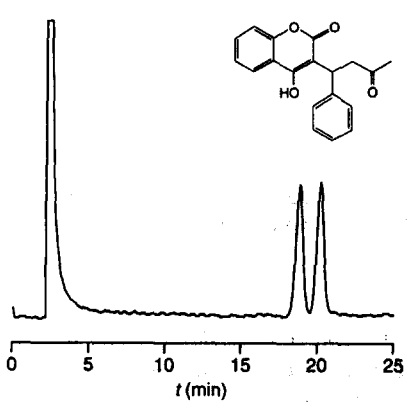

Fig. 5. SFC–FID of the separation of (±)-warfarin using a CSP consisting of a copolymeric permethyl β-cyclodextrin methyloctylsiloxane. Open tubular column: 5 m × 50 μm I.D., $d_f \approx 0.25$ μm. Conditions: carbon dioxide, 70°C, density programmed from 0.50 to 0.62 g ml^{-1} at 0.005 g ml^{-1} min^{-1} after a 1-min isopycnic period. From ref. 17.

2.1.3. CSPs based on amides or amino acids

Among these CSPs there are several so-called Pirkle phases, *i.e.*, side-arm-substituted CSPs having chiral selectors with π-electron-accepting or π-electron-donating functional groups such as 3,5-dinitrobenzamide or napthylamino acid esters [24,35–38]. This type of CSP has, with few exceptions, been used for the separation of derivatized amino acids. A CSP which has been studied by several groups is the Chirasil-Val phase (**5**) [10,11,39], a side-arm-substituted siloxane having L-valine-*tert*.-butylamide as the chiral selector originally developed for GC by Frank *et al.* [40] in 1977. This CSP has also mainly been used for the separation of derivatized amino acids. Other examples of CSPs based on amides are the copolymeric phases consisting of alternating chiral (cyclohexylenebisbenzamide) and achiral (methylsiloxane) blocks, *e.g.*, **6**, described by Petersson and co-workers [14,22,25,41,42]. These CSPs have proved to be suitable for the separation of enantiomers of underivatized diols in particular, of which some examples are shown in Fig. 6, but also for compounds such as *trans*-stilbene oxide and mephenytoin (Fig. 2).

2.1.4. CSPs based on polysaccharides

The use of this type of chiral selector has previously been restricted to packed-column LC and SFC as the cellulose derivatives are rigid high-molecular-mass polymers not suitable as stationary phases in open-tubular column chromatography. However, it is possible to coat open-tubular columns with a mixture of an achiral polymer and a cellulose derivative, as described by Juvancz and co-workers [26,43,44], and thereby combine the chiral selectivity of the cellulose derivative and the efficiency of a more

