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Review

Counter-current chromatography: instrumentation, solvent selection and some recent applications to natural product purification

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Abstract

Modern instruments for counter-current chromatography (CCC) and centrifugal partition chromatography (CPC) are reviewed, and a description of biphasic solvent systems is provided, together with some simple rules for finding the best system for a given purification. Applications include the purification of acetogenins, 10-deacetyl-baccatin III, amphotericin B, anthocyanins, hop bitter acids, alkaloids and partially depolymerized fucans. CPC–NMR coupling is briefly discussed.

Keywords: Reviews; Counter-current chromatography; Centrifugal partition chromatography; Solvent systems; Alkaloids; Acetogenins; Bitter acids; Deacetylbaccatin; Polysaccharides

Contents

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1. Generalities and instrumentation

1.1. Introduction

Modern counter-current chromatography originates from the pioneering studies of Ito et al. [1], who first constructed an apparatus in Japan designed to differentiate particles in suspension or solutes in solution in a solvent system subjected to a centrifugal acceleration field. This first machine opened the way in two main directions: one, pursued by Ito in the USA, is based on a wide variety of 'CCC apparatuses' using a variable-gravity field produced by a two-axis gyration mechanism and a rotary seal-free arrangement for the column; the other, pursued by Nunogaki in Japan, is based on the 'centrifugal partition chromatography (CPC) apparatus' and uses a constant-gravity field produced by a single-axis rotation mechanism and two rotary-seal joints for inlet and outlet of the mobile phase. The theoretical and instrumental contributions of Y. Ito have resulted in approximately 200 papers. Several books have been published [2–6], in which many examples of separations in various fields can be found. Though the first widely distributed commercial model of droplet CCC produced an unfavourable impression of the CCC technique as time-consuming and inefficient, modern instruments are much more efficient and merit the designations of HSCCC for high-speed CCC or HPCPC for high-performance CPC. The efficiency of CCC and CPC apparatuses, if evaluated by the number of theoretical plates, is still far from that encountered in HPLC. However we should not forget that the goal of a separation is the resolution between peaks. Thus, the high selectivity found in CCC and CPC (the two liquid phases can be finely tuned, as shown in Section 2) and the high stationary to mobile phase ratio characteristic of modern instruments largely compensate for this apparent weakness. In fact, it is in the optimization of selectivity that CCC and CPC has the most to offer, although this optimization may appear obscure to the beginner. In this paper, we will try to highlight some simple rules for selecting and modifying a biphasic solvent system and discuss some recent examples found in the literature. Very recently Ito introduced a new mode of purification of ionizable compounds by CCC, referred to as pH-zone refining CCC. This mode is close to the displacement mode already known in chromatography, and we will see that it can also be applied to ionic compounds in a liquid–liquid ion-exchange displacement mode.

1.2. Instrumentation

1.2.1. CCC and CPC companies since the beginning

The most well known companies producing instruments are P.C. Inc. (Potomac, MD, USA), Pharmatec (Baltimore, MD, USA) and Sanki Eng. (Kyoto, Japan), which have had booths at the Pittsburgh Conference for many years. In business since 1982, P.C. Inc. has pioneered the development of centrifugal counter-current equipment. Its CCC instrument is versatile and 'elegantly simple'. A triple coil allows a preliminary test of the solvent system with a 15-ml mini-coil and subsequent separation of larger samples with a 75-ml or a 215-ml coil. These coils can be connected in series. Pharmatec manufactures various prototypes of CCC instruments, generally with three coils in series, and interested scientists should contact the company for information about its latest model. Sanki is the only company which has manufactured CPC instruments for thirty years. Its analytical HPCPC has one disk pack with 2136 partition channels for a volume of 230 ml; the rotational speed is limited to 2000 rpm and the backpressure to 60 bar. Two preparative-scale CPC instruments are available, with rotors of 1.3 l and 5.4 l for around 1000 partition channels. Larger instruments (up to 30 l) can be produced upon request.

1.2.2. New instruments

Scientists interested in CCC techniques will be pleased to know that two new European companies are manufacturing CCC instruments. AECS (Bridgend, UK) proposes the Quattro, which has four coils on two holders. The coils are wound by pairs: 50 and 250 ml on one bobbin, and 100 and 200 ml on the other. The coils may be interconnected in various combinations or used to run four independent samples. A novel feature is the absence of a central shaft, which allows higher values of β (the ratio of the planet radius to the orbital radius), the key parameter in CCC [2,3,6]. The Quattro is temperature-controlled. Last spring, SEAB (Villejuif, France) introduced the Kromaton III, a large CCC instrument with two pairs of coils on two holders. Two analytical columns (200 ml each) and two preparative ones (1 l each) can be used independently or interconnected. This arrangement of coils allows an average β value higher than 0.7, which should result in very good retention of most biphasic systems. The Kromaton III is temperature controlled. Otherwise a CCC apparatus has been built at the Vernadsky Institute of Geochemistry and Analytical Chemistry in Moscow (Russia), CCC prototypes produced by Shimadzu have been used in Japan, and many CCC apparatuses have been built by Professor T. Zhang at the Beijing Institute of New Technology Application (China).

2. Solvent selection

2.1. Diversity of solvent systems

The selection of a two-phase solvent system for CCC and CPC is similar to the choice of a column and an eluant for HPLC. Important criteria are the polarity of the sample and its solubility, charge state, ability to form complexes, etc. The purpose of solvent optimization is first to find a solvent combination for which the sample is freely soluble, since the goal of CCC and CPC is preparative rather than analytical, and then to adjust this solvent combination to ensure that the distribution ratio of the species to be separated differs and is generally centered around 1. Most often, optimization of a separation involves optimization of chromatographic selectivity, and it is precisely here that CCC and CPC has the most to offer. Both phases are directly accessible, and their compositions can be fine-tuned to achieve the desired resolution. Fig. 1 compares how CCC and HPLC are used to separate (or resolve) two unresolved peaks. In CCC, resolution is achieved by improving selectivity, whereas HPLC is efficient for peak slimming. A phase diagram may be used, when available, to understand how the effects of varying the composition of one phase act on the composition of the other. Ternary diagrams for many solvent systems have been compiled by Sørensen and Arlt [7], and 82 of these diagrams have been selected and published (in the vol.% diagrams) in reference [4]. As shown in Fig. 2, these systems belong to type 1 (widely used, e.g. $CHCl_3$–MeOH–water), type 2 (e.g. EtOAc–BuOH–water) and type 0 (only one example with water–DMSO–THF).

2.2. Strategy of solvent selection: best solvent

Since most systems used in CCC and CPC are type 1 systems, the primary role of the solvent that is soluble in the other two needs to be highlighted. This solvent, generally the ordinate on ternary diagrams, partitions in the other two exactly as we would like our sample to partition. Thus, it must be a good solvent of the sample with which we are working, and therefore we allow it to go into the two phases of the selected biphasic system. In other words, when a sample is to be fractionated by CCC and CPC, the first requirement is to find the best solvent for the sample, i.e. the solvent in which the sample is most

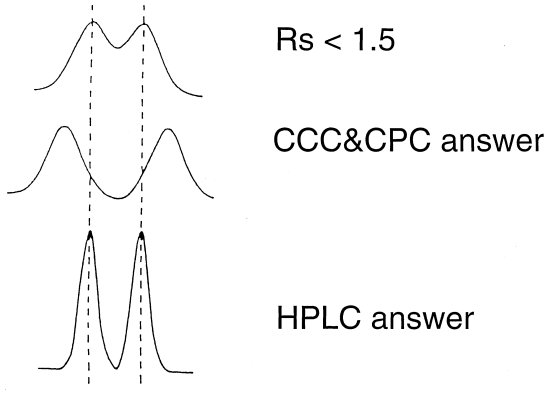

Fig. 1. Two ways of improving resolution.

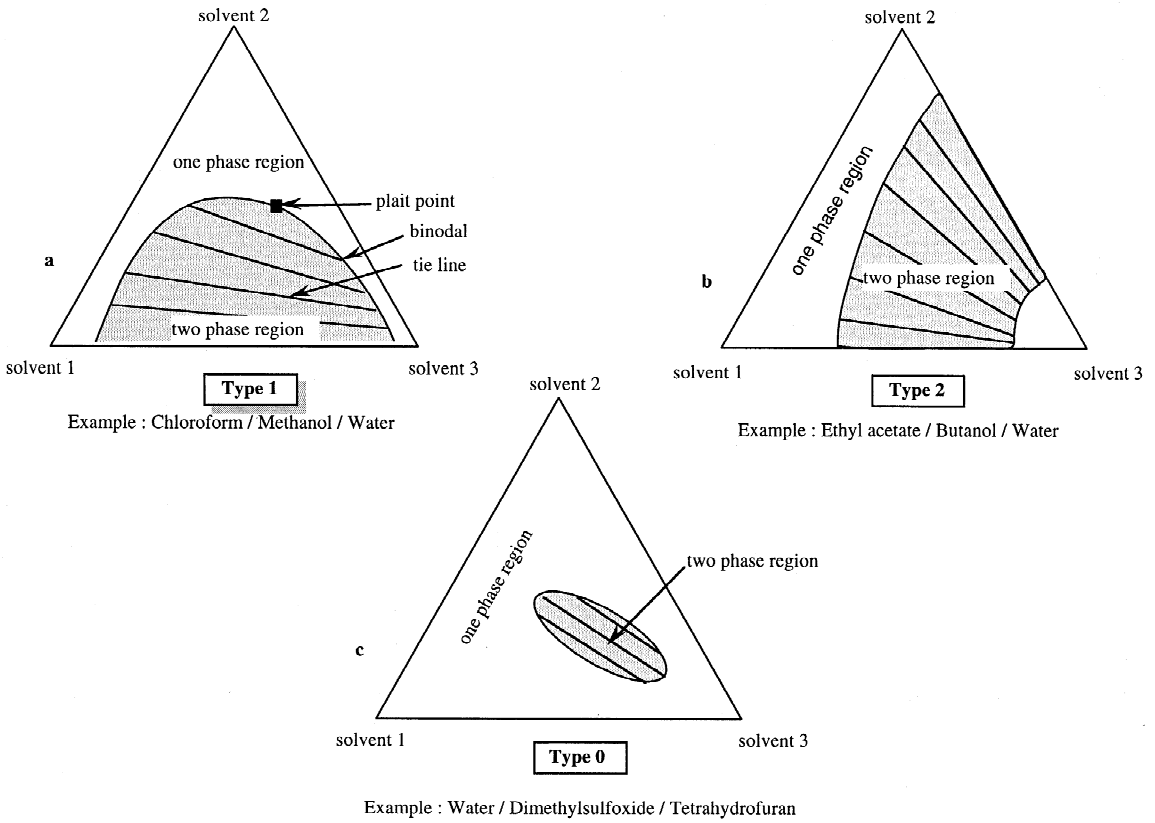

Fig. 2. Three types of ternary diagrams encountered in CCC and CPC.

soluble, since the goal is often preparative chromatography. Then, we need to partition this best solvent into two other solvents in order to build a biphasic system; that is, we have to look for ternary diagrams where the best solvent plays the role of solvent 2 in Fig. 2. Table 1 gives some examples of the 'best solvents' between two columns of less and more polar solvents, leading to biphasic systems (more examples can be found in reference [4]).

