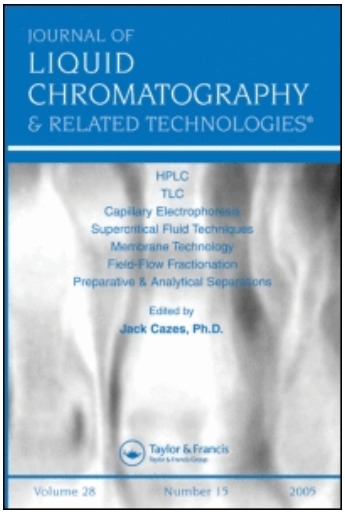

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Gradient Elution Centrifugal Partition Chromatography: Comparison with HPLC Gradients and use of Ternary Diagrams to Build Gradients

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GRADIENT ELUTION CENTRIFUGAL PARTITION CHROMATOGRAPHY: COMPARISON WITH HPLC GRADIENTS AND USE OF TERNARY DIAGRAMS TO BUILD GRADIENTS

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Abstract

Just as with HPLC, gradient elution provides to CPC an easy way to fractionate solutes of widely differing polarities and partition coefficients, and to reduce run times. The easiest way to design a gradient for CPC is to refer to ternary diagrams corresponding to the ternary liquid mixtures generally encountered in centrifugal partition chromatography.

Introduction

CPC and HPLC are very similar in many aspects, (i) the fundamental principle, *i.e* partition of solutes, which will govern where a molecule will prefer to stay, in one or in the other phase, (ii) the goal, which is to differentiate solutes in a mixture, in order to isolate one or several of them, (iii) the way to do it, which is to move one phase through the other one from the beginning to the end of an apparatus designed for keeping one phase stationary, (iv) to collect eluates if they come out, and (v) the viability of the process, which means when one produces a pure compound and wants to sell it, one must deal with the costs with respect to the market prices.

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As for optimization of the experimental conditions, both isocratic and gradient elutions are very useful for HPLC; we were very interested in determining if gradients could be used with CPC for many applications.

Two examples have been published recently[1], using the very simple solvent system Hexane/Methanol/Water, (which is very similar, chromatographically, to commonly used reversed-phase HPLC), and the solvent system Chloroform/Methanol/Water, for which the composition of the stationary phase (chloroform rich) is varied during a gradient run (enrichment with methanol), resulting in a small degree of instability, but not enough to make the stationary phase elute.

These examples have been pooled with some new results obtained in our laboratory, and they are reported here.

Experimental

CPC separations :

A Centrifugal Partition Chromatography apparatus (Model CPC LLN) manufactured by Sanki Engineering (Nagaokakyo, Kyoto), and currently available in the U.S. from Sanki Laboratories (Sharon Hill, PA, USA) was used. It consists of a continuous-flow centrifuge containing 6 partition cartridges (type 250 W; total volume 128 ml), a valve connection unit (FCU II) equipped with a 4 ml PTFE sample loop injector, an electric power supply unit (PCB II). Each partition cartridge contains four polychlorotrifluoroethylene plates engraved with 100 channels, giving a total of 2400 channel for 6 cartridges.

The pump was a Perkin Elmer series 4, controlled with a PE 7000 laboratory computer. The pump has four solvent reservoirs, so that one can be used to fill the CPC instrument with the stationary phase and two other for running a gradient. The detector was a UV Vis detector (Linear Instruments, model 200, Reno, NV) with a variable pathlength illuminated volume preparative scale flow cell (model 0203-7083). The detector was connected to a recorder (Fisher), or to the Perkin Elmer laboratory computer.

Chemicals :

Water was deionized, and solvents were HPLC or ACS grade (Aldrich Chem. Co., Milwaukee, WI).

Solutes were from various sources; peptides were from Sigma Chem. Co., St Louis, MO.