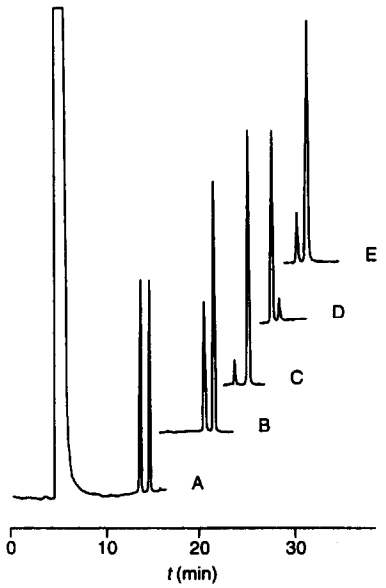

Fig. 6. SFC–FID of enantiomers of diols with different shapes and functionality using an open-tubular column (5 m × 50 μm I.D., $d_f \approx 0.20$ μm) coated with a copolymeric poly[(1*R*)-*trans*-N,N'-1,2-cyclohexylenebisbenzamide]-methylsiloxane and carbon dioxide as the mobile phase. Test compounds and conditions: (A) (±)-diethyl tartrate, 60°C, density programmed from 0.30 to 0.40 g ml^{-1} at 0.01 g ml^{-1} min^{-1} after a 5-min isopycnic period; (B) methyl-*cis*-1,2-dihydroxycyclohexane-1-carboxylate, 60°C, density programmed from 0.30 to 0.50 g ml^{-1} at 0.01 g ml^{-1} min^{-1} after a 5-min isopycnic period; (C) ethyl (2*R*,3*S*)- and (2*S*,3*R*)-dihydroxyoctanoate, 50°C, density programmed from 0.30 to 0.50 g ml^{-1} at 0.01 g ml^{-1} min^{-1} after a 5-min isopycnic period; (D) 3,3-dimethyl-1,2-butanediol, 55°C, density programmed from 0.20 to 0.55 g ml^{-1} at 0.01 g ml^{-1} min^{-1} after a 5-min isopycnic period; (E) methyl (2*R*,3*S*)- and (2*S*,3*R*)-dihydroxy-3-phenylbutanoate, 50°C, density programmed from 0.30 to 0.60 g ml^{-1} at 0.01 g ml^{-1} min^{-1} after a 5-min isopycnic period. From ref. 14.

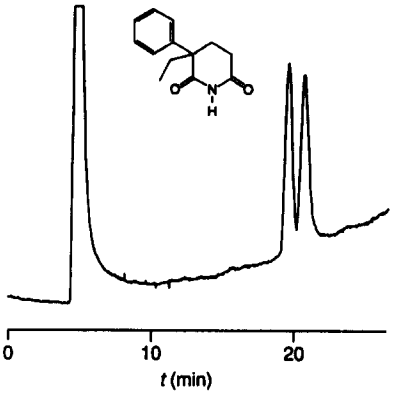

Fig. 7. SFC–FID separation of (±)-gluthetimide using a *m*-methylbenzoyl derivative of cellulose mixed with a methylphenylsiloxane as CSP (5:95, w/w). Open-tubular column: 10 m × 50 μm I.D. Conditions: carbon dioxide, 70°C, density programmed from 0.40 ml^{-1} at 0.005 g ml^{-1} min^{-1} after a 5-min isopycnic period. From ref. 44.

flexible siloxane polymer. The applicability of these CSPs in SFC has been illustrated by the separation of polar aromatic compounds such as 1-(4-phenyl)phenylethanol, glutethimide (Fig. 7) and 2-phenylpropionic acid phenylamide using benzoyl derivatives of cellulose (**8**) as the chiral selector.

2.2. Packed columns

Chiral separations by packed-column SFC or SubFC are usually performed by the use of commercially available LC columns having an I.D. of 4.6 mm and a length of 150–250 mm. The lower viscosity of the supercritical fluid should, however, permit the use of both longer and narrower columns packed with smaller particles than what is normally possible in LC. This was recently shown by Berger and Wilson [45], who used up to eleven LC columns (200 mm × 4.6 mm I.D. packed with 5-μm silica particles) coupled in series and thereby obtained a total efficiency of 220 000 plates under supercritical conditions.

The packing material in chiral packed-column SFC usually consists of silica particles having a diameter of 5–10 μm to which the chiral selector has been covalently anchored or, alternatively, on which a chiral polymer, insoluble in the mobile phase, has been coated. The use of silica as packing material requires the addition of polar modifiers to the mobile phase as the number of free silanol groups, even with end-capping, is significant and thus may result in undesirable interactions and long retention times. The former conclusion is supported by a study in which it was found that end-capping results in an increased chiral selectivity [20]. The polar modifier, usually an alcohol, competes with the analytes in their interactions with the silanol groups and hence suppresses such interactions. Unfortunately, the critical temperature and pressure of

the mobile phase increase as a modifier is added and the calculation of the mobile phase density becomes less straightforward. Further, modifiers are not generally compatible with FID (with the possible exception of water and formic acid). There is consequently a need for the development of inert packing materials which would decrease the demand for polar modifiers and extend the applicability of packed-column SFC.

