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Review

Enantioseparations in counter-current chromatography and centrifugal partition chromatography

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Abstract

Examples of chiral separations in counter-current chromatography (CCC) and centrifugal partition chromatography (CPC) are not numerous, due to the difficulty of finding chiral selectors highly selective in the liquid phase as well as a combination of solvents that does not destroy the selectivity and retains the capacity to elute chiral isomers of interest. New ideas and new chiral selectors generally come from other separation techniques, as will be highlighted in this review.

Keywords: Reviews; Enantiomer separation; Counter-current chromatography; Centrifugal partition chromatography; Chiral selectors; Vancomycin; Albumin; Cyclodextrins; *N*-Dodecanoyl-L-proline-3,5-dimethylanilide

Contents

1. Introduction

A total of 18 papers in 18 years, including those dealing with the 'CPC chiral reactor' which is not chiral chromatography stricto sensu, constitutes a modest output compared with the literature for other chiral separation techniques. Moreover, only three or four efficient chiral selectors are found in these 18 papers, as compared to hundreds for HPLC, GC or CE! A review of the literature shows that chiral

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separations are very often characterized by a separation factor, α, in the 1.05 to 1.3 range. Counter-current chromatography and centrifugal partition chromatography (CCC&CPC), although powerful preparative techniques because of their high capacity, low cost of stationary phases (i.e. solvent mixtures) and low solvent consumption (10 times less than for HPLC), show rather poor efficiency (more than 1000 theoretical plates are seldom found). For this reason, they need to be used with a separation factor greater than, say, 1.4. The major difficulty is to find chiral selectors highly selective in the liquid phase as well as a combination of solvents that does not destroy this selectivity and retains the capacity to elute chiral isomers of interest. Since the CCC&CPC community constitutes a small group, new ideas and new chiral selectors will generally come from other separation techniques, and we would like to highlight the origin of use of the three chiral selectors which have proved most efficient in chiral CCC&CPC, and which are described in this review:

- *N*-Dodecanoyl-L-proline-3,5-dimethylanilide was introduced by Oliveros, who was among the first to synthesize a chiral L-proline bonded silica for HPLC in 1979 and has a thorough knowledge of chiral chromatography. He extrapolated certain data of Pirkle and innovated with a simple method of synthesizing *N*-dodecanoyl-L-proline-3,5-dimethylanilide.
- Sulfated β-cyclodextrin was introduced by Breinholt after remarkable results were obtained in capillary electrophoresis (a resolution factor greater than 100 or, alternatively, a world record 10 s separation time) and then had to be applied to preparative needs.
- Vancomycin was introduced by Margraff, a specialist in the purification of natural products, who was impressed by Armstrong's experiments in HPLC with vancomycin and other chiral macrocyclic antibiotics grafted to silica.

These three scientists shared the same idea, i.e. to borrow a chiral selector from different separation techniques that displayed high selectivity and then to determine whether it would remain robust for chiral CCC&CPC.

We will describe here the few, but relevant, examples found in the literature, and we will take advantage of this review to develop some analytical considerations.

2. CCC&CPC: basic theory, methods and instrumentation

Modern counter-current chromatography originates from the pioneering studies of Ito et al. [1], this first machine leading to two main applications: one based on a wide variety of 'CCC apparatus' using a variable-gravity field produced by a two-axis gyration mechanism and a rotary seal-free arrangement for the column; and the other based on 'centrifugal partition chromatography (CPC) apparatus' and using a constant-gravity field produced by a single-axis rotation mechanism and two rotary-seal joints for inlet and outlet of the mobile phase. Several studies have been published [2–6] concerning examples of separations in various fields. Basically, CCC and CPC refer to support-free liquid–liquid chromatography with two immiscible liquids prepared by mixing two or more solvents or solutions. The instrument keeps one liquid stationary while the other is pumped through it, and the chromatographic process occurs between the two liquid phases.

Most applications are based on liquid–liquid partition between two liquid phases, so that solute retention depends on one parameter only, the distribution ratio, D:

$$V_R = V_M + DV_S$$

where V_R, V_M, and V_S are the solute retention volume, the mobile phase volume, and the stationary phase volume inside the CCC 'column', respectively. The distribution ratio, D, is the ratio of the total analytical concentration of a solute in the stationary phase (regardless of its chemical form) to its total analytical concentration in the mobile phase.

An advantage of CCC&CPC is that the volume ratio of the stationary phase to the total volume is much higher than that found in chromatography with solid support. Direct consequences of this better balance between the two phases in CCC&CPC are (1) the range of partition coefficients useful for CCC&CPC purification is centered around 1, and (2) the capacity of the CPC column is high when compared with an HPLC column with the same total volume.

Ito's classification distinguishes two basic types of instruments:

1. The hydrodynamic equilibrium system (HDES).

The column (or bobbin) is made by winding PTFE tubing around a cylindrical holder hub to make multiple layers of coils. The holder revolves around the central axis of a centrifuge and simultaneously rotates around its own axis at the same angular velocity. The motion of the coil causes vigorous agitation of the two solvent phases and a repetitive mixing and settling process, ideal for solute partitioning, occurs at over 13 times per second. These instruments are commonly called CCC instruments.

2. The hydrostatic equilibrium system (HSES). This system differs from the HDES system in that the column units are fixed in a centrifuge and do not themselves rotate, i.e. they only have a single axis. The 'column' consists of channels connected in series by ducts, engraved on disks or cartridges, and arranged around the rotor of a centrifuge with their longitudinal axes parallel to the direction of the centrifugal force. These instruments are commonly called CPC instruments.

The selection of a two-phase solvent system for CCC&CPC is similar to the choice of a column and an eluant for HPLC. Most often, optimization of a separation involves optimization of chromatographic selectivity, and it is precisely here that CCC&CPC has the most to offer. Both phases are directly accessible, and their compositions can be fine-tuned to achieve the desired resolution [2–6].

Modern CCC instruments are manufactured by Pharmatec [Baltimore, MD, USA (http://www.pharma-tech.com/)], Conway Centrichrom [Buffalo, NY, USA (http://www.centrichrom.com/Index.htm)], AECS (Bridgend, UK), and SEAB [Villejuif, France (http://www.kromaton.com/)]. Modern CPC instruments are manufactured by Sanki (Kyoto, Japan), and SEAB.