2.3. Multi-solvent systems: a logical approach for medium polar compounds

When routinely working with new but similar samples such as plant extracts or pharmaceuticals, it may be a good idea to have on one's bench a collection of selected biphasic systems that will allow rapid determination of the one that is suitable for successful fractionation. This is why multisolvent systems have been developed which can cover a broad range of polarities through variation of the ratio of each solvent. One of these systems, which is very well-known, is the heptane–EtOAc–MeOH–water system.

2.3.1. Heptane–EtOAc–MeOH–water system

The methodological description of this system has been made by Margraff (see [4,8]). This versatile quaternary system can be considered as a combination of the two binary heptane–MeOH (1:1) and EtOAc–water (1:1) systems. Hence, in moving from A, the middle of the EtOAc–water (1:1) edge of the tetrahedron representing the quaternary system (Fig. 3), to Z, the middle of the opposite heptane–MeOH edge, all the polarities between these two systems will be covered. Thus, if a compound of interest, Σ, is mainly in the 'nonpolar' phase of system A (i.e. the EtOAc-rich phase) but mainly in the 'polar'

Table 1
The 'best solvent table': starting with a new sample, first find the best solvent, and then try to put it between two other solvents, as shown

Less-polar solvent	Best solvent	More-polar solvent
Heptane, $CHCl_3$	THF	Water
Heptane, toluene, MiBK, $CHCl_3$, EtOAc	AcO	Water
Heptane	Methyl ethyl ketone	Water
THF	DMSO	Water
Toluene, MtBE, MiBK, EtOAc	MeCN	Water
Heptane, toluene, $CHCl_3$, EtOAc	BuOH	Water
Heptane, toluene, $CHCl_3$, EtOAc	PrOH	Water
Heptane, $CHCl_3$, EtOAc	EtOH	Water
Heptane, toluene, $CHCl_3$, EtOAc, BuOH	MeOH	Water
Heptane, toluene, $CHCl_3$, MiBK, EtOAc, BuOH	HOAc	Water
$CHCl_3$	HCOOH	Water
Nonaqueous systems		
Heptane	THF, dimethylformamide, EtOAc, PrOH, EtOH	MeOH, MeCN

phase of system Z (i.e. the MeOH-rich phase), a suitable system is sure to be found in which the distribution ratio of Σ will be around 1. Margraff has selected 23 systems (from A to Z, with letters E, I and O omitted). The corresponding plots (Fig. 3) are nearly equidistant from each other, and the corresponding integer ratios (volume ratios) are given in Fig. 3. The following control by TLC of the partition of one compound in the J to Q systems shows that a precise step-by-step variation can be obtained with these rational mixtures (Fig. 4).

This system has proven very useful but may have some limitations for scaling up a purification, because of the solubility of the sample. Systems A to Z have a constant ratio of heptane+water of 50%; and in the middle of the range (say J to S), i.e. when the sample is highly soluble in MeOH and/or EtOAc but poorly soluble in heptane or water, this 50% may limit the use of the quaternary system to the analytical scale. For this reason, we developed another system with five solvents for use with medium polar compounds [9].

Systems	Heptane	EtOAc	MeOH	Water
A	0	1	0	1
B	1	19	1	19
C	1	9	1	9
D	1	6	1	6
F	1	5	1	5
G	1	4	1	4
H	1	3	1	3
J	2	5	2	5
K	1	2	1	2
L	2	3	2	3
M	5	6	5	6
N	1	1	1	1
P	6	5	6	5
Q	3	2	3	2
R	2	1	2	1
S	5	2	5	2
T	3	1	3	1
U	4	1	4	1
V	5	1	5	1
W	6	1	6	1
X	9	1	9	1
Y	19	1	19	1
Z	1	0	1	0

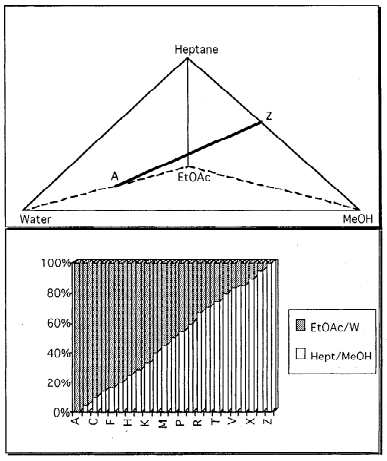

Fig. 3. Margraff's [4,8] approach for a logical composition of the heptane (Hept.)–EtOAc–MeOH–water (w) system.

2.3.2. Heptane–MeOH (1:1)+methyl tert.-butyl ether–ethylene glycol dimethyl ether–water (1:2:1) system

Although some solvent polarity tables indicate that ethylene glycol dimethyl ether (glyme) is close to EtOAc, there is a major difference since glyme is fully miscible with both heptane and water. A closer look at the properties of glyme indicates that it partitions evenly in the biphasic methyl *tert*.-butyl ether (MtBE)–water system where up to 60% (v/v) can be added without loss of biphasic properties. Finally, we found that the two biphasic heptane–MeOH (1:1) and MtBE–glyme–water (1:2:1) systems can be mixed in any proportion to give a biphasic system with roughly equal volumes of lower and upper phases. 'Any proportion' means that volumes v_1 of the lower phase+v_1 of the upper phase of the heptane–MeOH (1:1) system are mixed with volumes v_2 of the lower phase+v_2 of the upper phase of the MtBE–glyme–water (1:2:1) system. We can thus define 21 systems, ranging from the less

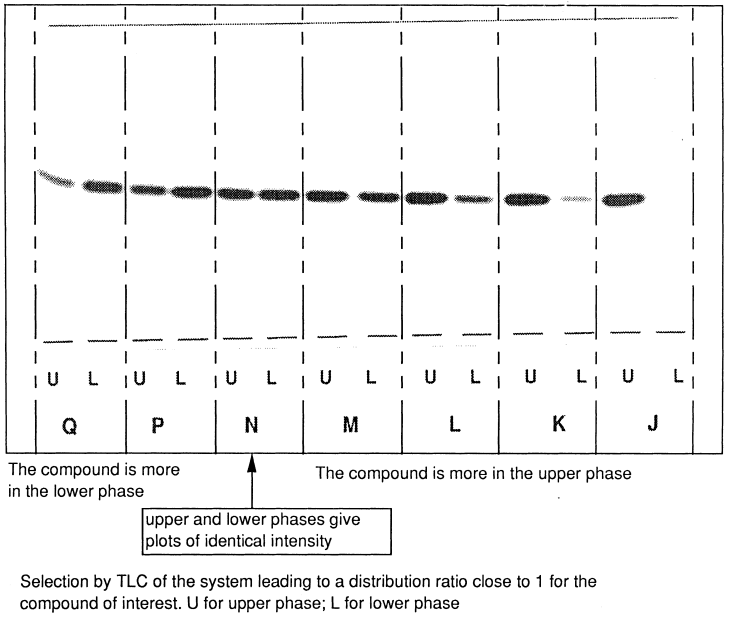

Fig. 4. Selection by TLC of the system leading to a distribution ratio close to 1 for the compound of interest. U for upper phase; L for lower phase.

polar heptane–MeOH (1:1) system (system 1) to the more polar MtBE–glyme–water (1:2:1) system (system 21), with a 5% (v/v) variation between each system. The advantage of this five-solvent system as compared to the four-solvent heptane–EtOAc–MeOH–water system is that over the entire range of polarity (except for system 1) the ratio of medium polar solvents (MtBE+glyme+MeOH) is higher than 50% (v/v), as shown in Fig. 5. In practice, interpolations can be made between systems n and $n+1$, and some tests followed by TLC have shown that variations in the distribution ratio can be very precisely controlled.

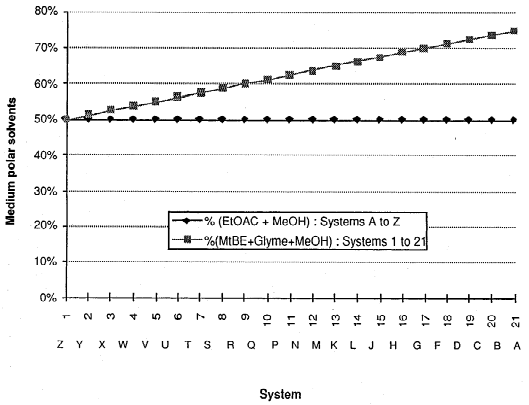

Fig. 5. Comparison of the ratio of medium-polar solvents for the systems heptane–EtOAc–MeOH–water and heptane–MeOH (1:1)+MtBE–glyme–water (1:2:1).