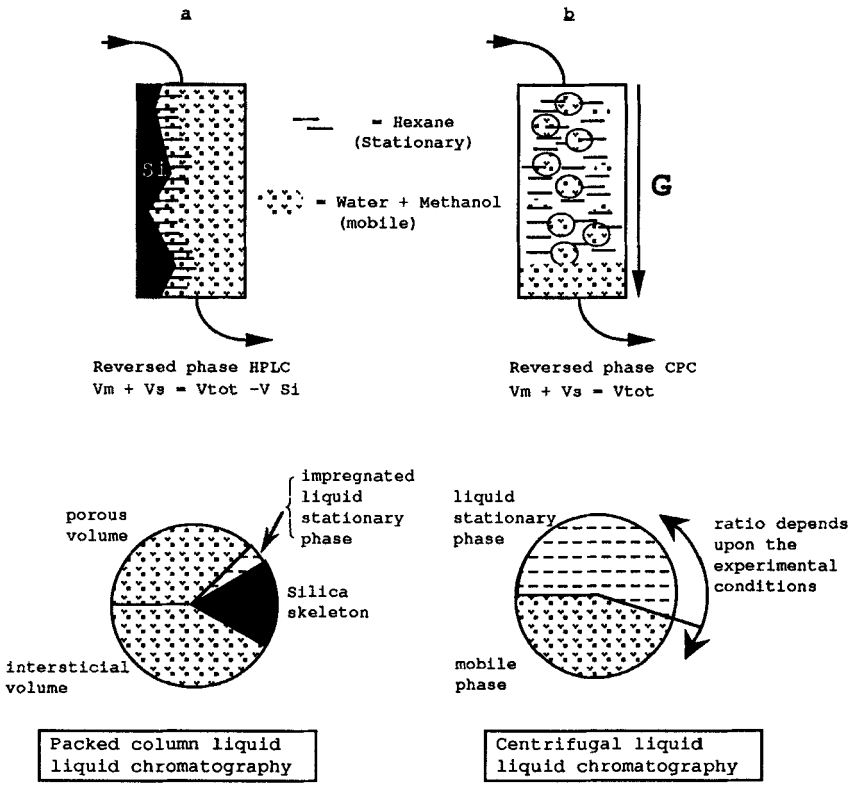

Figure 1 : Comparison between conventional liquid liquid reversed phase chromatography and centrifugal liquid liquid reversed phase chromatography.
a : Surface of Silica is bonded with hexyl silane, and saturated with methanol, which constitutes the impregnated liquid stationary phase of hexane/methanol (only ≈5% of the total volume) in equilibrium with the water/methanol mobile phase (which occupies the interstitial and most of the porous volume, *i.e.* ≈ 75% of the total volume). The bulk volume of silica, which does not participate in the partition process, occupies ≈20% of the volume.
b : Silica is eliminated and a strong gravity field allows the heavy droplets of water/methanol to flow through the hexane (saturated mainly with methanol) light stationary phase. The total volume is used for the partition process, and the ratio $\frac{\text{stat.}}{\text{mob.}}$, depending upon the experiment, is approximately 50/50.

Results and Discussion

Figure 1 shows a comparison between HPLC and CPC. Reversed-phase chromatography with hexyl moiety and Methanol/Water solvent system for HPLC, and Hexane/Methanol/Water for the CPC, are chosen because of their popularity. With the solid support, the total volume is shared by the bulk volume of the solid support (≈20%, v/v) (which does not participate in the partition process), by the $-C_6H_{13}$ grafted moiety, fully solvated with methanol and providing an impregnated liquid stationary phase rich with this solvent (5%, v/v), and by the mobile phase, *i.e.* a mixture of water and methanol, which occupies the major part of the porous volume in the silica beads (≈35%, v/v), and all the interstitial volume between them (40%, v/v)[2]. With the gravity field, in CPC, the total volume is shared by the liquid stationary phase, hexane saturated mainly with methanol, and the mobile phase, a mixture of methanol and water saturated with hexane. The volume ratio of the stationary phase is approximately 50%; the actual value depends upon the experimental conditions and upon the composition of the ternary mixture which is used to prepare the mobile and stationary phases.

Some favorable situations may occur, when 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, which are graphic representations of ternary mixtures, with their monophasic and biphasic regions (if there are any), and which allow one to calculate the composition of upper and lower phases when a biphasic system exists.

Figure 2 shows two ways to represent the composition of a mixture of 3 solvents ①, ② and ③. The axis may be expressed in volume%, mass%, or mole%, the two first unit being more convenient for chromatographic purposes. Representation 2a is widely used, but is not readily compatible with spreadsheets and computers, which prefer to deal with orthogonal representations for a given set of data; moreover it is redundant, as ①+②+③ = 100.