The first example of chiral separations performed by SFC and SubFC was reported by Mourier *et al.* [46] in 1985. They used a commercially available Pirkle-type LC column and mobile phases composed of carbon dioxide and alcohols or water for the separation of phosphine oxides. In this and other studies in which the chromatographic properties of chiral LC columns were compared under LC and SubFC conditions, it was concluded that SubFC provides, for a given retention time, significantly improved resolution or, for a given resolution, significantly shorter retention times, often one third to one tenth of the retention time required in LC. These results, a decreased consumption of organic solvents and the fact that often it is possible to use UV detection at shorter wavelengths than are normally possible in LC should make the replacement of normal-phase LC with SubFC attractive.

2.2.1. CSPs based on cyclodextrins

LC columns based on α-, β- and γ-cyclodextrin and an acetylated β-cyclodextrin have been used for the separation of enantiomers of aromatic amides, aromatic phosphine oxides, α-methylene-γ-lactone, oxazepam, etc., under SubFC conditions [47,48]. In these studies it was found that the different modifiers evaluated, methanol, ethanol and 2-propanol, give the same resolution, but also that increasing polarity of the modifier results in better peak shapes and shorter retention times, *i.e.*, methanol should be a more favourable modifier than ethanol.

2.2.2. CSPs based on amides or amino acids

The dominant type of CSP used in packed-column SFC or SubFC is undoubtedly π-electron-accepting CSPs derived from N-(3,5-dinitrobenzoyl)amino acids, *e.g.*, **7**, (Pirkle phases) for which charge-transfer interactions between π-electron-accepting and -donating groups play an important role in the chiral recognition mechanism [3,7,6,9,46,48–52]. The enantiomers of compounds having aromatic groups, *e.g.*, phosphine oxides, sulphoxides, lactams and benzodiazepines (Fig. 1), can be directly separated using this type of CSP. More polar compounds such as amines and carboxylic acids generally require a conversion into less polar derivatives prior to injection (Fig. 8). Recent studies by Tambuté and co-workers [6,7] showed that it is even possible to separate the underivatized enantiomers of 1,2-amino alcohols (β-blockers) directly by SubFC (Fig. 9). The investigation of this result by LC, SubFC, NMR and molecular modelling suggest that the carbon dioxide acts as a complexing agent which forms a bridge between the hydroxyl and amine group of the analyte and gives rise to a less polar and more rigid complex that may be responsible for the chiral discrimination. Other CSPs in this group are either Pirkle phases having π-electron-donating groups [20,53] or CSPs based on amino acids whose chiral discrimination is mainly based on the formation of hydrogen bonds [54–56]. For this group of CSPs the efficiency often decreases and the selectivity often increases with increasing size and/or decreasing polarity of the modifier

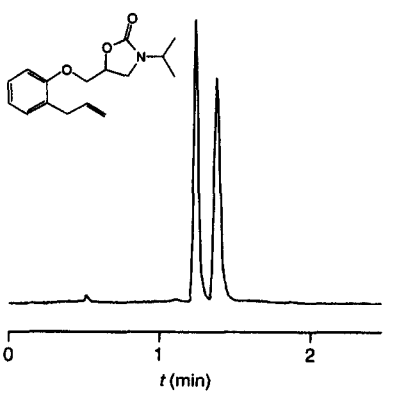

Fig. 8. SFC–UV separation of a (±)-alprenolol derivative using a packed microbore column. Packed column: 150 mm × 1.2 mm I.D., 5-μm silica particles, CSP derived from a 3,5-dinitrobenzoyl derivative of (1*R*,2*R*)-diaminocyclohexane. Conditions: carbon dioxide–dioxane–methanol (70:15:15, v/v/v), 50°C, flow-rate 360 μl min^{-1}, average column pressure 128 bar. From ref. 49.

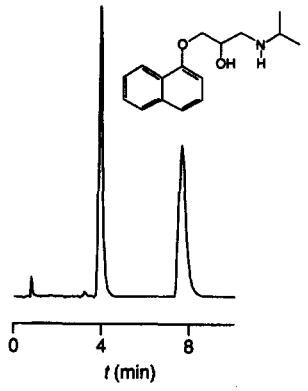

Fig. 9. Direct separation of the enantiomers of the β-blocker propranolol using SubFC–UV. Packed column: 150 mm × 4.6 mm I.D., 5-μm silica particles, CSP derived from N-(3,5-dinitrobenzoyl)tyrosine. Conditions: carbon dioxide–methanol containing 1% (v/v) *n*-propylamine (80:20, v/v), 25°C, flow-rate 4 ml min^{-1}, detection at 224 nm, average column pressure 200 bar. From ref. 6.