3. Applications of CCC and CPC in purification of natural products

3.1. Use of the heptane–EtOAc–MeOH–water system and its limitations

3.1.1. Purification of annonaceous acetogenins, a work performed by Duret et al. [10]

This application is presented to show how the composition of the biphasic heptane–EtOAc–MeOH–water system can be logically modified for successful purification of very close molecules. Annonaceous acetogenins are recently discovered polyketides isolated until now only from tropical and subtropical plants of the Annonaceae family. They are characterized by common features: a long chain of 35–37 carbon atoms, including one or two tetrahydrofuran rings, several oxygenated functions

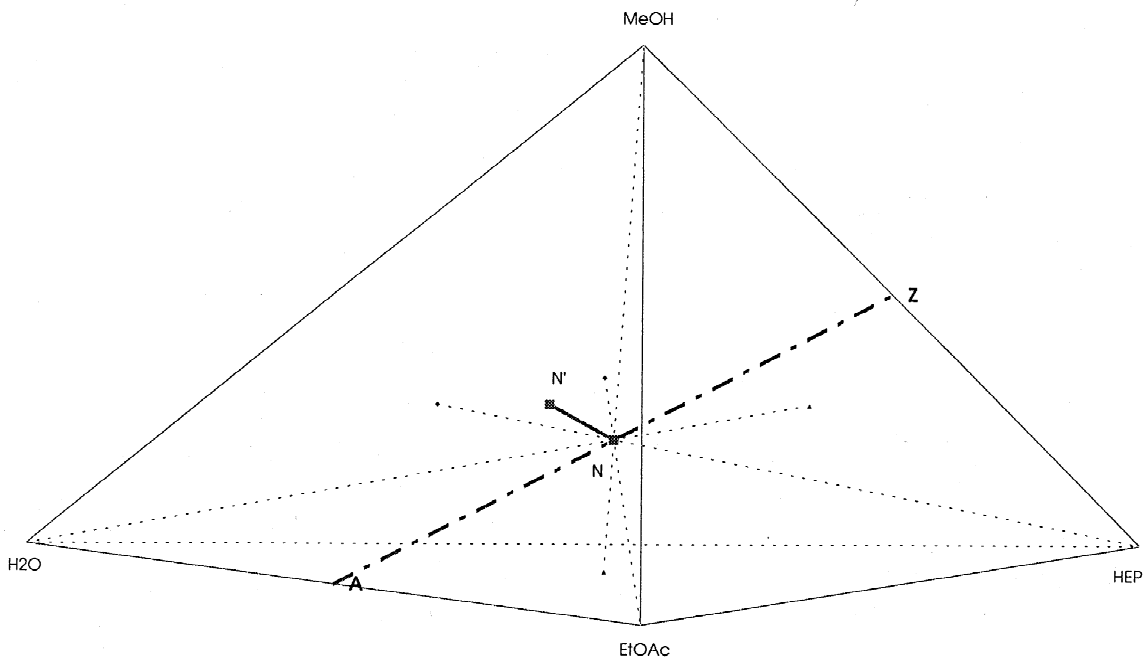

Fig. 6. A logical way to increase solubility of the acetogenin mixture while keeping an average distribution ratio around 1, as it was for N. N′ contains more EtOAc and more MeOH. HEP = Heptane.

(hydroxyl, ketone) and a γ-methyl-γ-lactone terminal group. Most of these compounds exhibit interesting cytotoxic activity with promising antitumor potential, and some have parasiticidal, insecticidal and immunomodulating properties. Various successive and repeated chromatographic steps on silica gel, Sephadex LH-20, preparative TLC, and normal- and reversed-phase HPLC were required to purify acetogenins, with generally poor overall yields. This procedure can be dramatically shortened, and the yields improved, by use of CCC. Duret et al. [10] tested the heptane–EtOAc–MeOH–water system to partition their sample and found that the N system (1:1:1:1, Fig. 3), was suitable to obtain an average distribution ratio of around 1 for their mixture. However, they were disappointed by the very low solubility of their sample, whereas it was freely soluble in MeOH or EtOAc. As previously explained, this was due to too large a percentage of heptane+water, which is always 50% in systems A to Z. At this point they decided not to search for another biphasic system, but to increase simultaneously, the ratio of EtOAc (which is more soluble in heptane than in water) and that of MeOH (which is more soluble in water than in heptane), starting from system N, as shown in Fig. 6. Some fine-tuning led to lowering the ratio of heptane more than that of water, so that the final mixture was heptane–EtOAc–MeOH–water (3:10:10:7) (point N′, Fig. 6), for which the ratio of EtOAc+MeOH was 67% (v/v), i.e. 17% more than for system N. Fig. 7 shows the CCC elution order of acetogenins, with their chemical formulae. Two pure compounds were obtained in one step, using the N′ system after injection of 500 mg of the sample, i.e. squamocin (tubes 58 and 59) and rolliniastatin-2 (tubes 67 to 69), which differ only in the position of one hydroxyl group. In addition, four pairs of acetogenins were obtained and further purified by HPLC in one step for each pair. This work by Duret et al. [10] shows that the heptane–EtOAc–MeOH–water solvent system, as described by Margraff, can lead to very selective separations and be logically modified to overcome some problems with solubility.

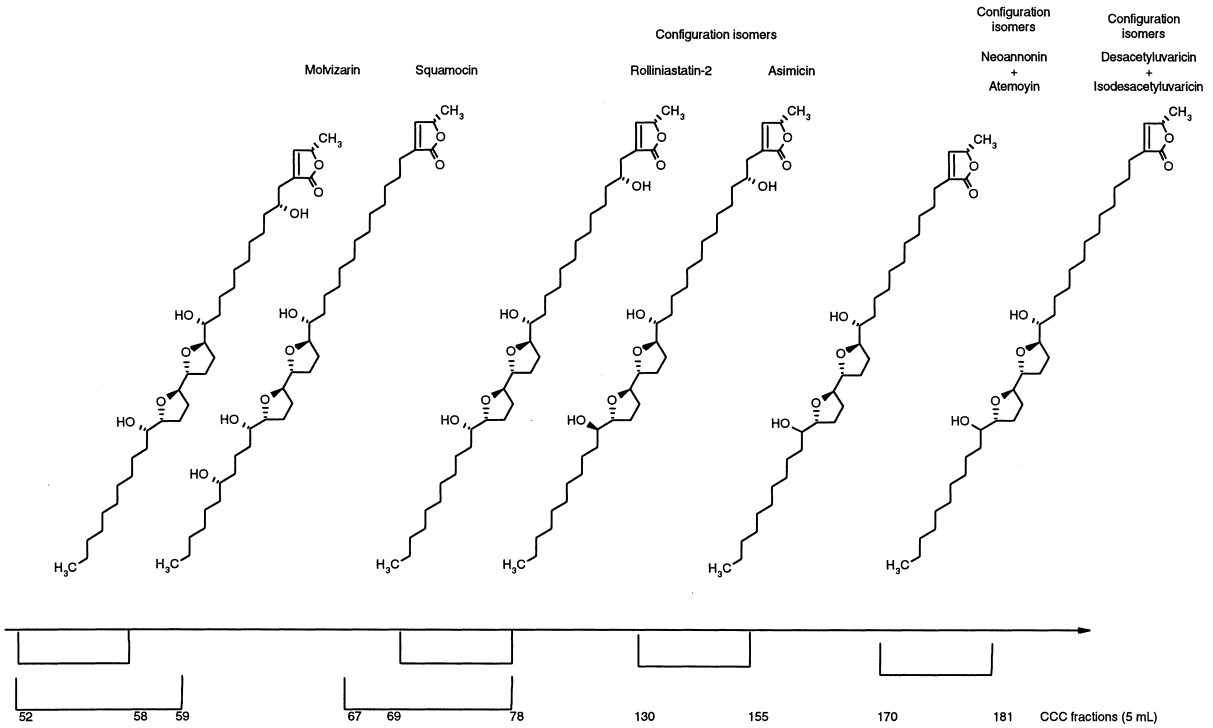

Fig. 7. CCC elution order of acetogenins. This work was performed on a P.C. Inc. CCC system, with a coil of 240 ml. The rotational speed was 860 rpm, and the flow-rate 4 ml/min, in the head to tail mode. The lower phase of solvent system N′ (see Section 3.1.1) was the mobile phase. The stationary phase ratio was 64% and 500 mg were injected in 5 ml of mobile phase [10].

3.1.2. Purification of 10-deacetyl-baccatin III, a work performed by Margraff [11]

This example is presented to show how some solubility problems can be overcome by switching from one system to another. 10-Deacetyl-baccatin III, or '10-DAB', the starting material for docetaxel (Taxotère) semisynthesis, is produced by the European yew *Taxus baccata*, together with its 19-hydroxy analog, or '19-OH-10-DAB' (Fig. 8). To obtain a distribution ratio close to 1 for 10-DAB,

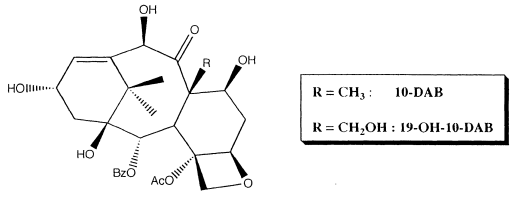

Fig. 8. 10-Deacetyl-baccatin III and its 19-hydroxy analog. Reprinted from Ref. [11], p. 335, by courtesy of Marcel Dekker.