It is easier to use the representation 2b, where only the percentage of solvents ① and ② (or ② and ③, or ① and ③), are displayed. In this way, it is very easy to use computers to draw the tie-lines and the binodial, and to get the volume% graph from mole% or mass% data. The binodial is the line separating the monophasic and biphasic zones. Any point along a tie-line will give the same composition for the upper and

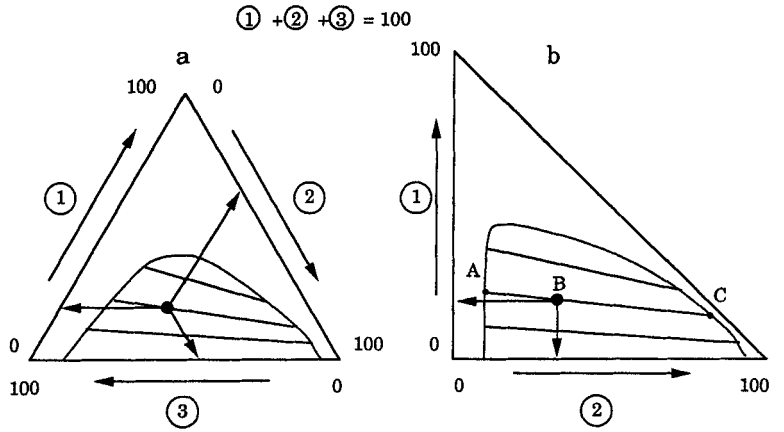

FIGURE 2. Composition of a ternary solvent system.
a/ ternary representation, difficult to manipulate with computers and spreadsheets.
b/ orthogonal representation, giving the same amount of information, and very easy to manipulate with computers.

lower phases, but with different volume ratio. A point very close to the binodial and in the biphasic zone will produce roughly one phase, saturated with few droplets of the other.

Hundreds of ternary diagrams have been described in the literature; a large compilation was published by Sørensen and Arlt[3] in a collection entitled Liquid Liquid Equilibrium Data Collection, which is large, expensive, and gives the data in mole%.

Ternary diagrams are very useful to understand how to vary the composition of a ternary mixture to significantly change the properties of a biphasic system, and to predict the consequences of varying the composition of one phase upon the composition of the other.

Figure 3 compares three ternary diagrams chloroform/alcohol/water, with the alcohol as ordinate and water as abscissae. Tie-line orientations clearly indicates where an increase of the alcohol content of a mixture will lead. (i) For methanol (3a) the composition of the chloroform-rich phase will vary only slightly when the alcohol content of the mixture is increased from M to M', while the composition of the water rich phase will vary from ≈10 to ≈40% methanol (v/v).

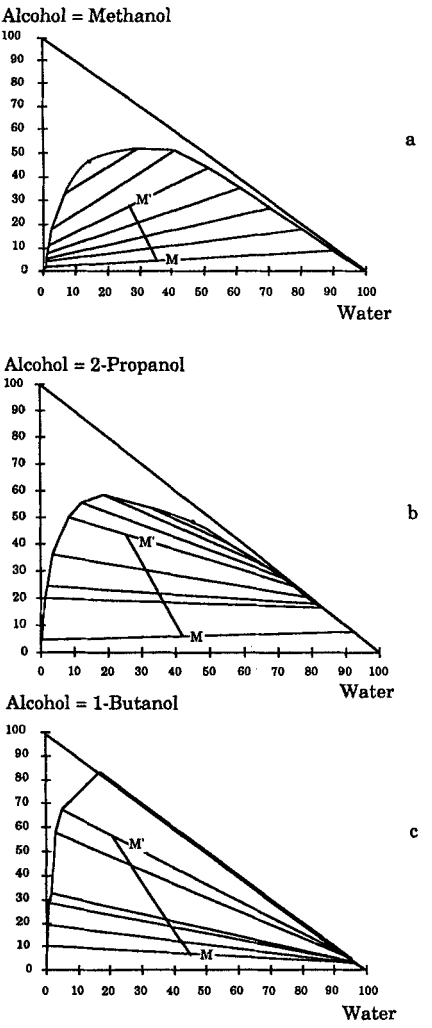

FIGURE 3. Ternary diagrams for 3 chloroform/alcohol/water solvent systems. Tie lines orientation clearly indicates where will preferentially go an increase of the alcohol content of a mixture (*e.g.* from M to M').