3. Chiral CCC: the pioneers

To our knowledge, the first attempt to resolve a pair of enantiomers was in 1982 in Switzerland by Hostettmann and Prelog [7]. Based on good results obtained by liquid–liquid partition [8], these researchers used (*R,R*)-di-5-nonyltartrate as a chiral selector to partly resolve (±)-norephedrine as its hexafluorophosphate salt. A 1,2-dichloroethane–water system was used, with the chiral selector in the organic mobile phase and sodium hexafluorophosphate in the aqueous stationary phase. Although no baseline separation was achieved, the researchers showed that practically pure enantiomers could be obtained by CCC. The instrument was a rotation locular counter-current chromatograph (RLCCC), which showed poor efficiency and was extremely slow (4 days for a run). It would be interesting to redo these experiments with a modern instrument.

The second attempt, in Japan in 1984, was a transposition to CCC of ligand exchange chromatography, a technique quite popular at the time (see the contribution of Kurganov in this issue). Taking their inspiration from Davankov [9], Takeuchi et al. [10] synthesized *N*-*n*-dodecyl-L-proline (C_{12}-PRO), which remains in the organic phase of the *n*-butanol–water system. By adding Cu(II) ions, which were extracted in the organic phase as complexes with C_{12}-PRO, they partly resolved several D,L-amino acids by 'ligand exchange CCC'. Baseline resolution was achieved for D,L-isoleucine, but the separation time (2.5 days) was excessive due to the use of a prototype of a droplet counter-current chromatograph made by connecting 400 pieces of Teflon tubing of two sizes alternately in series, which gave a total volume of around 2 L. As for the first attempt, it would be interesting to reinvestigate 'ligand exchange CCC' using recent instruments.

The third and last attempt of pioneer research, concerning the resolution of various substituted bicyclo[2.2.1]hept-5-ene-2-carboxylic acids, took place in 1986 in the USA [11]. Oya and Snyder adopted CCC after several attempts with more traditional methods such as fractional crystallization or esterification to form diastereoisomers (a rather tedious and inefficient approach). (−)-(*R*)-2-Aminobutanol proved to be a powerful chiral selector in conjunction with chloroform–methanol–aqueous phosphate buffer, where it stays in the upper aqueous phase. Resolution was complete using a DCCC instrument with a volume of around 280 mL and a run time of about 2.3 days.

4. *N*-Dodecanoyl-L-proline-3,5-dimethylanilide

Oliveros finalized an easy method of synthesizing *N*-dodecanoyl-L-proline-3,5-dimethylanilide, a chiral

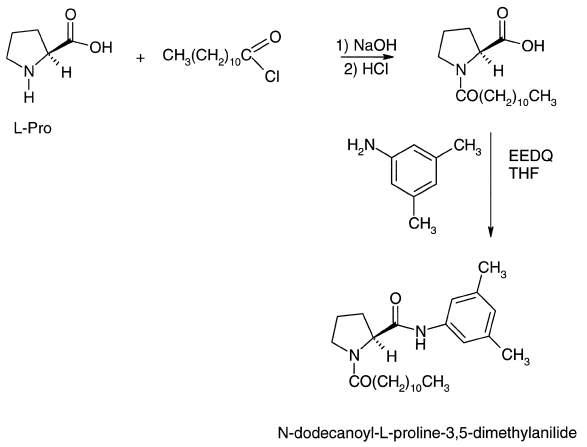

Fig. 1. Synthesis of *N*-dodecanoyl-L-proline-3,5-dimethylanilide. EEDQ, 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline; THF, tetrahydrofuran. Room temperature.

π donor molecule similar to that used by Pirkle [12], and very useful in charge-transfer complexation (Fig. 1) [13]. This π donor allowed the first speedy (80 min) and complete resolution of two neutral DNB-amino-acid derivatives, namely *N*-(3,5-dinitrobenzoyl)-*tert*.-butylvalinamide and *N*-(3,5-dinitrobenzoyl)-*tert*.-butylleucinamide, using the heptane–ethyl acetate–methanol–water (3:1:3:1) biphasic system and a CPC apparatus with a column volume of 240 mL.

This π donor was then used in an authoritative way by Ito et al. to perform complete analytical studies and introduce the pH-zone-refining mode of CCC for chiral discrimination [14–17]. These authors verified that the peak resolution of the racemates was increased by (1) augmenting the net amount or concentration of the chiral selector in the organic stationary phase and (2) adjusting the hydrophobicity of the solvent system so that the mean partition values for the racemates fell between 0.6 and 0.8 (except for the pH-zone-refining mode). They were able to fractionate up to 1 g of DNB-amino acids using a CCC apparatus with a column volume of 330 mL in the classical elution mode, and up to 2 g in the pH-zone-refining mode (vide infra). Fig. 2 shows a nice separation of four DNB-amino-acid racemates using the π donor selector. The instrument, a prototype of a semi-analytical CCC equipped with a set of three column holders for a total volume of 180 mL, was manufactured at the NIH machine shop (a similar apparatus is available from Pharma-Tech Research). One drawback is the rather long time required for experiments, which indicates the need for faster apparatus.

4.1. Chiral separation by pH-zone-refining

Ito's introduction of the pH-zone-refining [18] mode in CCC as a variant of displacement chromatography [14] was highly successful. This approach concerns the purification of compounds whose electric charge depends on pH (HA/A^- or BH^+/B pairs). One characteristic of pH-zone-refining is the release of products from the column by continuous blocks arranged according to their pK_a values and partition coefficients. Another characteristic of the pH-zone-refining mode of CCC&CPC is its powerful preparative capabilities. This has been dramatically demonstrated by Renault et al. [19], who injected 7 g of a crude mixture of *Catharanthus roseus* alkaloids onto a 250 mL CPC column, using HCl as retainer in the aqueous stationary phase and triethylamine as displacer in the organic mobile phase. The four major indole alkaloids were isolated from this crude mixture in one run.