Margraff tested the heptane–EtOAc–MeOH–water system and found system K (1:2:1:2) to be suitable, giving a value of 0.7 [$D = C_{(\text{upper phase})}/C_{(\text{lower phase})}$] for 10-DAB, with a selectivity coefficient of 1.75 between 10-DAB and 19-OH-10-DAB. Unfortunately, the solubility of 10-DAB in this system was far too low for preparative works. 10-DAB solubility was then measured in various solvents and found to be high enough in tetrahydrofuran (THF), MeOH, EtOH and acetone (AcO). This led Margraff [11] to test the biphasic methyl isobutyl ketone (MiBK)–AcO–water system, since both MiBK and AcO are rather popular in industry. The ternary diagram of this system is shown in Fig. 9. System (2:3:2) was selected since it leads to a distribution ratio of 3 for 10-DAB and a selectivity coefficient of 6 between 10-DAB and 19-OH-10-DAB. A distribution ratio of 3 instead of 0.7 means that this system is slightly more polar than the K system previously selected. Various 'bridges' between systems A to Z and some

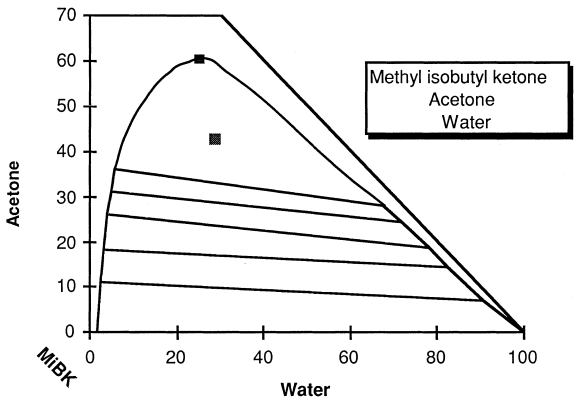

Fig. 9. Ternary diagram of the MiBK–AcO–water system.

well-known ternary systems would be definitely useful for a fast and logical means of selecting a solvent system. Finally, it was found that 10-DAB solubility in the upper phase of the MiBK–AcO–water system was at least three times as high as in pure AcO. We have also observed higher solubility for many compounds in a mixture of solvent than in each solvent separately, probably because of preferential solvation of part of the compound by one solvent and part by another. Fig. 10 shows the CPC chromatogram obtained in dual-mode elution after injection of 10 mg of crude 10-DAB in 500 μl of upper phase. The scale up was done by injecting 5 g in 60 ml of upper phase, using a CPC instrument with a volume of 240 ml.

3.2. A specific case of highly insoluble compound: the WDT system [12]

The search for a solvent system providing high solubility for a mixture of interest can be disappoint-

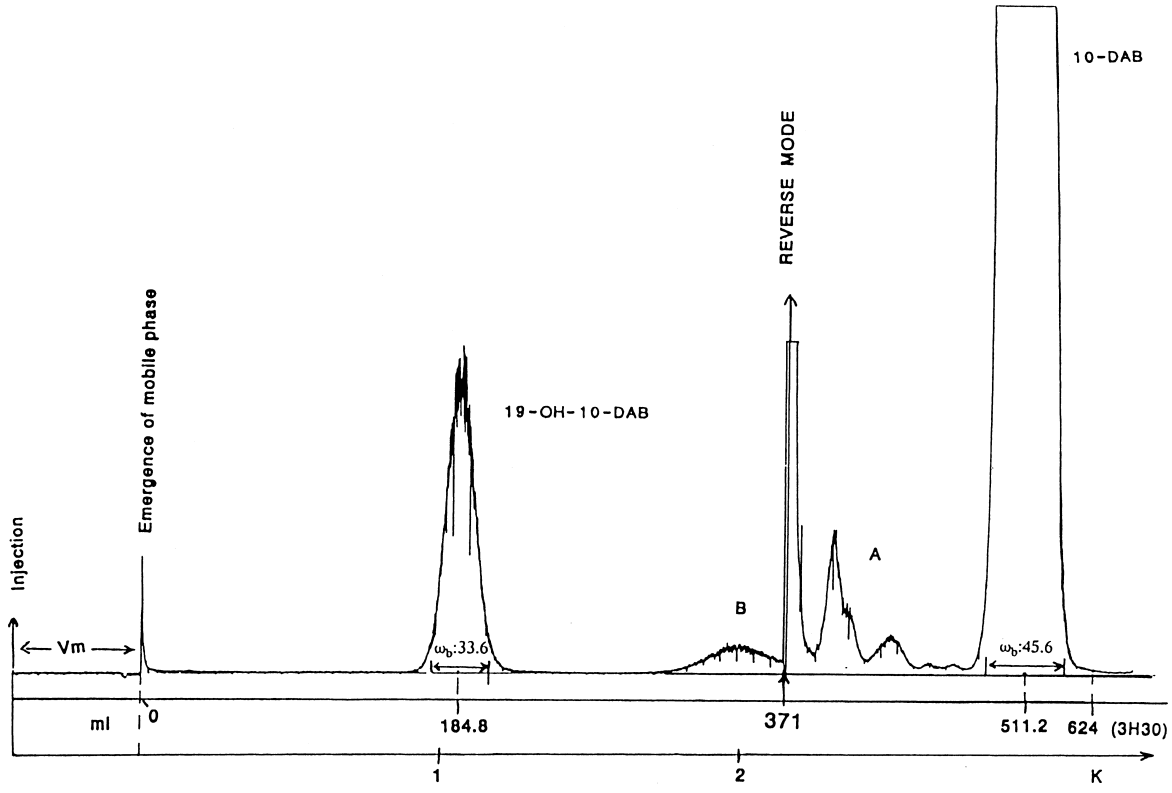

Fig. 10. CPC chromatogram with evaporative light-scattering detection (ELSD) after injection of 10 mg of crude 10-DAB in 500 ml of the upper phase. This work was performed on a Sanki HPCPC system, with a rotor of 240 ml. The rotational speed was 1300 rpm, and the flow-rate 3 ml/min. The biphasic system was MiBK–AcO–water (2:3:2). The mode was first descending, then ascending after 371 ml. The stationary phase ratio was around 73% at the beginning of the run. Reprinted from Ref. [11], p. 343, by courtesy of Marcel Dekker.

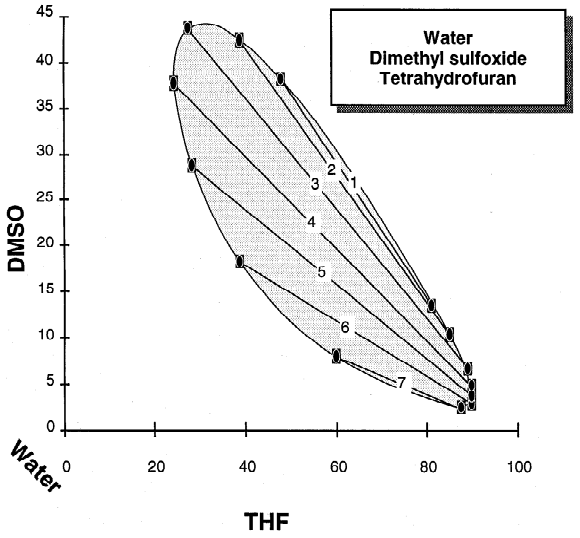

Fig. 11. The water–DMSO–THF system.

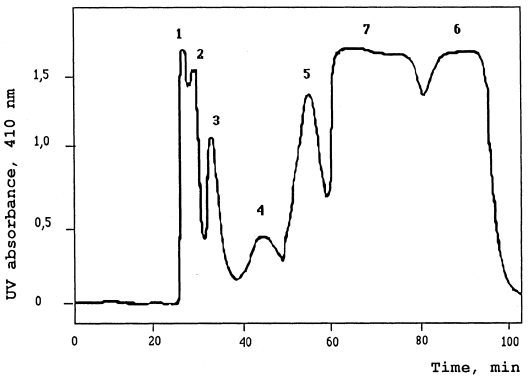

Fig. 13. CPC chromatogram after injection of 500 mg of crude Amphotericin B (perchlorate salt). This work was done on a Sanki HPCPC system (240 ml). The biphasic system was water–DMSO–THF (24.6:16.2:59.2, v/v/v). The rotational speed was 1400 rpm and the flow-rate 7 ml/min, in the ascending mode. The stationary phase ratio was 50%. Pure Amphotericin B is compound 7 [13].

ing because of a long succession of negative results. In these circumstances, the original biphasic system consisting of water, dimethyl sulfoxide (DMSO) and THF, known as the WDT system, would appear useful. This system is a type 0 liquid–liquid equilibrium system, i.e. the three solvents are miscible in all proportions when taken by pairs, while a zone exists in the ternary phase diagram (Fig. 11) where a biphasic system occurs when the three solvents are mixed in an appropriate ratio. The good solvating properties of THF and DMSO should make WDT systems very useful when sample solubility is a problem. We experimented with the WDT system to purify amphotericin B (Fig. 12), a polyene macrolide antibiotic whose commercial form contains a mixture of heptaenes, tetraenes and pentaenes. Its solubility in water is only 0.1 g/l at pH 2 or pH 11. However, it becomes very soluble in the WDT system when converted in its perchlorate salt, and up to 10 g/l can

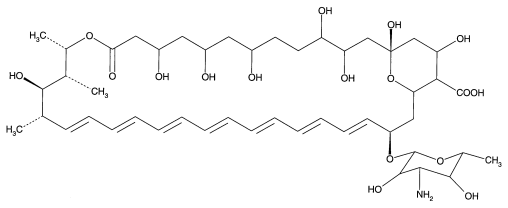

Fig. 12. Amphotericin B, (M_r = 924.11).

be dissolved in both phases of the system water–DMSO–THF (24.6:16.2:59.2, v/v/v). The CPC chromatogram corresponding to the injection of 500 mg of crude amphotericin B is shown in Fig. 13 [12,13]. Other polyene macrolides or peptide-like macrocycles have been purified since then, and in each case good solubility has been obtained.