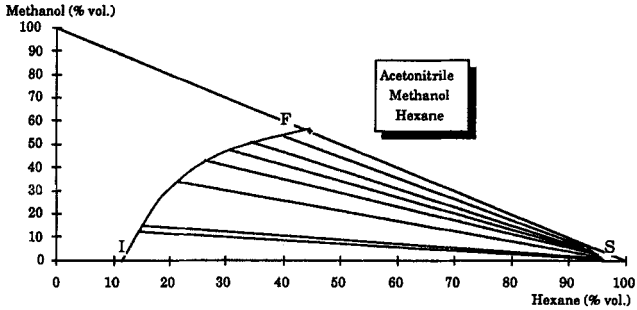

FIGURE 4. Ternary diagram for the solvent system acetonitrile/methanol/hexane, an example of non aqueous solvent system which can be used to build up gradients

(ii) In the case of isopropanol (3b), the composition of the two phases varies greatly with the level of alcohol, which distributes evenly in water and chloroform.

(iii) For 1-Butanol (3c), the composition of the chloroform rich phase will vary extensively (≈10 to ≈70% 1-butanol (v/v)), while the water rich phase will remain approximately constant. In this case, if we fill the CPC apparatus with the water rich phase, we should be able to vary the polarity of the other phase by adding butanol in chloroform, provided we stay close to the binodial for the composition of the mixture used as the mobile phase. We can then go from 10 to 70% 1-butanol in chloroform (v/v), saturated with water, to elute from a "water column", and run, in this case, a gradient of increasing polarity on a "normal phase column".

Many ternary diagrams indicate this kind of behavior, most with water as one of the constituents; but some of them are non aqueous, *e.g.* the system acetonitrile/methanol/hexane, the ternary diagram of which is shown on Figure 4. The hexane rich phase (S) can be used as the stationary phase; and the other (mobile) phase can be varied from I (≈90% acetonitrile, 10% hexane) to F (≈55% methanol, 45% hexane), approximately following the binodial.

The first gradient we tried was the system water/methanol/hexane, with hexane as the stationary phase (Figure 5). This system is very close to the widely used methanol water elution with a reversed-phase column in HPLC. We started by first filling the CPC with a mixture of stationary +

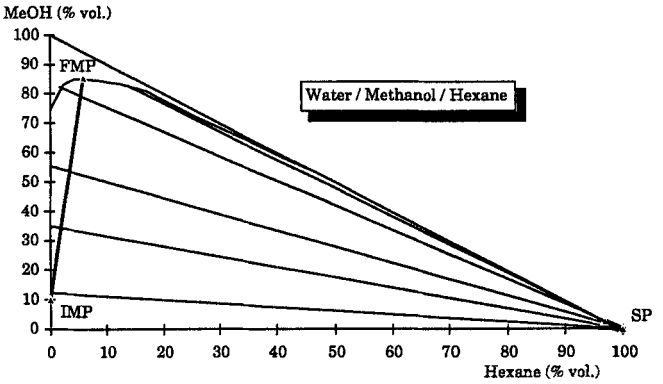

FIGURE 5. Ternary system Hexane/Methanol/Water
Compositions of the liquids phases used to generate a gradient of methanol in water on an hexane-rich liquid stationary phase :
IMP : Initial mobile phase
FMP : Final mobile phase
SP : Stationary phase

initial mobile phase, with a delivery ratio equal to the volume ratio we wanted to use for the run, using a high flow rate and no rotation for the CPC rotor; then, when it was full, we started rotation and pumped the initial mobile phase, and the CPC was equilibrated within a few minutes.

A typical result is shown in Figure 6, where we injected approximately 1 g of a mixture of aromatic alcohols, aldehyde and polynuclear hydrocarbons. The baseline is very stable, and we have repeatedly run several gradients, one after the other, each time returning to the initial conditions without special care, as is done with HPLC. During this run, the flow rate was varied from 3 to 4 ml/min, and the rotational speed from 800 to 1000 rpm because the back pressure decreases during a gradient, not because the volume of the stationary phase decreases, but the density difference between the two phases decreases, and the interfacial tension decreases too. This has often been observed. So we can increase the flow rate to increase the efficiency, as described by D. Armstrong *et al.*[4], and increase the rotational speed to favor the stability of the stationary phase. In all these experiments, the volume of the stationary phase remained extraordinarily constant, which means the way we use the ternary diagram to build up the gradient seems to be valid.