In the absence of a complexing agent in the stationary phase, the separation of the analytes and their elution order are expressed by the following equation:

$$\mathrm{pH} = \mathrm{p}K_a + \log\left(\frac{K_D}{D} - 1\right) \quad (1)$$

where K_D is the partition ratio of the neutral analyte, and D the distribution ratio for the retainer acid or base. For chiral applications, a chiral selector (CS) is added in the stationary phase (assuming that it does not partition in the mobile phase), which complexes the analyte with a complex formation constant $K_{\pm}$. Consequently, Eq. (1) must be modified to:

$$\mathrm{pH} = \mathrm{p}K_a + \log\left(\frac{K_D}{D}(1 + (\overline{CS})K_{\pm}) - 1\right) \quad (2)$$

where $K_{\pm}$ is the complex formation constant of the $+$ or $-$ isomer and $(\overline{CS})$ the concentration of the chiral selector in the stationary phase. Since both the pK_a and K_D values of the two enantiomers are identical and D is governed by the ratio of the

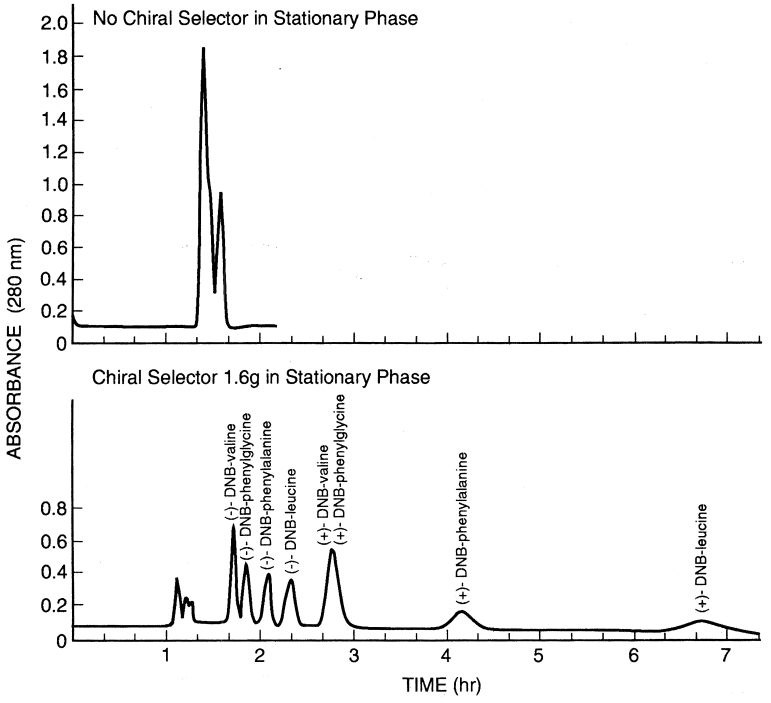

Fig. 2. Separation of four DNB-amino-acid racemates by counter-current chromatography using *N*-dodecanoyl-L-proline-3,5-dimethylanilide as chiral selector. Solvent system, hexane–ethyl acetate–methanol–10 m*M* HCl, 8:2:5:5. CCC column volume, 180 mL; flow-rate, 1 mL/min; rotation, 1000 rpm. Detection, UV 280 nm; sample, 10 mg each of (±) DNB-amino acid. Reproduced, with permission, from Ref. [16].

retainer and of the displacer, the separation of the two enantiomers is governed by the difference between K_+ and K_- and the concentration of CS in the stationary phase. Ma et al. [14] showed the capabilities of chiral separation through pH-zone-refining by using the chiral selector introduced by Oliveros [13] to resolve 2 g of (±) DNB-leucine in about 3 h on a 330 mL CCC column, using trifluoroacetic acid as retainer in the organic stationary phase and ammonia as displacer in the aqueous mobile phase (Fig. 3). Chiral pH-zone-refining for the resolution of ionizable racemates is probably the most promising preparative technique with CCC&CPC.

5. Sulfated β-cyclodextrin

The following example is a very interesting demonstration of the complementarity of CCC with other separation techniques, which indicates what the thinking of scientists not initially involved in CCC can bring to the technique when the scaling up of analytical results has to be done.

Breinholt et al. noted that when ''using sulfated β-cyclodextrin (S-β-CD) as the chiral selector, resolution factors (R_s) greater than 100, or, alternatively, a world-record 10-s separation time, were obtained for the enantiomeric pair of 7-*des*-methylormeloxifene (7-DMO) (**1** and **2**), of which **1** is a major metabolite in vivo of (−)-(3*R*,4*R*)-levormeloxifene (**3**), a partial estrogen receptor agonist'' (Fig. 4) [20,21]. However, this was capillary electrophoresis (CE)! The authors then added: ''an inherent limitation of CE is sample capacity, and the technique lacks in its present technical shape a direct preparative counterpart. ... A fundamental feature of CE (a separation mechanism governed by solution phenomena involving no solid support) is shared by counter-current chromatography (CCC). ... The