3.3. Preparative purification by gradient elution CPC, a work performed by Renault et al. [14]

As with HPLC, gradient elution provides CCC and CPC with an easy way to fractionate solutes of widely differing polarities and distribution ratios, and to reduce run-times. Some favourable situations can occur in which the composition of one phase may be systematically varied while the composition of the other remains relatively constant. The most direct way to predict this condition is to refer to ternary-phase diagrams [4,15]. The ternary system, EtOAc–1-BuOH–water (Fig. 14), fulfils the criteria with its converging tie-lines to point S, corresponding to the composition of the lower aqueous phase, which is roughly identical for all biphasic mixtures and immiscible with the corresponding organic phases. Gradients can be run in the normal phase mode by varying the composition of the organic mobile phase from point ‘I’, the initial mobile phase, to point ‘F’,

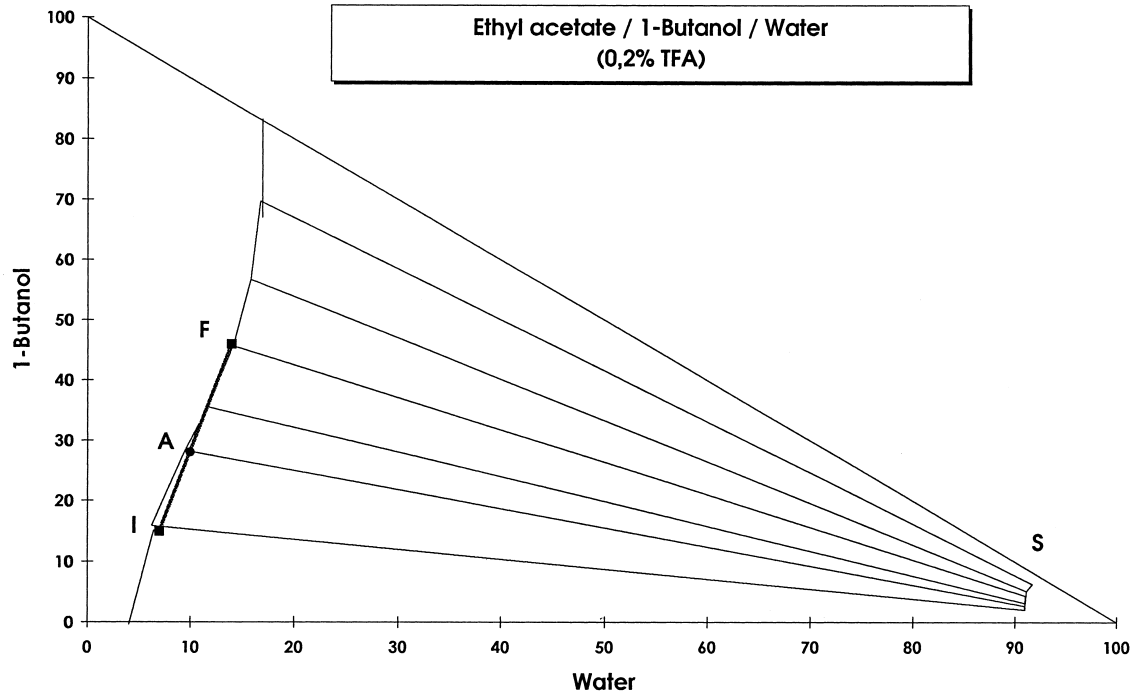

Fig. 14. The EtOAc–1-BuOH–water system.

the final mobile phase. Such a gradient has been used by Renault et al. [14] to purify anthocyanins from champagne vintage by-products (*Vitis vinifera*) using a 5-l pilot CPC system. Anthocyanins differ in respect to the number and location of hydroxy and methoxy groups, the nature and the number of sugars and their position on the aglycon. Moreover, sugars can be esterified by aliphatic or aromatic acids. The structures of isolated anthocyanins are shown in Fig. 15. As these polar and fragile molecules can be easily adsorbed or degraded on solid supports, they are good candidates for CPC purifications. In a preliminary report [16], the same scientists successfully purified anthocyanins on an analytical scale. The originality of the present work is the combined use of a gradient and a large-scale CPC apparatus. This instrument is a stacked disk type rotor with 1040 cells, for a total volume of 5.4-l (Sanki, Kyoto, Japan). Its overall dimensions and mass are 60×81× 106 cm and 300 kg. This chromatograph usually accommodates feeds of 100 to 150 g in up to 1.2 l. For the described experiment, the flow-rate was 60 ml/min and the rotational speed 1140 rpm. The gradient was run from I to F (Fig. 14), and 600-ml fractions were collected. Starting from 7.5 kg of wetted blue-grape marc (skins, seeds and stalks), the injected sample (24.5 g in 500 ml of stationary phase) was estimated to contain a total of 2.8 g anthocyanins. Post-run quantitation was done by integration of the H-4 protons from ^{1}H-NMR spectra recorded in $C^{2}H_{3}O^{2}H$ at 300 MHz. The reconstructed chromatogram is shown in Fig. 16. Fractions 16–21, containing a mixture of the two major anthocyanins, were rechromatographed on an analytical CPC using an isocratic run corresponding to point A, so that the final amount isolated was 0.637 g of Peonidin 3-glucoside, 1.256 g of Malvidin 3-glucoside, and 0.250 g of minor anthocyanins [acylated anthocyanins (fractions 9–12) were not quantified]. The total of 2.14 g anthocyanins obtained in a 0.028% yield from 7.5 kg marc was similar to that reported in the literature.

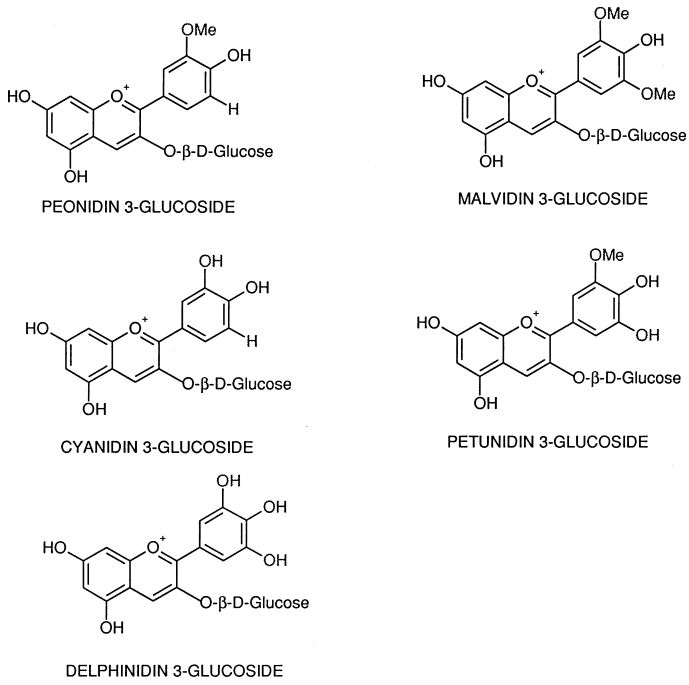

Fig. 15. Structures of isolated anthocyanins from blue grapes.

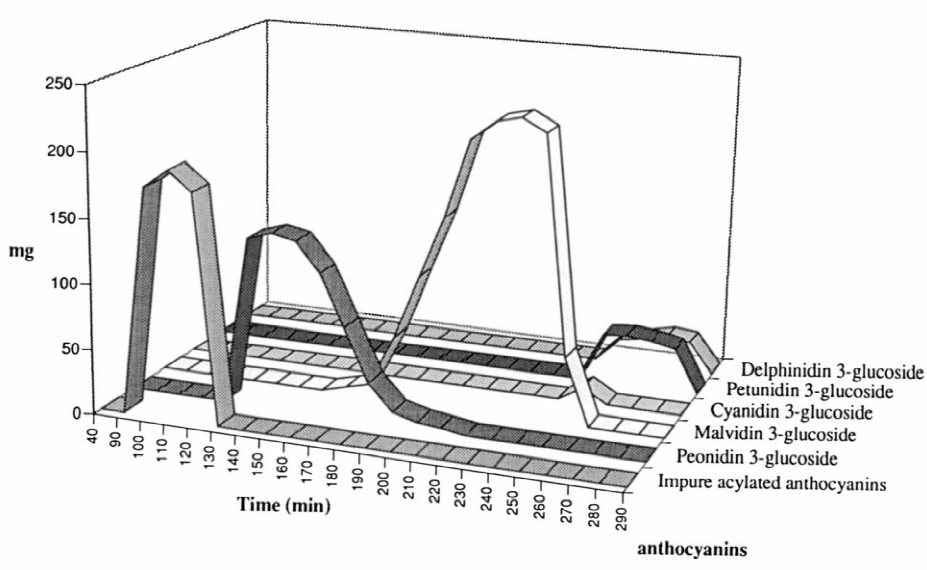

Fig. 16. Elution profile of anthocyanins from blue grapes in a 5-l CPC gradient run of a 24.5 g extract containing an estimated total amount of 2.8 g anthocyanins. The flow-rate was 60 ml/min at 1140 rpm and fractions were collected every 10 min. Post-run NMR quantification. From Ref. [14], with permission.