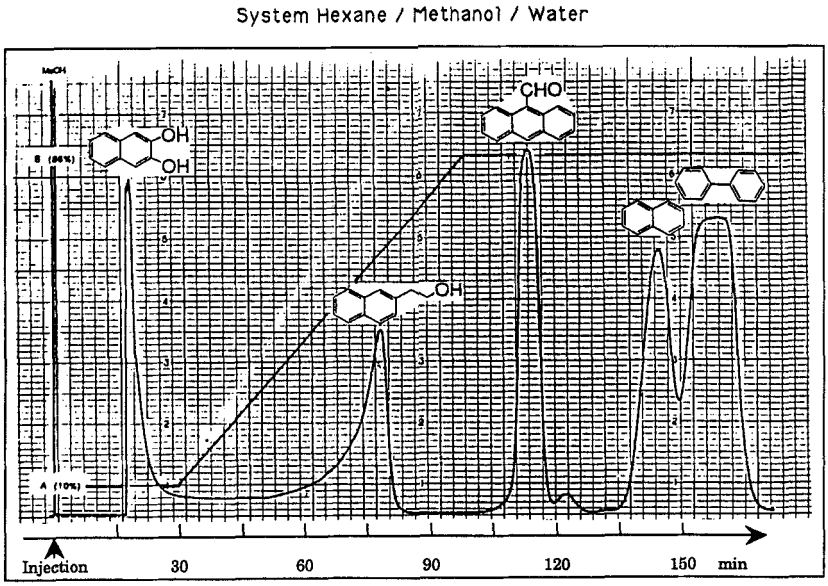

FIGURE 6. Gradient elution with the Hexane/Methanol/Water system
Compositions initial (IMP) and final (FMP) mobile phases, and of stationary (SP) phase are calculated from the diagram of Fig. 5.

	C_6H_{14}	CH_3OH	H_2O
IMP	sat.	10	90
FMP	≈6(sat.)	85.9	8.1
SP	≈100%, saturated with A		

Vo (mobile phase volume) : 45 to 50 ml
Mobile phase : CH_3OH/H_2O-rich phase, descending mode

Rotational speed : 800 to 1000 rpm
Flow rate : 3 to 4 ml/min
Pressure drop : 50 to 40 bars
Detection UV at 254 nm, 1.28 AUFS

Sample in 4 ml	Naphtalene diol	238 mg
	Naphtalene ethanol	386 mg
	9-Anthraldehyde	27 mg
	Naphtalene	225 mg
	Biphenyl	114 mg

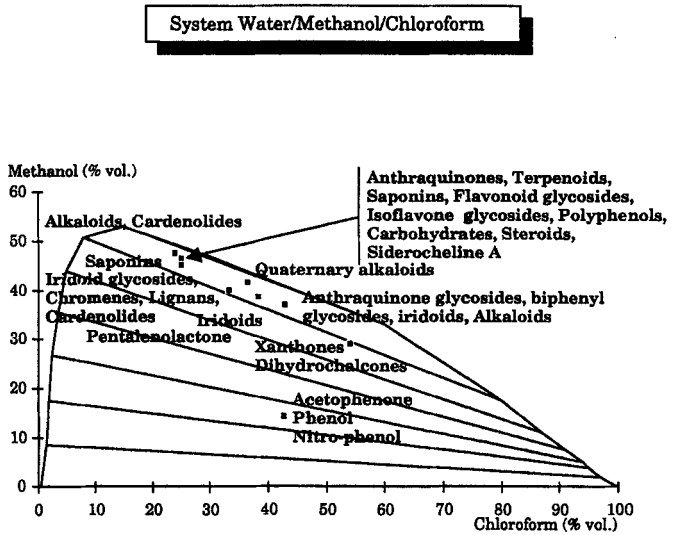

FIGURE 7. Data found in the literature for compounds and families purified using the ternary system Chloroform/Methanol/Water

The second system we examined was a bit less well behaved, the system water/methanol/chloroform. This solvent system has been widely used for isolation of natural products, and it was one of the favorites when droplet countercurrent chromatography was popular. Various mixtures have been used[5], and points corresponding to their composition have been indicated in Figure 7.

Most of the points are located between the top two tie-lines; they all produce roughly the same biphasic system, with the same lower and upper phase compositions.