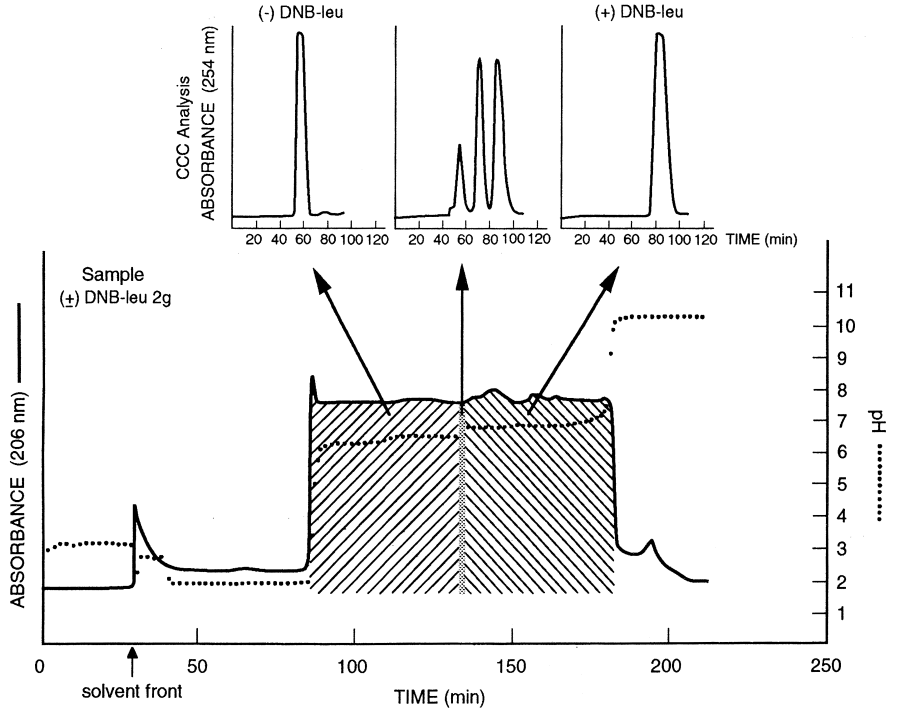

Fig. 3. Separation of (±) DNB-leucine by pH-zone-refining CCC. Solvent system, methyl-*tert*.-butyl ether–water; stationary phase, upper organic containing trifluoroacetic acid (40 m*M*) and *N*-dodecanoyl-L-proline-3,5-dimethylanilide (40 m*M*); mobile phase, lower aqueous containing ammonia (20 m*M*); CCC column volume, 330 mL; flow-rate, 3.3 mL/min; rotation, 800 rpm; detection, pH, and UV 280 nm for the analytical control; sample, 2 g of (±) DNB-leucine. Note that the analysis of the fraction from the mixing zone (middle chromatogram) shows three peaks corresponding from left to right to (−) DNB-Leu, impurity and (+) DNB-Leu. Reproduced, with permission, from Ref. [14].

true strength of CCC lies in its preparative applications, and we envision that the speed of CE, utilized for fast screening of several chiral selectors in order to identify particularly efficient ones, might be nicely complemented by the preparative aspect of CCC'' [21].

The idea is simple and brilliant. Moreover, as sulfated β-cyclodextrin with a degree of substitution of 7–11 is a commercial product (Aldrich 38,915-3) (Fig. 4), other scientists can easily test this powerful chiral selector. In fact, this is the first example in which the CS is in the aqueous phase, a property shared with vancomycin (described later in this review). Fig. 5 shows the influence of solvent composition on resolution. The formation of a 7-DMO–S-β-SC inclusion complex is believed to rely at least partially on hydrophobic interactions, and it is expected that such a complex could be stabilized if the polarity of the aqueous phase was increased, i.e. from (A) to (C) in Fig. 5.

It is to be hoped that this first innovative use of a sulfated-β-cyclodextrin will enlarge the CCC&CPC field for chiral preparative applications.

6. Bovine serum albumin (BSA)

To our knowledge, the first report of chiral separation on an immobilized BSA stationary phase was performed by Stewart and Doherty in 1973 [22]. A significant improvement in column performance and ease of operation was obtained through the introduction of a silica support for the immobilized albumin [23]. However, the low capacity of these chromatographic systems makes them mainly useful for analytical purposes.

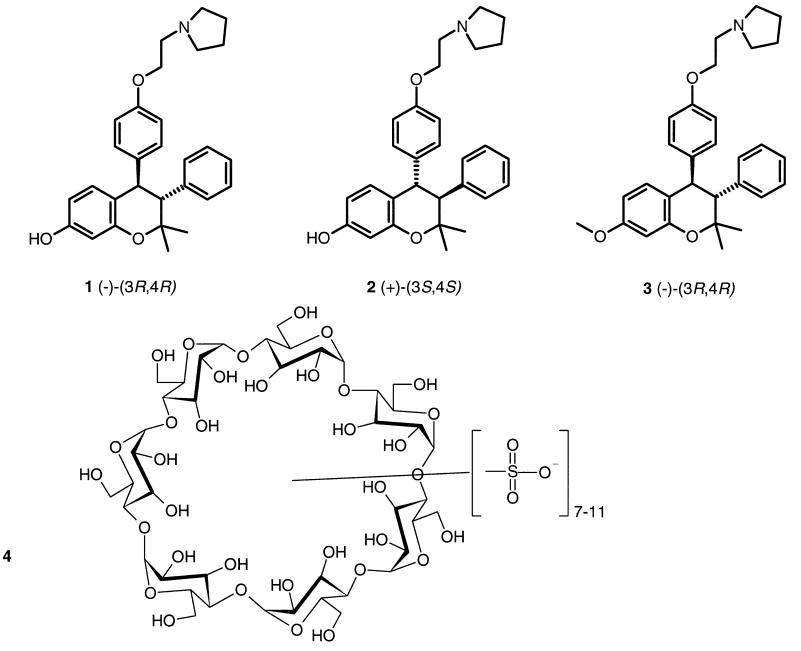

Fig. 4. (**1**) and (**2**): (−)-(3*R*,4*R*)- and (+)-(3*S*,4*S*)-7-*des*-methyl-ormeloxifene; (**3**) (−)-(3*R*,4*R*)-levormeloxifene; (**4**) sulfated β-cyclodextrin (degree of substitution, 7–11).

Partition in an aqueous two-phase system for direct chiral resolution using BSA in one phase has been tried in two ways: counter-current distribution and CCC. The phase system, consisting of two immiscible aqueous phases, is obtained by mixing two polymers, or one polymer and a salt, with water [24]. Under suitable conditions, a protein can be confined almost completely to one of the phases, while low-molecular-weight compounds partition more equally between the phases.