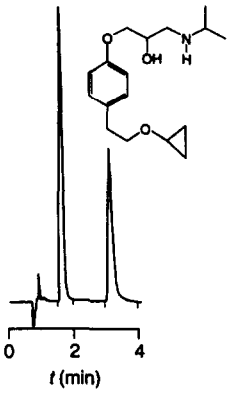

Fig. 10. Direct separation of the enantiomers of betaxolol using SubFC–UV. Packed column: 250 mm × 4.6 mm I.D., 10-μm silica particles coated with cellulose tri(3,5-dimethylphenylcarbamate). Conditions: carbon dioxide–methanol (80:20, v/v), 35°C, flow-rate 4 ml min^{-1}, detection at 254 nm, average column pressure 200 bar. From ref. 5.

[3,7,9,46,56]. The addition of a small amount of water to the organic modifier has also been found to improve both efficiency and selectivity, in addition to shortening the retention times [3,9,46].

2.2.3. CSPs based on polysaccharides

Derivatives of cellulose and amylose coated on silica particles, normally used for LC, have been successfully used for the separation of enantiomers of compounds with different functionality. Cellulose tribenzoates (**8**) have, for instance, been used for the separation of α-methylene-γ-lactams and lactones, aromatic amides, 3-thienylcyclohexyl glycol acid and esters, 1,2-amino alcohols (β-blockers) (Fig. 10), etc. [5,48,57], whereas cellulose triphenylcarbamates and amylose triphenylcarbamate have been used for the separation of *trans*-stilbene oxide, benzoin, flavanone, etc. [58,59]. It has been found that higher chiral selectivities are obtainable with branched alcohols (see Fig. 14) as modifiers, but the use of short linear alcohols results in higher efficiencies [48,57,58].

2.2.4. CSPs based on polymethacrylates of helical conformation

These CSPs, whose chirality is a result of a helical secondary structure, have been designed for the separation of enantiomers having aromatic substituents. Macaudière *et al.* [48] used bi-β-naphthol and α-methylene-γ-lactone as model substances for an investigation of how the amount and type of organic modifier influence chiral separations performed under SubFC conditions using (+)-polytriphenylmethyl methacrylate (**9**) coated on silica particles. It was concluded that among the investigated modifiers, methanol, ethanol, 2-propanol and acetonitrile, an increasing polarity resulted in higher chiral selectivity whereas increased amounts of modifier in the mobile phase resulted in decreasing selectivity for the lactone and an increased selectivity for the bi-β-naphthol.

3. CHIRAL MOBILE PHASES AND CHIRAL DERIVATIZATION

The use of chiral stationary phases is undoubtedly the most favourable chromatographic method for the determination of enantiomeric purity. Nevertheless, it is sometimes necessary to use the alternatives, a chiral mobile phase or derivatization, to form diastereomers. The following example of the latter approach illustrates well the advantages of SFC over GC for the analysis of thermally labile compounds. The enantiomers of a precursor to an HIV-inhibiting drug could not be separated using chiral open-

tubular column GC and the enantiomers were therefore converted into diastereomeric derivatives which subsequently were analysed by non-chiral GC. However, as these compounds proved to be thermally labile (Fig. 11A), the separation was finally performed using SFC and a non-chiral open-tubular column at relatively low temperature (Fig. 11B) [60]. One advantage connected with the use of chiral derivatization is the possibility of changing the elution order of the diastereomeric derivatives by using the (+)-form of the derivatization reagent instead of the (−)-form, or *vice versa*, thereby improving the measurement. There are, of course, also drawbacks with chiral derivatizations as the reaction must be quantitative, the enantiomeric purity of the derivatization reagent must be high and known and racemization of the analyte during the reaction must be avoided.