How can we imagine a gradient with this system (Figure 8)? If we want to go from IMP to FMP for the mobile phase, the stationary phase will have to go from ISP to FSP to be equilibrated. This will generate a decrease in the chloroform content of the stationary phase, mostly replaced by methanol. This can result in a degree of instability with appearance of droplets of chloroform in the effluent Typically, the variation involves an increase of the content of the mobile phase by about 15%. If the CPC

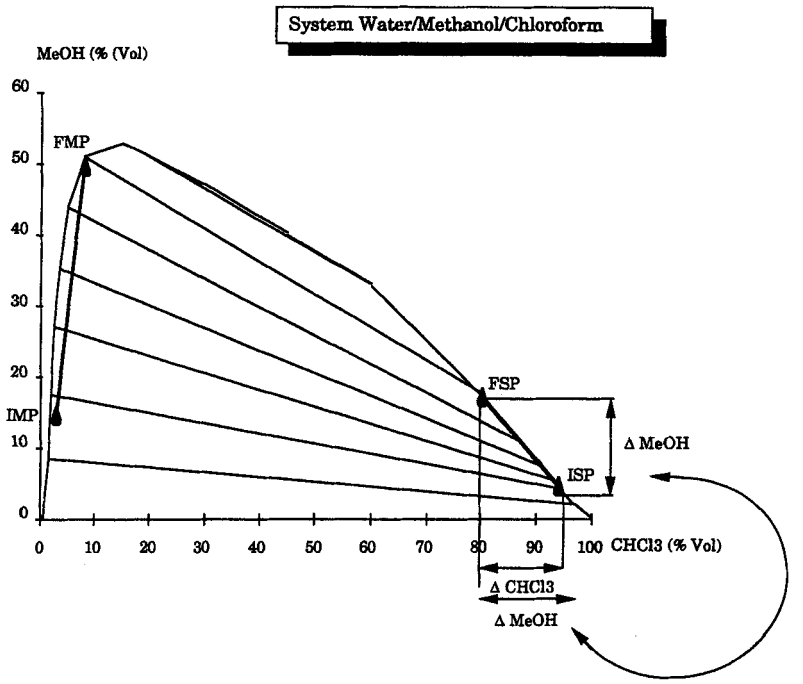

FIGURE 8. Ternary system Chloroform/Methanol/Water. Compositions of the liquids phases used to generate a gradient of methanol in water on a chloroform-rich liquid stationary phase :

IMP : Initial mobile phase
FMP : Final mobile phase
ISP : Initial stationary phase
FSP : Final stationary phase

instrument is eluted in the reverse manner at the end of the gradient, the volume ratio of mobile to stationary reverts to what it was at the beginning, and the next injection can be performed without refilling the instrument.

Figure 9 shows the chromatogram for 3 test solutes using this gradient. About 1.7 g was injected. The shape of the phenol peak is characteristic of a solute eluted at saturation in the mobile phase;