BSA has been used in counter-current distribution to resolve D,L-tryptophan [25] and Ofloxacin [26], and in CCC to resolve commercial D,L-Kynurenine [27]. However, the resolution is poor in each case. It is said that liquid–liquid partition is easy to scale-up and that the aqueous polymer phase system should be of interest for large-scale separation of enantiomers, yet a significant increase in efficiency is required to accomplish this. This is not easy since the polymer phase systems are rather viscous and mass transfer during the chromatographic process is mediocre. Another factor making scale-up difficult is the molecular weight of BSA ($6.6 \cdot 10^4$). Regardless of the stoichiometry of the complex, liquid–liquid partition involving a complexation equilibrium commonly generates Langmuirian isotherms, the plateau of which is governed by the concentration of the complexing molecule (here BSA). Given its molecular weight of $6.6 \cdot 10^4$, a large amount of BSA in one phase does not give a large concentration. That is why CCC and CPC may be of interest for interaction studies between free protein and other molecules rather than for their preparative capabilities, in this specific case.

7. Vancomycin

Macrocyclic antibiotics have recently been introduced by Armstrong et al. as powerful and ‘general’ chiral selectors in liquid chromatography (LC) [28], thin-layer chromatography (TLC) [29] and capillary electrophoresis (CE) [30]. Native or derivatized vancomycin, rifamycin B and thiostrepton have been used in both normal and reversed-phase mode in LC, with acetonitrile–1% triethylammonium acetate buffer, or 2-propanol–hexane [28] as mobile phases. A tremendous variety of racemates has been resolved

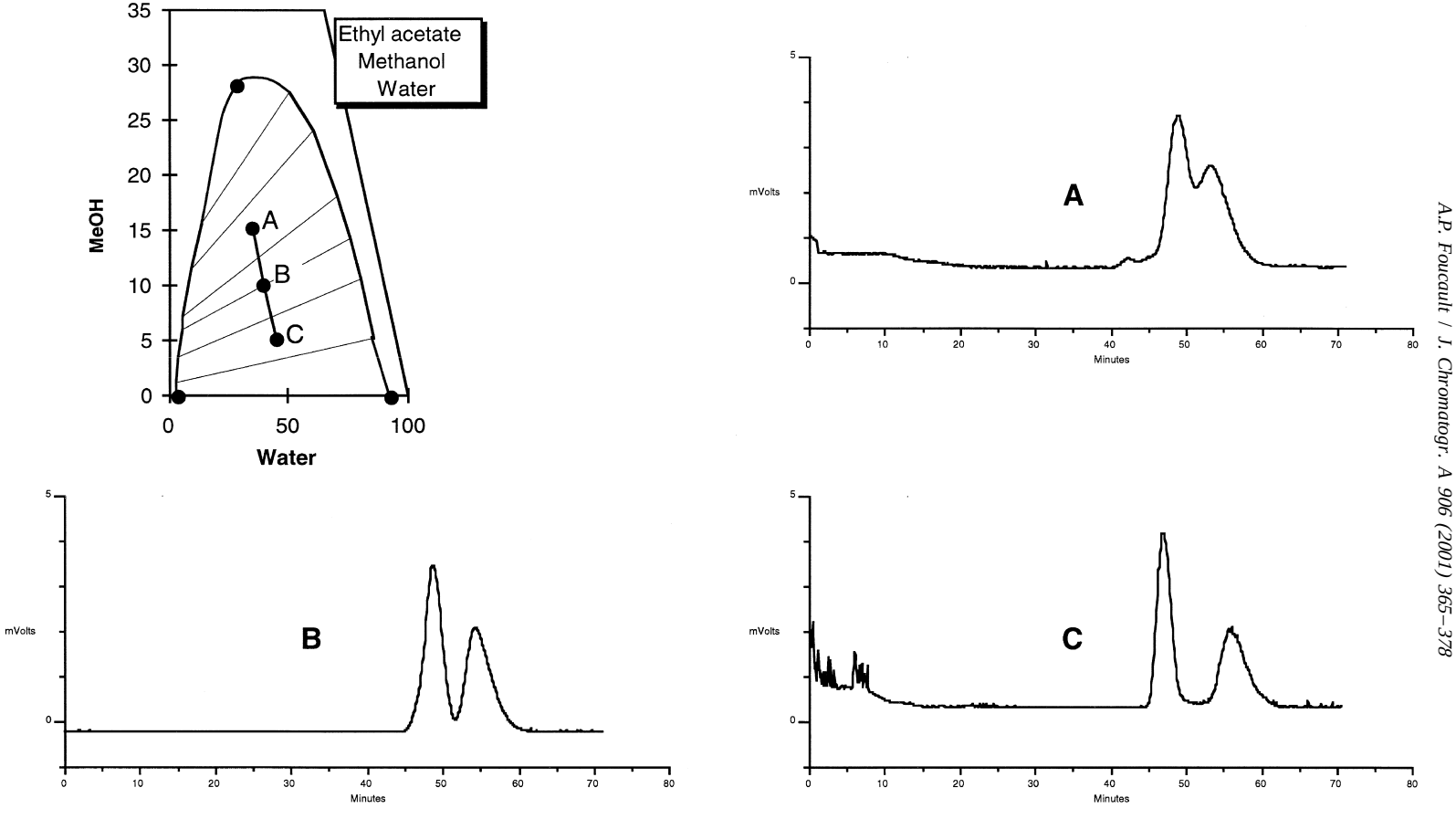

Fig. 5. Resolution of 7-*des*-methyl-ormeloxifene using sulfated β-cyclodextrin as chiral selector. The solvent systems are ethyl acetate–methanol–aqueous triethylammonium acetate, 10:3:7 (A), 10:2:8, (B), and 10:1:9 (C) containing 2% (w/v) S-β-CD in the aqueous stationary phase. The first peak represents the (+)-enantiomer of 7-DMO.

on these chiral stationary phases, such as coumachlor and warfarin, devrinol, 5-methyl-5-phenylhydantoin, various derivatized amino acids, with higher separation factors (α) of 2.42 (coumachlor, pH 3.6). Vancomycin has been used as a chiral additive in a water–acetonitrile mixture to resolve 6-carbamylquinolyl N-protected amino acids (AQC), racemic drugs and dansyl amino acids by TLC on diphenyl-type stationary phases [29].

Vancomycin is soluble in water (>140 mg/mL), somewhat soluble in methanol, and insoluble in higher alcohols and other less-polar organic solvents. Thus, a typical biphasic system, suitable for chiral CCC&CPC, consists of a water-rich stationary phase containing vancomycin and a less-polar mobile phase in which the racemates preferentially stay, so that their partition ratio is in the 0.5 to 1 range, favoring rapid recovery.