3.4. Preparative separation of bitter acids from hop extracts by CPC, a work performed by Hermans-Lokkerbol et al. [17,18]

The Dutch School of CPC is very innovative, with Van der Wielen and Van Buel et al. in Delft working on flow regime in CPC and modeling isocratic and gradient elution in CPC, and Verpoorte et al. in Leiden working on preparative applications. The following example, highlighting the specific behaviour of ionizable species when injected in large concentrations, provides an introduction for the subsequent discussion relating to pH-zone-refining and displacement chromatography. Hermans-Lokkerbol et al. [17,18] have used a CPC system model LLN (Sanki), consisting of 6 cartridges (total internal volume 125 ml for 2400 channels), and a preparative separation of α- and β-bitter acids from a crude supercritical carbon dioxide extract of hop cones has been optimized on this instrument for further development on a large-scale CPC system. Extracts of hop cones, the female flowers of *Humulus lupulus* L., are used in the beer brewing process. The main components of hop extracts are the two groups of bitter acids: α-acids or humulones, and β-acids or lupulones (Fig. 17). pK_a values for the α-acids are 4.7, 5.5 and 5.7 for cohumulone, humulone and adhumulone, respectively, and the difference in pK_a values for the α- and β-acids is about 2. Thus, a one-step gradient was necessary to purify both α- and β-acids [18]. The stationary phase was toluene saturated with water, and the mobile phase was successively a 0.1 *M* triethanolamine (TEA) in water, brought to pH 8.4 with HCl, and a 0.2 *M* diethanolamine (DEA) in 20% (v/v) MeOH in water, with phosphoric acid added to pH 9.75. After the development of this gradient, Hermans-Lokkerbol et al. studied the optimization of the yield/cost ratio for the isolation of α-acids by varying the quantity, concentration and volume of the sample injected [18]. An overview of the most informative CPC runs is given in Fig. 18. In all cases, cohumulone, humulone and adhumulone eluted in that order, i.e. by increasing pK_a. The peaks show a rather characteristic sharp fall-down at the moment the next compound starts to elute. Increasing the amount of hop extract injected (from top to bottom in Fig. 18) results in tailing and an increase in fronting, while the maxima are flattened. Very poor resolution was obtained after injection of 20.5 g of hop extract in 40 ml of stationary phase, but square-wave features are still noticeable. The 20.5 g in the 125 ml CPC–LLN is enormous since it would correspond to 820 g injected in a 5-l large-scale CPC! If we look at the dynamic process, the α-acids injected in the protonated form (neutral) in stationary phase at the start of the CPC run are partitioned over the two phases according to their respective distribution ratio at pH 8.4. Consequently, the pH of the mobile phase decreases, as does the ionization of the weak acids, since hop acids are much more concentrated than the TEA buffer, which in fact no longer plays the role of a buffer. This means that a stronger acid (e.g. cohumulone) forces a weaker acid (e.g. humulone) in the stationary phase. This self-regulating process is repeated in the next channels of the CPC and results in the resolution of the α-acids, with the strongest acid moving fastest. The skewed shape of the peaks could be a result of the limited availability of nonprotonated TEA in the mobile phase, while the sharp decline at the end of the peak is a result of the logarithmic relation between *D*, the distribution ratio, and pH [19]. This replacement mechanism differs from pH-zone-refining and displacement chromatography (see below) in that no external displacer is used. However, it is already a kind of displacement chromatography since solutes of the injected sample displace each other due to the variation of the distribution ratio with pH which is

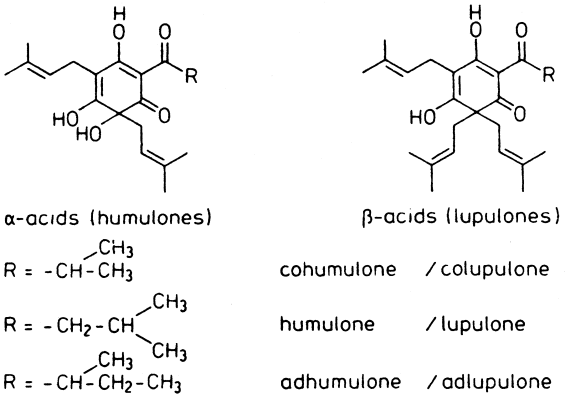

Fig. 17. Chemical structure of the main hop bitter acids. From Ref. [18], with permission.

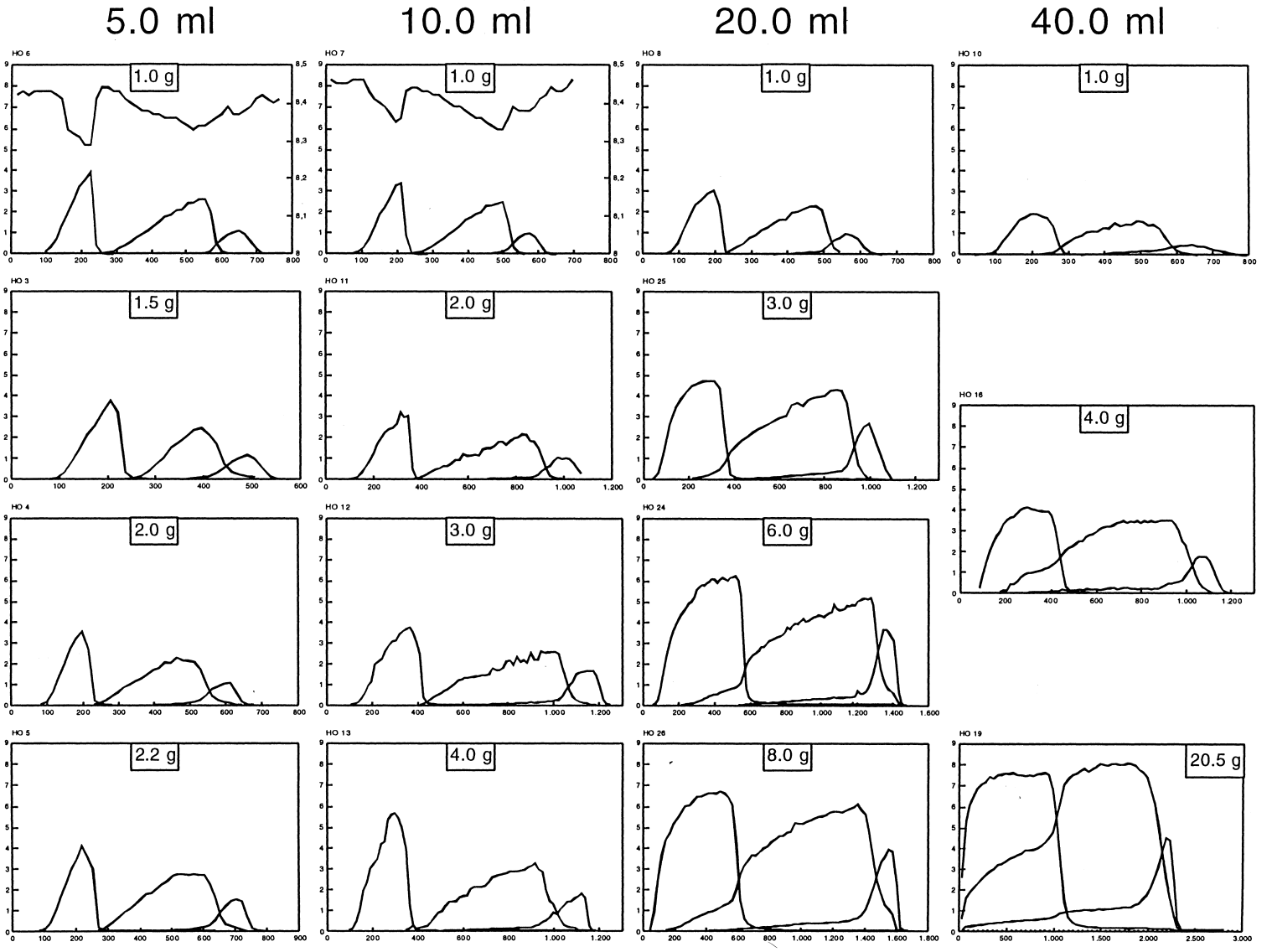

Fig. 18. Overview of CPC runs with variation in injection volumes and amount injected. Hop extract was dissolved in stationary phase. The injection volume is indicated on top of each column and the amount injected is given in each diagram. Horizontal axes: elution volume (ml). Vertical axes: left = concentration of the bitter acid in arbitrary units and right = pH of the eluate. CPC separation was monitored by HPLC analysis of the fractions. Order of elution: cohumulone, humulone, adhumulone. A CPC model LLN was used (total volume 125 ml for 2400 channels) with the two-phase system toluene–0.1 *M* TEA–HCl pH 8.4 in water. Descending mode. Rotational speed: 1100–1300 rpm. Flow-rate: 2 ml/min. From Ref. [18], with permission.

more imposed by these solutes than by the buffer present in the mobile phase.

3.5. pH-zone-refining CCC: displacement of ionizable molecules

Ito, the historical inventor of CCC, is also the inventor of pH-zone-refining, and/or the first to introduce the displacement mode in CCC. The elution pattern of pH-zone-refining CCC bears a remarkable resemblance to that observed in displacement chromatography and isotachophoresis: the chromatogram consists of an isotachic train of rectangular peaks of major components, each with minimum overlap. To clarify the nomenclature and respect the terminology of Ito, we will use pH-zone-refining when referring to ionizable molecules (e.g. carboxylic acids, amines), and displacement chromatography when referring to ionic species (e.g. sulfate, quaternary ammonium).

How does pH-zone-refining CCC work? Typically, a biphasic system such as MtBE–water is used, and the two phases are separated after equilibration. The upper organic phase is then mixed with a proper amount of a 'retainer' acid, such as trifluoroacetic acid (TFA) and used as the stationary phase. A proper amount of an eluant base, such as NH_3, is added to the lower aqueous phase and constitutes the mobile phase. For an experiment, separation is initiated by filling the entire CCC column space with the acidified organic phase followed by introduction of the sample, which is dissolved in the stationary phase. For this example, the sample will be a mixture of carboxylic acids R_iCOOH, with i from 1 to 3 and $pK_{a_1} < pK_{a_2} < pK_{a_3}$. The mobile phase is then pumped through the column while the apparatus is run at optimum speed. If the CCC column is long enough, the system will reach an isotachic state moving at the velocity of the NH_3 border, with the more acidic R_1COOH appearing first in the isotachic train (Fig. 19). At the front of the train (TFA border), acidic TFA protonates R_1COO^- and forces it into the stationary phase. At the end of the train, the most retarded R_3COOH is forced into the mobile phase through deprotonation, leading to R_3COO^- and NH_4^+. R_3COO^- then moves with the mobile phase until it reaches the border between R_3COOH and R_2COOH where it returns to the stationary phase by taking a proton from R_2COOH, displacing it in the mobile phase, and so on from zone to zone. In this example the retainer is an acid and the displacer a base, but we can easily imagine an opposite example in which the retainer would be a base and the displacer an acid. The role of organic and aqueous phases can also be inverted, thus providing four ways of performing pH-zone-refining CCC of ionizable molecules. A complete bibliography can be found in Ito's papers [20,21].