The use of chiral mobile phases in SFC has been described by Steuer *et al.* [61], who applied ion-pairing methods ordinarily used in LC for the separation of 1,2-amino alcohols (β-blockers) as diastereomeric ion pairs under SubFC or SFC conditions. This ion-pairing method extends the use of open-tubular column SFC to the separation of ions and primary amines, compounds which normally cannot be eluted with this technique using carbon dioxide as the mobile phase.

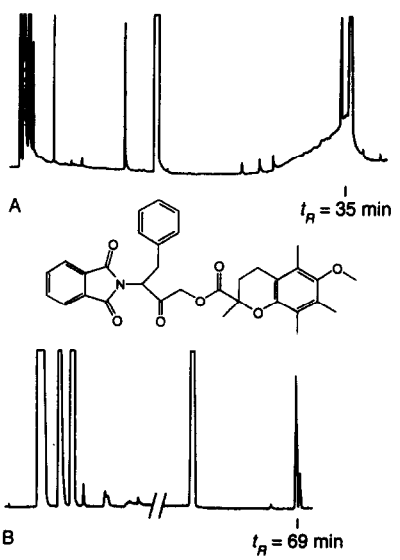

Fig. 11. (A) GC– and (B) SFC–FID of thermally labile diastereomeric derivatives of a precursor to an HIV-inhibiting drug. (A) Open-tubular column: 15 m × 320 μm I.D., cyanopropylmethylsiloxane. Conditions: hydrogen, temperature programmed from 150 to 320°C at 3°C min^{-1}. (B) Open-tubular column: 10 m × 50 μm I.D., biphenylmethylsiloxane. Conditions: carbon dioxide, 100°C, density programmed from 0.20 to 0.75 g ml^{-1} at 0.0075 g ml^{-1} min^{-1}. From ref. 60.

4. PREPARATIVE SEPARATIONS

A direct preparative chromatographic separation of enantiomers provides an alternative to asymmetric synthesis or recrystallizations of diastereomeric salts for the preparation of enantiomerically pure compounds. At present, most preparative separations of enantiomers are still carried out by LC. The use of SFC or SubFC may, however, result in higher production rates as the resolution per unit time in general is better in SFC. SFC should also facilitate the elimination of the solvent as the major part of the supercritical fluid, *e.g.*, carbon dioxide, is simply removed by depressurization.

The sample capacities of the preparative separations that have been performed by SubFC were of the order of 10–100 mg per solute [62–64]. This relatively low sample capacity can fortunately be compensated for by the short retention times and the use of repetitive injections (*e.g.*, Fig. 12). This strategy has been shown to allow a production rate of up to 750 mg h^{-1} [63].

5. CHOICE OF EXPERIMENTAL CONDITIONS

In SFC several variables, temperature, mobile phase composition and density of the mobile phase, have a large influence on retention, selectivity and resolution. Hence the choice of suitable experimental conditions becomes essential.

In several studies it has been concluded that the retention, expressed in terms of the capacity factor, k', decreases with increasing temperature at constant density, *i.e.*, $\ln k' \propto 1/T$ [65–68]. There is, consequently, an analogous expression for the dependence of chiral selectivity on temperature, *i.e.*, $\ln \alpha \propto 1/T$. If the slopes and intercepts of such plots are interpreted in terms

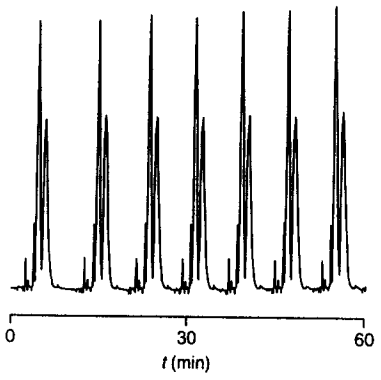

Fig. 12. Preparative SubFC–UV separation of the enantiomers of a "Pirkle reagent" performed by repetitive injections. Packed column: 250 mm × 9 mm I.D., 5-μm silica particles, CSP derived from N-(3,5-dinitrobenzoyl)phenylglycine. Conditions: carbon dioxide–2-propanol (1.48 ml min^{-1}), 28°C flow-rate 8 ml min^{-1}, detection at 290 nm, average column pressure 260 bar. From ref. 62.