Duret et al. [31] resolved racemic dansyl-norleucine (DNS-Nle) completely using a CCC apparatus (13 mL column) and a CPC apparatus (90 mL column). In both cases, the biphasic system was a mixture of toluene and an aqueous solution of 140 mg/mL vancomycin, adjusted to pH 4.7. The dual mode was performed since the D-enantiomer is strongly retained in the vancomycin aqueous solution. D,L-DNS-Nle is injected with the vancomycin aqueous solution as the stationary phase (tail to head for CCC, or ascending mode for CPC). When the L-enantiomer has been eluted, the mode is switched (head to tail, descending mode) and the D-enantiomer is eluted in the vancomycin aqueous mobile phase. Fifty milligrams of racemate are resolved in 50 min using CPC. Despite this success, the conclusions of Duret et al. are pessimistic:

1. ''The experimental conditions where vancomycin in solution will effectively recognize an enantiomer are extremely narrow:
   - an aromatic solvent is needed to make a biphasic system with water containing vancomycin;
   - addition of a non-aromatic solvent destroys chiral recognition;
   - pH range is narrow;
   - the concentration of vancomycin must be high to get a high selectivity.
2. The high molecular weight of vancomycin (MW≈1400) is a major drawback when considering preparative applications. Since two molecules of vancomycin are needed to complex one molecule of enantiomer, and since the Langmuirian shape of the isotherm suggests that it could be a supra-molecular organization involving four to six additional molecules of vancomycin, we must conclude that even with large amounts of vancomycin in solution, injected quantities of racemate will remain small, even if the selectivity is high. We feel now that an ideal chiral selector for CCC&CPC must have a low molecular weight, in order to get a high molarity in a solvent by dissolving a reasonable mass of it. Since chiral chromatography involves the formation of a complex between an enantiomer and the chiral selector, it is probable that we will always encounter Langmuirian shaped isotherms, and one way to extend the linear zone of these isotherms is to use higher molarities of chiral selector, the limit being that this chiral selector will be one of the solvents making the biphasic system.''

8. Chiral CCC&CPC reactor

The chiral CPC reactor was first mentioned in June 1990 in San Mateo, CA, by Bruening, a pioneer in CPC when he was working in Nakanishi's laboratory at Columbia University in New York with Derguini [32]; the talk was entitled 'The CPC reactor, a new tool for continuous organic synthesis' [33]. The abstract noted that ''the concepts of two-phase reaction media and phase-transfer catalysis are well established in synthetic organic chemistry. Moreover, they reflect the reaction conditions found in biological systems, where the 'active sites', as in enzymes for example, are frequently lipophilic domains in a hydrophilic environment. A system where the catalytic component could be held stationary, while reactants are introduced at the same time as products are removed, would allow to run certain chemical reactions continuously. Many attempts in this direction have failed, however, mainly because of the problems connected with the necessary mixing and demixing of the components in order to guarantee phase-transfer conditions. In CPC, thorough mixing of the two phases is produced at the inlet of each 'column', while demixing occurs concomitantly

at the outlet; thus, a single 'column' in a CPC cartridge could be seen as a microreaction vessel for phase-transfer catalysis ''. The example was the enzymatic deesterification of D,L-*sec*-phenethyl acetate. However, Bruening never published these results, probably because he was very busy at the University of Hawaii at Manoa. During the same symposium, Armstrong gave an example of an off-column enzyme reactor, followed by CPC separation of L-tryptophan from D-tryptophan-methyl ester [34]. As this was not directly related to the CPC reactor concept, 5 years passed before the first paper was published on the topic by Bousquet et al. [35]. Their example was the enantioselective hydrolysis of 2-cyano cyclopropyl-1,1-dicarboxylic acid dimethyl ester by Esterase 30000. The biphasic system was 1,1,1-trichloroethane–aqueous phosphate buffer (0.1 *M*, pH 7.5), with 7 g/L of the enzyme in the upper aqueous stationary phase. Deesterification of one isomer makes it go into the stationary phase, while the unreacting isomer remains in the mobile phase, thus allowing 100% recovery. One drawback is that the diester must remain 8 h in the column to achieve an enantiomeric purity of 80%. This paper failed to indicate the characteristics of the CCC instrument or provide details such as the temperature, flow-rate, stationary phase holdup, and elapsed time before the stationary phase needs to be regenerated. No subsequent papers on the subject have been published by this scientific group.

A major contribution was made by the Kluyver Institute for Biotechnology in Delft (Netherlands) headed by Luyben and van der Wielen. After fundamental research in CPC reported in the thesis [36] and corresponding papers [37–41] of van Buel, this group began studies of the CPC reactor for the production of chiral amino acids (corresponding to papers by den Hollander et al. [42,43]). The concept of a chromatographic reactor for preparative purposes has been studied in great detail [44,45], and a systematic approach for the potential of CPC to function as a chromatographic reactor was indispensable. A complete mathematical model was obtained by starting from van Buel's data [40] and expanding them with a reaction in the stationary phase depicted by μ_i, the stoichiometry coefficient of component *i*, and *R*, the rate of enzymatic conversion (mol s^{-1} m^{-3}). This led to two differential equations which can be studied by numerical simulation.

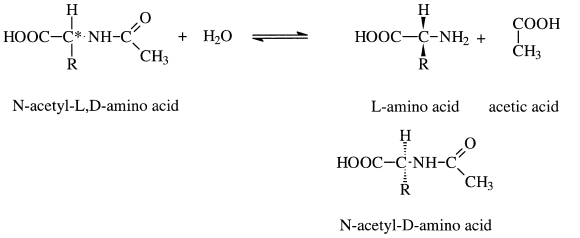

Fig. 6. Selective hydrolysis of *N*-acylated amino acids (R is the amino-acid side chain).