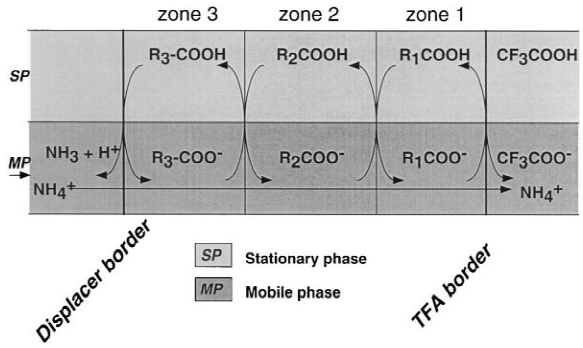

Fig. 19. Schematic representation of an isotachic state obtained in pH-zone-refining CCC. Each zone contains only one species and is demarcated by two well-defined boundaries. TFA is the retainer acid, and NH_3 the displacing base.

3.5.1. Purification of alkaloids by pH-zone-refining CCC, a work performed by Ma et al. [22]

One important application of pH-zone-refining CCC is to isolate biologically active compounds such as alkaloids from plant extracts [22]. Fig. 20 shows the separation of 3 g of three basic alkaloids from a crude extract of *Crinum moorei* using the acidic aqueous phase (Fig. 20A) or the basic organic phase (Fig. 20B) as mobile phase. In both cases, a binary MtBE–water solvent system was used, where $(Et)_3N$ (5–10 m*M*) was added to the organic phase, and HCl (5–10 m*M*) to the aqueous phase. Of course, elution occurs in reverse order, but the interesting point is that in A (aqueous mobile phase) the alkaloids were eluted as HCl salts, while in B (organic mobile phase) they are eluted as free bases. More generally, with pH-zone-refining CCC, ionizable molecules can be purified as neutral ones, e.g. free acids or free bases, or as salts, and the co-ion can be chosen by selecting the appropriate acid or base as displacer,

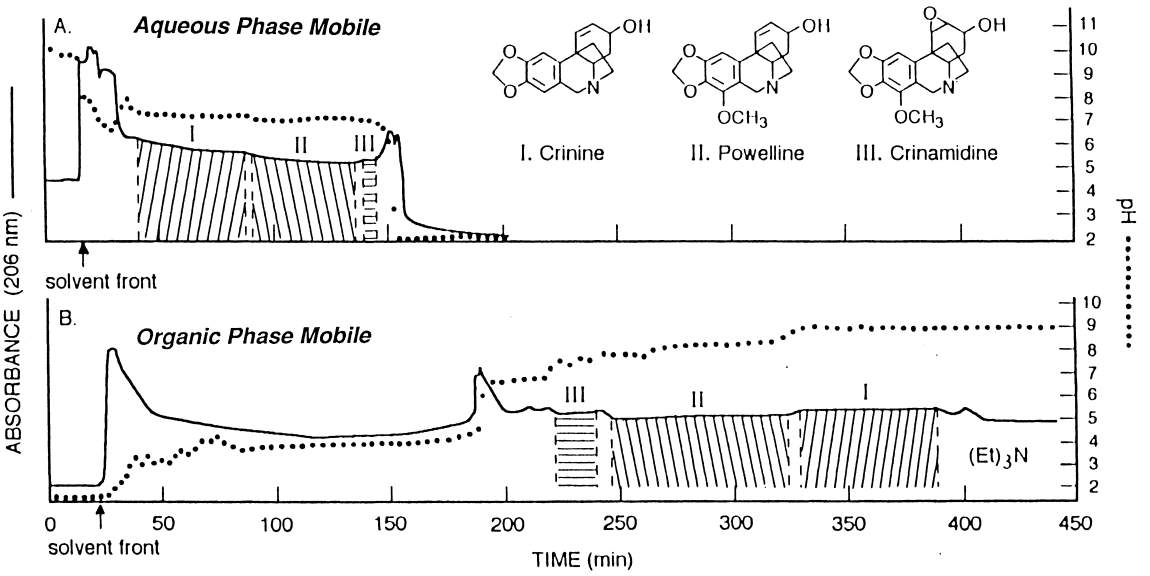

Fig. 20. pH-zone-refining CCC of crude alkaloid extract of *Crinum moorei*. This work was performed on a P.C. Inc. CCC system, with a coil of 325 ml. Solvent system was MiBK–water. Stationary phase is (A) upper organic phase with 5 m*M* $(Et)_3N$ and (B) lower aqueous phase with 10 m*M* HCl. Mobile phase was (A) lower aqueous phase with 5 m*M* HCl and (B) upper organic phase with 10 m*M* $(Et)_3N$. The flow-rate was 3.3 ml/min in (A) head to tail and (B) tail to head mode. 3 g dissolved in 30 ml of equal volume of each phase were injected in each case. Revolution is (A) 800 rpm and (B) 600 rpm [22].

provided that the retainer goes more into the stationary phase and the displacer more into the mobile phase.

3.5.2. NMR monitoring of pH-zone-refining CPC, a work performed by Spraul et al. [23]

In pH-zone-refining, solutes are not eluted as separate peaks but as contiguous blocks of constant (generally high) concentration, so that it is highly difficult or even impossible to monitor the separation by means of a UV detector. On-line pH monitoring is generally used, allowing the observation of transitions between solutes since each square wave zone has its own pH determined by the pK_a and the solute concentration it contains. Renault et al. experimented an original coupling of pH-zone-refining CPC with NMR in collaboration with the Bruker company. The choice of NMR as a detection and analysis method is adequate, even at modest field strength, since sensitivity is not a limiting factor. It gives maximum structural information and allows measurement of the relative concentrations of eluted compounds. In the following example, the biphasic system was MtBE–D_2O, with the upper organic phase acidified by TFA and the lower aqueous phase basicified by NH_3. The test mixture to be separated consisted of three dinitrophenyl (DNP)-amino-acids: Leu, Ala and Asp. NMR data were acquired on a Bruker DRX500 spectrometer equipped with a 4-mm inverse dual $^1H/^{13}C$ flow probe with triple axis gradients. Other details can be found in Ref. [23]. The experiment was carried out in the ascending mode on stop-flow mode with time intervals of 3 min between the stops (to allow the bleeding stationary phase to decant partially away from the NMR observation zone). The result is summarized in Fig. 21. The distribution of the solutes as a function of elution time, which was quantified by the value of the integral of the α-proton signals, is shown in Fig. 22. DNP-Ala and DNP-Leu are mono-acids and were eluted in their acidic neutral form. Their concentrations were approximately those of the displacer (i.e. TFA). DNP-Asp was eluted partly in its monoanionic form and partly in its diacidic form, due to the relative pK_a values of DNP-Asp and TFA. This coupling of CPC with NMR, successfully achieved using currently avail-

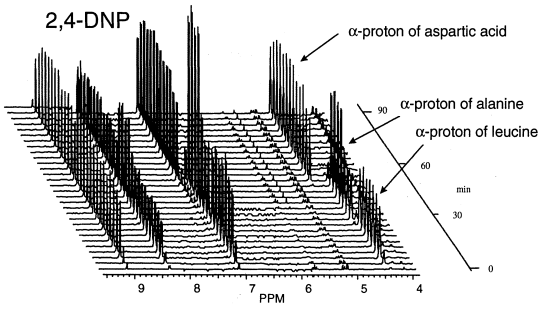

Fig. 21. Stacked plot of the ^{1}H-NMR of the CPC effluent, in the zone of aromatic and α-protons. This work was performed on a Sanki HPCPC system, with a rotor of 240 ml. Solvent system was MtBE–water, with TFA (13 m*M*) in the upper organic phase and NH_3 (22 m*M*) in the lower aqueous phase. The ascending mode was used at 2 ml/min and 800 rpm. Injected sample: 0.46 mmol DNP-Leu + 0.41 mmol DNP-Ala + 0.55 mmol DNP-Asp in 9.5 ml of stationary phase + 0.5 ml of MtBE. From Ref. [23], with permission.

able HPLC–NMR instrumentation technology, opens the way to new applications of CCC and CPC, e.g. for fast structural determination of labile molecules.

3.6. Ionic molecules: ion-exchange displacement CPC, a work performed by Chevolot et al. [24]

In contrast with the ionizable molecules usually purified by pH-zone-refining, the following example concerns ionic compounds, i.e. those which keep their charge at any pH in aqueous solution. The sample was a mixture of partially depolymerized fucans (F_i^-, whatever the number of negative charges), a family of polysulfated polysaccharides

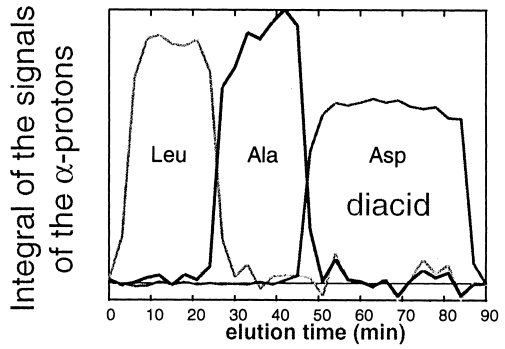

Fig. 22. pH-zone-refining CPC of DNP-Leu, DNP-Ala and DNP-Asp. Concentrations were measured as the integral of the signals of the α-protons. From Ref. [23], with permission.