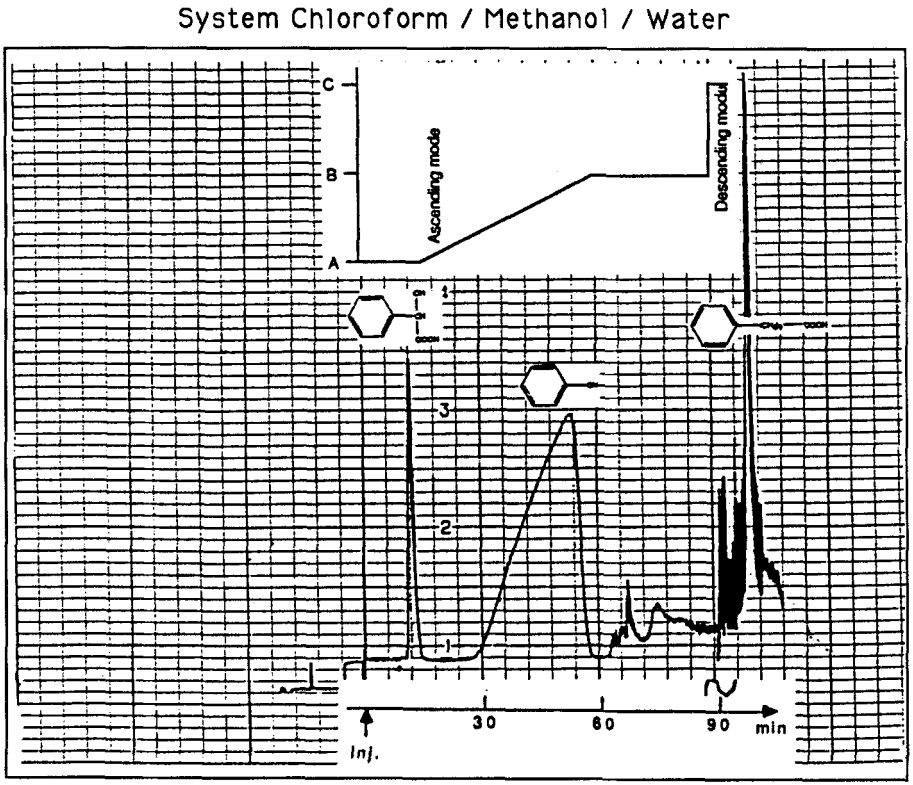

FIGURE 9. Gradient elution with the Chloroform/Methanol/Water system Compositions initial (IMP) and final (FMP) mobile phases, and of stationary (ISP) phase are calculated from the diagram of Fig. 8.

	$CHCl_3$	CH_3OH	H_2O
IMP	2	15	83
FMP	8	50	42
ISP	94	5	1

Vo (mobile phase volume) : 45 to 60 ml
Mobile phase : CH_3OH/H_2O-rich phase, ascending mode

Rotational speed : 500 rpm
Flow rate : 4 ml/min
Pressure drop : 50 to 30 bars
Detection UV at 254 nm, 1.28 AUFS

Sample in 4 ml	Mandelic acid	410 mg
	Phenol	871 mg
	4-Phenyl butyric acid	398 mg

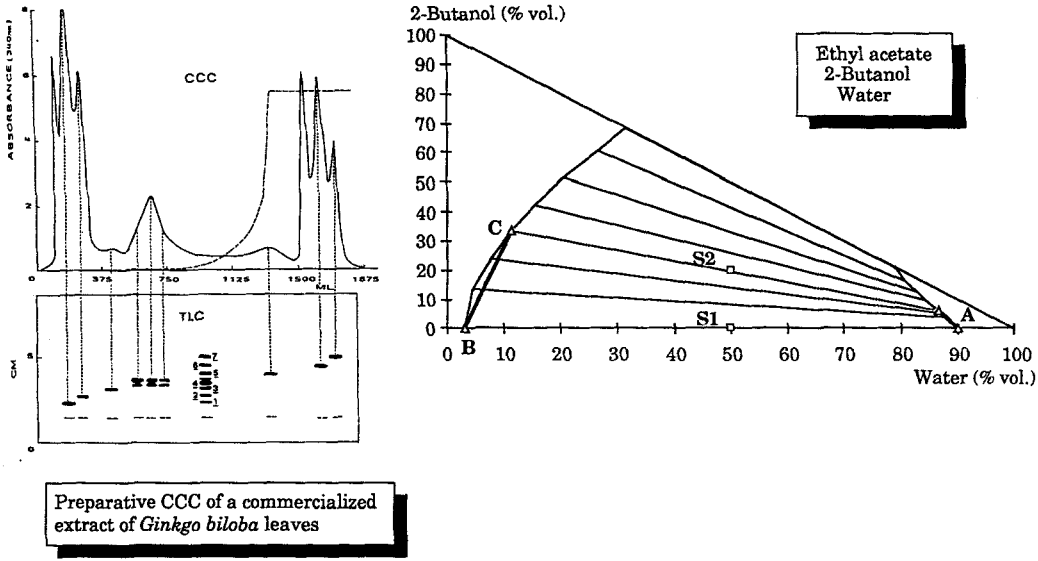

FIGURE 10. Ternary system Ethyl acetate/2-Butanol/Water Compositions of the liquids phases used to generate a gradient of butanol in ethyl acetate on a water-rich liquid stationary phase :

S1 and S2 : Ternary systems prepared by Vanhaelen *et al.* [6] to generate A, B and C