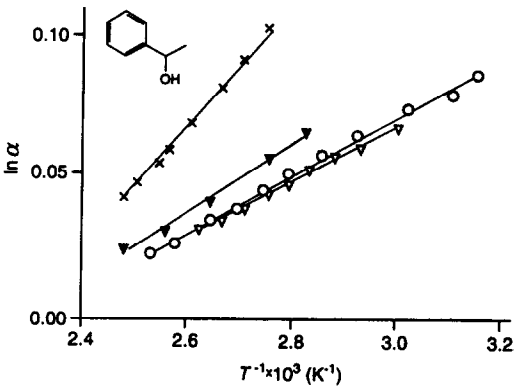

Fig. 13. Influence of temperature and pressure on chiral selectivity for the SFC–FID separation of (±)-1-phenylethanol on a CSP based on side-arm-substituted methylsiloxane having permethylated β-cyclodextrin as the chiral selector. Open-tubular column: 10 m × 100 μm I.D. Conditions: carbon dioxide at various temperatures and pressures: × = 1 (GC–FID, nitrogen), ▼ = 55, ○ = 77 and ▽ = 100 bar. From ref. 13.

of entropy and enthalpy variations associated with the retention process [8], one would expect co-elution of the enantiomers at high temperatures. At even higher temperatures the chiral selectivity should increase again after a reversal of elution order. This theory is supported by a limited number of chiral separations performed by GC [69–71], but to our knowledge not with SFC. In other words, from a practical point of view the chiral selectivity should decrease with increasing temperature, as mentioned in the Introduction (Fig. 13).

The capacity factor decreases with increasing density of the mobile phase at constant temperature as a result of an increased solvation of the analyte, *i.e.*, $\ln k' \propto \ln \rho$ [72]. In general, the chiral selectivity also decreases with increasing density (Fig. 13). One exception has been reported, however, namely the separation of the enantiomers of 1-phenylethanol on a CSP based on a nickel complex. In this case the chiral selectivity increased with increasing density under the experimental conditions investigated [13]. This increase in chiral selectivity may be explained by a coordination of carbon dioxide molecules which accentuates the differences in stability of the diastereomeric complexes formed by the analytes.

According to Foley and Crow [73], it should be possible to describe the influence of the amount of organic modifier in the mobile phase, χ, on the retention at constant temperature and density by a second-order polynomial, *i.e.*, $\ln k' \approx c_0 + c_1\chi + c_2\chi^2$, where the c represent solute- and system-specific constants and χ is expressed as % (w/w), % (v/v) or mole fraction. There are no general rules for how the type of organic modifier influences retention and chiral selectivity, as this depends on the type of CSP and analyte in question [3,7,9,46,48,56–58]. Fig. 14 gives some examples of how the chiral selectivity changes with amount and type of organic modifier in the mobile phase at constant pressure and temperature for the separation of an aromatic amide on a CSP based on cellulose. The maximum at low alcohol content can be explained by increased competition from modifier molecules at higher alcohol content in the interaction with the chiral selectors.

As relatively small changes in temperature or density can result in large changes in retention and resolution [73,74], it would be desirable to have access to retention time and peak width models that can aid in the optimization of these variables. Such models have recently been proposed for separations performed by non-programmed [74] and programmed open-tubular column SFC [75].