extracted from brown seaweed. Fucans can be roughly described as polymers of sulfated fucose units also bearing galactose, xylose and uronic acids. These polymers are highly polar and bear many negative charges, so that they first appear to be water-soluble only and unsuitable for a CPC purification. However, they are when ion-exchange is used in liquid–liquid systems. A liquid anion-exchanger called Amberlite LA2 (Rohm and Haas) has been used. It is a high-molecular-mass, oil-soluble secondary amine with excellent solubility in most organic solvents and extremely low solubility in aqueous solutions. LA2 is dispersed in an organic solvent such as MiBK and protonated by an aqueous HCl solution: this water-saturated organic phase will be the stationary phase. The sample is solubilized in 10 to 20 ml of the same organic phase ($LA2H^+$, Cl^- + MiBK), where it is surprisingly well-soluble (a few ml of MiBK-saturated water are added to complete dissolving), and injected into the CPC instrument. The ion-exchange displacement chromatographic run then begins, using OH^- as a displacer, which displaces all anions from the stationary phase to the mobile phase through deprotonation of the weak anion-exchanger. Provided that the selectivity coefficients between anions are different from 1, the displacer will force the desorption of fucans, which develop into adjacent square wave zones of homogeneous material. If the CPC column is long enough, the system will reach an isotachic state moving at the velocity of the displacer, with the least strongly adsorbed fucan appearing first in the displacement train (Fig. 23). At the front of the train, Cl^- anions are displaced by the less retained fucan, F_1^-, and eluted as NaCl if Na^+ has been chosen as the co-ion in the mobile phase. At the end of the train, the most retarded fucan, F_n^-, is forced into the mobile phase through deprotonation of the weak anion-exchanger. In the whole train, the conservation of the ionic charge means that the molarity (in equivalent/l) is that of the displacer in the mobile phase (OH^-). Size-exclusion chromatography (SEC) was performed for every fraction collected. Fig. 24 shows five typical SEC patterns of CPC fractions, while Fig. 25 shows the relative peak molecular mass, M_p, superimposed on the pH curve of the effluent. It can be seen that fucan elution starts with low-molecular-mass polysaccharides (tubes 22 and 24), followed immediately by a transition zone

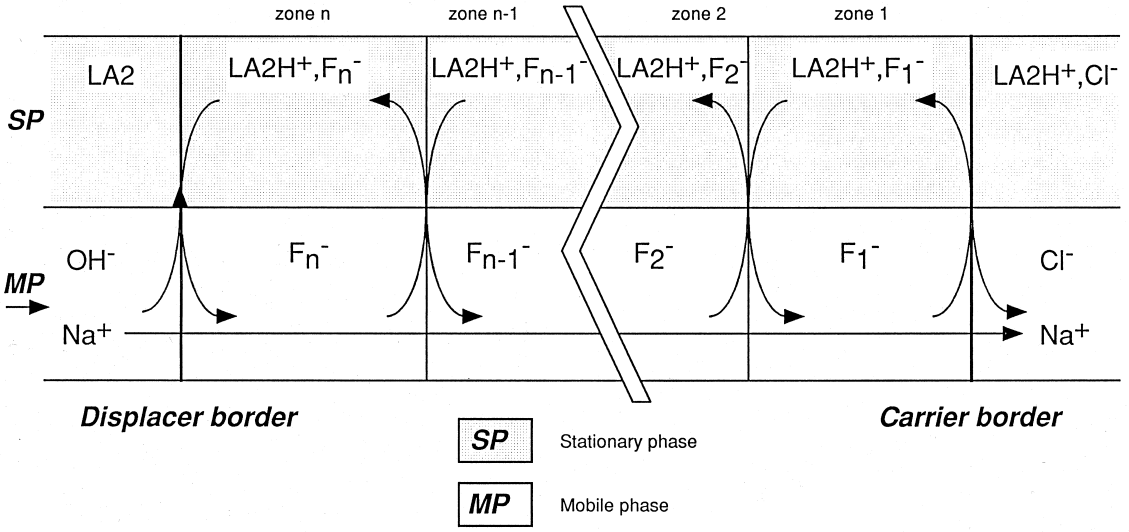

Fig. 23. Schematic representation of an isotachic state obtained in ion-exchange displacement chromatography. If the column is long enough, each zone contains only one species and is demarcated by two well-defined boundaries. Cl^- is the carrier ion (the less retained ion) and OH^- is the displacer ion, which displaces the most retained ion through deprotonation of the weak anion-exchanger.

(e.g. 27) in which both the low-molecular-mass and the highest-molecular-mass polysaccharides are found. Three characteristic families are then eluted (tubes 32–54) close to each other, considering their peak molecular mass, but different in both their SEC shapes (all remarkably symmetrical) and the corresponding pH of their elution zones (pH 5.13, 5.44, 7.2). Owing to the low difference in molecular mass,

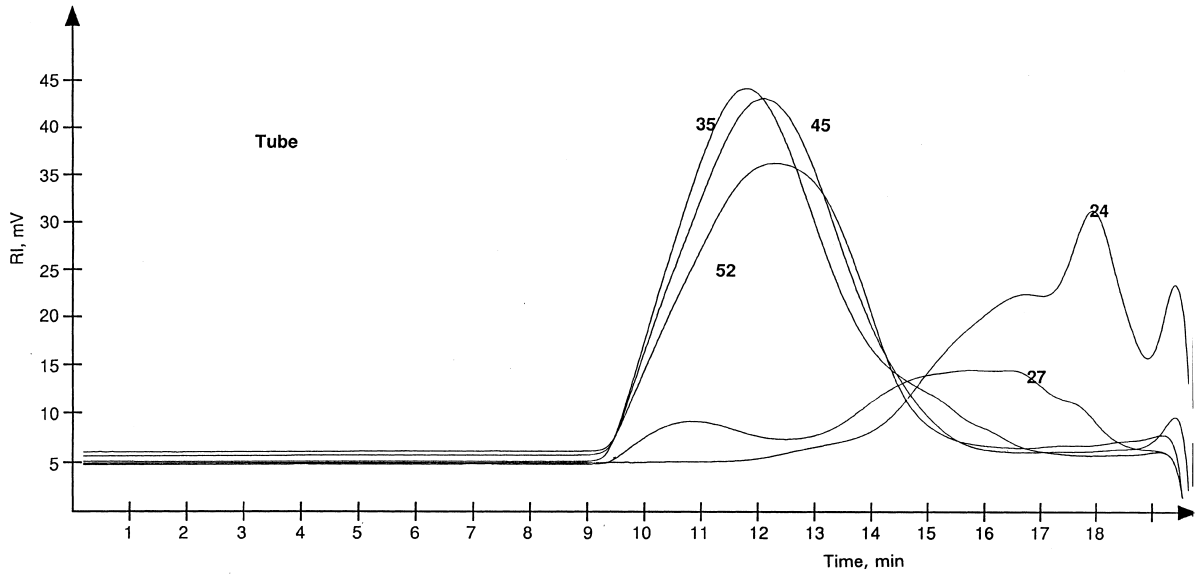

Fig. 24. SEC chromatograms of 5 CPC fractions representative of five typical zones of the CPC run. Tube numbers refers to the *x* scale of Fig. 25. A mass of 800 mg of partially depolymerized fucans were injected in a Sanki HPCPC system (204 ml) and fractionated by ion-exchange displacement CPC. The stationary phase was 10% (v/v) $LA2H^+$, Cl^- in MiBK, saturated with water. The mobile phase was 0.025 *M* aqueous NaOH, saturated with 10% LA2 in MiBK. Descending mode, at 2 to 5 ml/min [24].

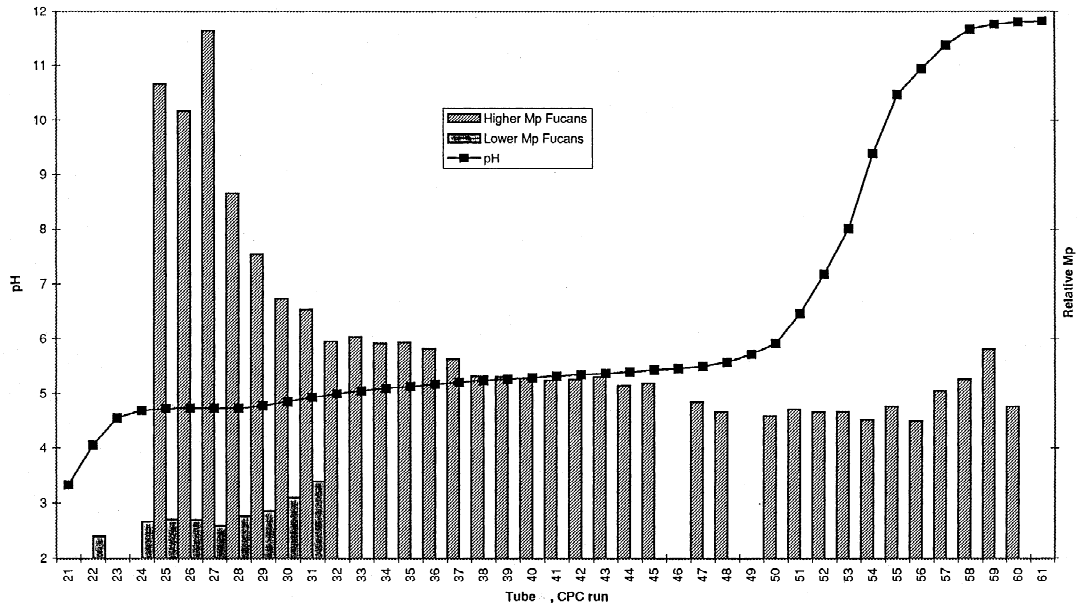

Fig. 25. Changes in peak molecular mass, M_p, of the fractionated fucans by ion-exchange displacement CPC. From tube 32 to 60, M_p varies little, but the corresponding SEC shapes are different (Fig. 24) [24].

it was impossible to separate these three families by preparative SEC. Anion-exchange chromatography on solid resins gave no satisfactory separation, probably because a rigid matrix cannot adopt the right shape to interact differentially with very similar molecules and thus to discriminate between them. Obviously, such a limitation does not exist with a liquid ion-exchanger. More data will be found in Ref. [24].

4. Conclusion

The improvement of CCC and CPC apparatuses and the arrival of new companies with analytical and preparative instruments have provided scientists with more tools to resolve their purification problems, provided that they are not discouraged by the very large number of solvent systems they can use. We hope that the few rules reported here, and the diversity of examples found in the recent literature, will prompt more scientists to get involved with CCC and CPC techniques. From the purification of very nonpolar fullerenes [25] to that of very polar fucans [24], from the 15-ml mini-coil to the 5-l rotor, from off-line monitoring by TLC to on-line detection by NMR, whether isocratic, gradient, dual-mode elution, pH-zone-refining or the displacement mode is used, it is to be hoped that everyone will find the best way to obtain his or her compound.

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