For this example, an aqueous two-phase system (ATPS), consisting of potassium phosphate (16%, w/w), PEG600 (16%, w/w) and water (68%, w/w) was used. Acylase was dissolved (0.2 g/L) in the stationary (PEG-rich) top phase, and the lower phase was used as the mobile phase. The substrate was injected (2 mL, 70 m*M*) onto the column, and the effluent profile was monitored with UV. The CPC column has 1068 individual cells for a total volume of 101 mL. The column was rotated at 800 rpm, and the flow-rate was 4 mL/min. With this setup, the stationary phase holdup was 61%. Fig. 6 shows the enzymatic reaction in the stationary phase. The enzyme cleaves the acyl moiety of the L-enantiomer in a racemic mixture of an *N*-acylated amino acid. This hydrolysis is equilibrium-limited at higher initial concentrations. Chromatographic reaction increases the conversion. Fig. 7 shows the effluent profile from the CPC column, indicating the simulta-

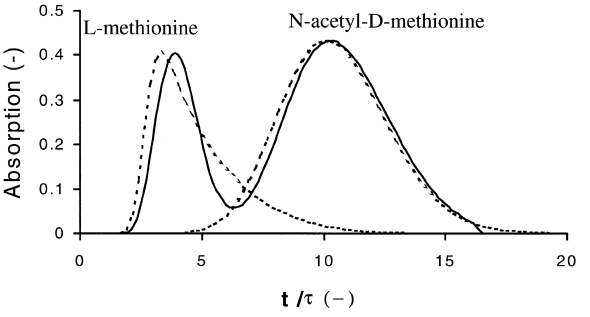

Fig. 7. Chiral CPC reactor, selective hydrolysis and chromatographic elution; solvent system, aqueous two-phase system PEG600 and potassium phosphate, containing acylase I (0.2 g/L); CPC column, 101 mL; flow-rate, 4 mL/min; rotation, 800 rpm. Injection of 70 m*M* *N*-(acetyl)-D,L-methionine. Calculated profiles of L-methionine and *N*-acetyl-D-methionine are also depicted (- - -). τ is the residence time for an unretained compound (V_M/F). Reproduced, with permission, from Ref. [43].

neous enzymatic hydrolysis in the stationary phase and the separation of the L-amino acid and the unconverted *N*-acylated amino acid. The predicted effluent profiles based on the mathematical model are also shown. Larger-scale production is being considered by this laboratory, and further improvements in the overall efficiency of the process can be expected owing to a better design of the CPC cells for adaptation to these rather viscous ATPS.

9. Analytical expressions

We would like to end this review by an analytical survey of equilibria and equations encountered in the literature useful for characterizing a chiral chromatographic separation. The presentation follows the IUPAC recommendations for nomenclature in liquid–liquid distribution [46], which designates the distribution ratio as the ratio of the total analytical concentration of a solute in one phase (regardless of its chemical form) to its total analytical concentration in the other phase (D), and the partition ratio as the ratio of the concentration of a substance in a single definite form in one phase to its concentration in the same form in the other phase at equilibrium (K_D or $D°$). The distribution ratio governs the retention of a solute, while the partition ratio is useful for several analytical calculations. These definitions are more precise than those found in the IUPAC recommendation for chromatography nomenclature [47] with respect to the distribution constant. In any event, the IUPAC rules for chromatography deal with all kinds of chromatography except CCC&CPC (not even a single comment).

The following are the nomenclature and symbols generally encountered in chiral CCC&CPC: $(\overline{CS})_{ini}$ is the initial concentration of the chiral selector; $(\overline{CS})$ is the actual concentration of CS in the stationary phase; $(A_\pm)$, (A_+) and (A_-) is the concentration of both the + and − isomer, the + isomer, and the − isomer, respectively; an overlined compound indicates one in the stationary phase:

$$K_\pm = \frac{(\overline{CSA}_\pm)}{(\overline{CS})(\overline{A}_\pm)}$$

is the complex formation constant of $\overline{CSA}_+$ or $\overline{CSA}_-$ in the case of 1:1 stoichiometry:

$$D_\pm = \frac{(\overline{CSA}_\pm) + (\overline{A}_\pm)}{(A_\pm)}$$

is the distribution ratio of A_+ and A_-; and:

$$D_0 = \frac{(\overline{A}_\pm)}{(A_\pm)}$$

is the partition ratio of A_+ and A_- in the absence of CS.

As indicated by Ma et al. [15], the quadratic scheme originally used by Oliveros et al. [13] to describe the equilibrium between CS and $A_\pm$ in CCC&CPC (Fig. 8a) can be greatly simplified if we consider that CS and $CSA_\pm$ remain in the stationary phase only (Fig. 8b). In this case (1:1 stoichiometry), the distribution ratio of $A_\pm$ is expressed as follows:

$$D_\pm = D_0(1 + K_\pm(\overline{CS})) \quad (3)$$

and the separation factor, α, is expressed as (+ more retained than − isomer):

$$\alpha = \frac{D_+}{D_-} = \frac{1 + K_+(\overline{CS})}{1 + K_-(\overline{CS})} \quad (4)$$

From Eq. (4), it arises that α varies from 1 to the

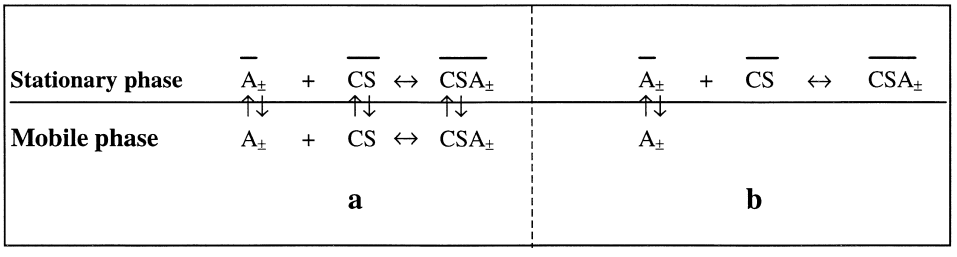

Fig. 8. Quadratic scheme describing the equilibrium involved in chiral CCC&CPC: (a) general scheme when CS is in both the mobile and stationary phases; (b) simplified scheme when CS is in only one phase, here the stationary phase.

maximum value of K_+/K_- (monotonous increasing function).