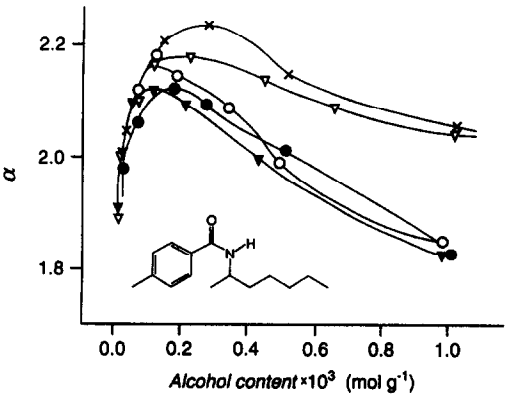

Fig. 14. Chiral selectivity as a function of type and amount of modifier in the mobile phase for the SubFC–UV separation of (±)-N-(2-heptyl)-*p*-tolylamide. Packed column: 250 mm × 4.6 mm I.D., 10-μm silica particles coated with cellulose tribenzoate. Conditions: carbon dioxide and various types and amounts of modifiers: ● = methanol, ○ = ethanol, ▼ = 1-butanol, × = 2-propanol and ▽ = 2-butanol; 25°C, flow-rate 4.5 ml min^{-1} at 0°C, detection at 229 nm, average column pressure 140 bar. From ref. 48.

For practical reasons, it would be preferable to use non-programmed conditions for the separation of enantiomers as fewer experiments are required in order to determine the parameters of the models and as the algorithms are faster and easier to implement in this case [76]. Fig. 15 shows one representative example of the agreement between calculated and experimental chromatograms for the enantiomers of ibuprofen under optimum conditions, *i.e.*, conditions resulting in baseline separation within the shortest possible time.

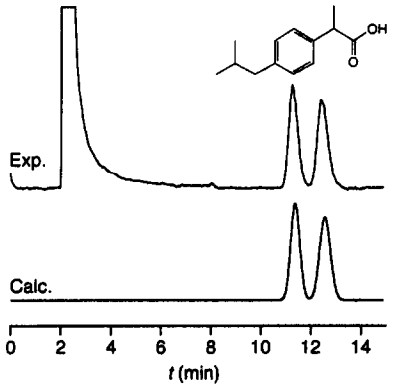

Fig. 15. (A) Experimental and (B) calculated chromatograms for the SFC–FID separation of (±)-ibuprofen under optimum conditions. Open tubular column: 5 m × 50 μm I.D., side-arm-substituted methylsiloxane having permethylated β-cyclodextrin as the chiral selector, $d_f \approx 0.25$ μm. Conditions: carbon dioxide, 60°C, isopycnic at 0.485 g ml^{-1}. From ref. 17.

In order to increase both resolution and signal-to-noise ratio, it is advisable to start both non-programmed and programmed separations with a focusing step in which the peak width is decreased via an injection at low density and a subsequent increase to optimum conditions after a short isopycnic period [75–77]. Such a focusing step often results in a 20–30% decrease in the peak width for non-programmed separations.

Other multivariate optimization strategies suggested for the optimization of temperature and density in separations performed by open-tubular column SFC are based on a modified simplex algorithm [78], overlapping resolution mapping [79] or the method of steepest ascent [42]. These strategies, however, usually require a relatively large number of experiments and/or the definition of response functions combining retention and resolution.

To our knowledge, no multivariate optimization strategies have been suggested for the use of organic modifiers or packed-column SFC. In the latter instance, it might be explained by the fact that not only do the linear velocity and efficiency change with altered temperature and density, as in open-tubular column SFC, but also the situation becomes even more complicated as there is a considerable pressure drop over the column, which results in different linear velocities and efficiencies at different parts of the column.

6. CONCLUSIONS

It can be concluded that chiral separation by SFC can offer faster separations than LC, milder and sometimes more selective separations than GC, reduced sample preparation procedures in comparison with GC and a larger number of detection methods than in both LC and GC. SFC should therefore seriously be considered for the separation of non-ionic chiral compounds of low to moderate molecular mass that are not readily analysable by GC owing to thermal instability or

lack of volatility or that are undesirable to derivatize.

Open-tubular columns are preferred for analytes soluble in carbon dioxide whereas packed columns should be used for analytes of higher polarity. At present, however, no chiral open-tubular columns are commercially available and the packed columns available have been designed for LC. It is therefore important that chromatographers demand the availability of chiral columns specially designed for these techniques.

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