Hence, if $K_+/K_- < \alpha_{lim}$, i.e. the minimum value needed to obtain a reasonable resolution with the CCC or CPC instrument under experimental conditions, then chiral separation is not possible. Ordinarily, K_+ and K_- are obtained easily by batch experiments with spectroscopic or HPLC controls.

Thus, if $K_+/K_- > \alpha_{lim}$, then what are the loading limits (sample size) for a given value of $(\overline{CS})_{ini}$, a value which is of the greatest interest because of the preparative qualities of CCC&CPC? To determine this, it is necessary to know the isotherms for A_+ and A_- in the given biphasic system with a given $(\overline{CS})_{ini}$. Most of the time, equilibrium such as that shown in Fig. 8 leads to Langmuirian isotherms (Fig. 9):

$$(\overline{A}'_{\pm}) = \frac{a_{\pm}(A_{\pm})}{1 + b_{\pm}(A_{\pm})}, \text{ with } (\overline{A}'_{\pm}) = (\overline{A}_{\pm}) + (\overline{CSA}_{\pm}) \quad (5)$$

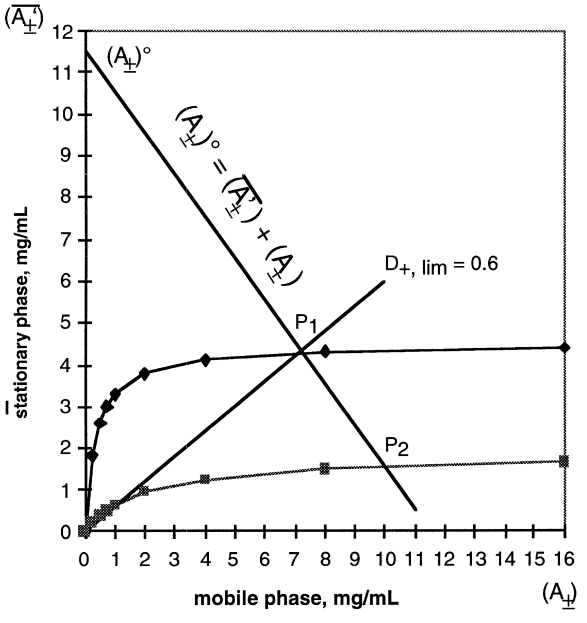

Fig. 9. Langmuirian isotherms and estimation of the operating conditions in chiral CCC&CPC. Parameters for the Langmuirian isotherms: $a_+ = 12.05$; $a_- = 0.95$; $b_+ = 2.67$; $b_- = 0.52$. The upper limit concentration of the injected racemic was calculated using the straight lines and the isotherms as described in the text.

Limit conditions for Eq. (5) lead to:

$$a_{\pm} = D_0(1 + K_{\pm}(\overline{CS})_{ini}) \quad (6)$$

slope at the origin:

$$\frac{a_{\pm}}{b_{\pm}} = (\overline{CS})_{ini} \quad (7)$$

or multiple of $\overline{(CS)}_{ini}$ stoichiometry-dependent (saturation of CS).

For an initial concentration $(A_{\pm})^\circ$ in one volume of each phase, there are thus two sets of two relationships:

$$(A_{\pm})^\circ = (\overline{A}'_{\pm}) + (A_{\pm}) \text{ (mass conservation)} \quad (8)$$

Eq. (5).

Combining Eqs. (8) and (5) gives:

$$-b_{\pm}(A_{\pm})^2 + (A_{\pm})((A_{\pm})^\circ b_{\pm} - 1 - a_{\pm}) + (A_{\pm})^\circ = 0 \quad (9)$$

Since the root product is negative, there is only one solution for $(A_{\pm})$, which can easily be extracted using mathematical tools (e.g., 'Goalseeker' in Excel).

A set of values (A_+), $(\overline{A}'_+)$, (A_-), $(\overline{A}'_-)$ is obtained for given values of $(A_+)^\circ$ and $(A_-)^\circ$ [in the case of a racemic starting material, $(A_+)^\circ = (A_-)^\circ$], and from there D_+, D_-, and $\alpha = D_+/D_-$. If we assume that the distribution ratio of the most retarded enantiomer must remain higher than a given value, e.g. 0.6, then for a given value of $(CS)_{ini}$, limited by solubility or for other reasons, the operating conditions can be optimized by finding the upper limit of $(A_{\pm})^\circ$ leading to the lower limits for D_+ and α.

A simpler way would be to use Fig. 9 directly to estimate $(A_{\pm})^\circ$: a lower limit of 0.6 can be assumed for D_+, represented by the straight line with a slope of 0.6 which crosses the D_+ isotherm at P_1. Then the straight line representative of Eq. (8) is positioned so as to cross the D_+ isotherm at the point P_1, which gives the values of both $(A_{\pm})^\circ$ (the intercept with the ordinate axis) and P_2 (corresponding to the equilibrium for D_-). In the example given in Fig. 9, it may be calculated that $D_- = 0.15$, $\alpha = 4$, and $(A_{\pm})^\circ = 11.5$ mg/mL (i.e. 23 mg/mL for the racemate).

These equations can be summarized as follows:

- α increases with (CS) and cannot go beyond the limit K_+/K_-.

- Due to the Langmuirian isotherms generally encountered, D_+, D_- and α decrease when $(A_\pm)^\circ$ increases, so that $(A_\pm)^\circ_{\lim}$ can be calculated by iteration to obtain final values of D_+ and α that are still higher than $D_{+,\lim}$ and $\alpha_{\lim}$, respectively, as imposed by the CCC or CPC experiment, thus giving the limit concentration of the sample to be injected.

10. Conclusion

The improvement of CCC and CPC apparatus, and the arrival of new companies with analytical and preparative instruments, have provided scientists with new tools to resolve their purification problems. Chiral separations are still challenges for CCC and CPC, due to the difficulty of finding chiral selectors highly efficient in liquid phases. The most promising way to perform chiral preparative purifications by CCC or CPC is probably the pH-zone-refining mode introduced by Ito, and which will soon give us new applications, once the choice of suitable chiral selectors has been solved

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