A : Initial stationary phase

B : Initial mobile phase

C : Final mobile phase

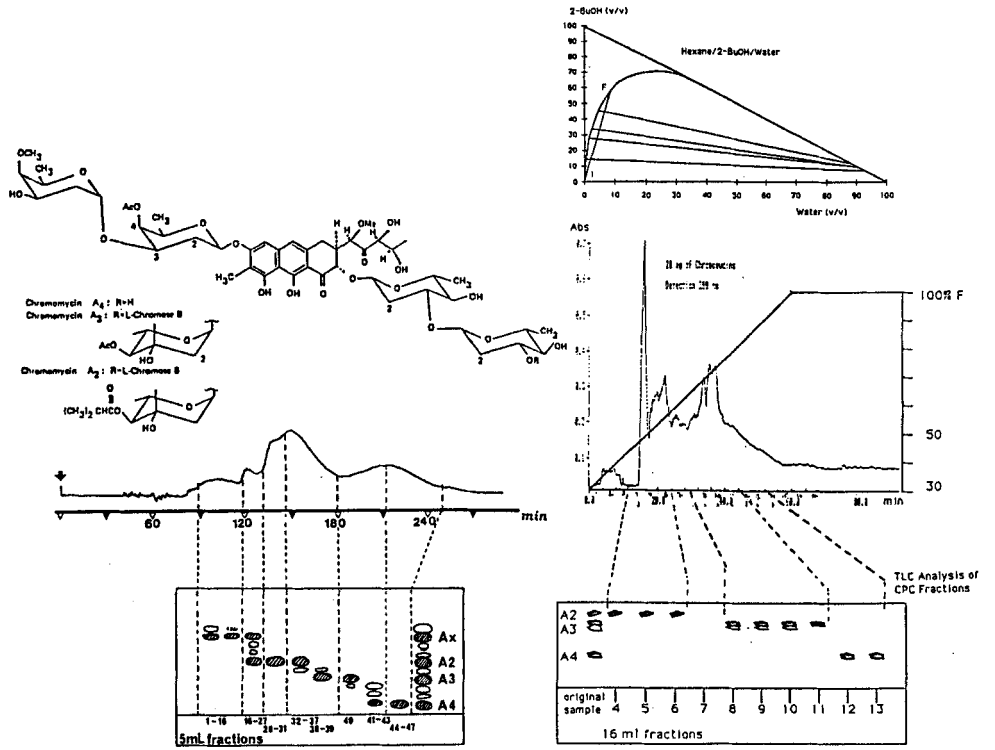

Figure 11 : Comparison between isocratic and gradient run, for fractionation of Chromomycins. :
Isocratic mode : system toluene/2-butanone/methanol/water
2 2 2 1
(from ref. 7)
Gradient mode : Hexane/2-Butanol/water solvent system, with "water"as the stationary phase and a gradient of BuOH.
Flow rate : 4 ml/min
Rotational speed : 700 rpm, ΔP from 50 to 42 bars
TLC analysis on silicagel, with EtOAc-Toluene-CH_3CN-H_2O (4-3-5-2)+ 0.1% Oxalic acid as eluent.

4-Phenyl butyric acid is strongly retained by the stationary phase; it must be eluted by inverting the elution mode, using the chloroform rich phase as the mobile phase, in the descending mode.

Some disturbances appear during the gradient run, between time 65 to 80 min, and some chloroform elutes as droplets in the mobile phase. This chloroform comes as a result of its displacement by methanol (see Fig.8),

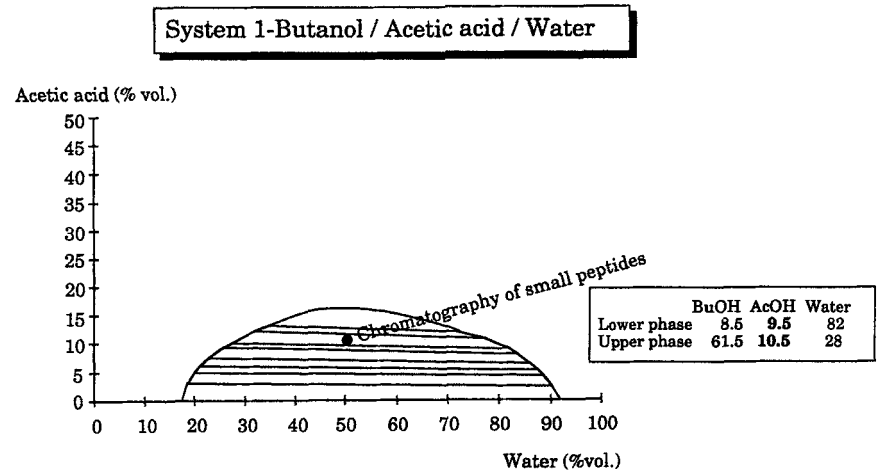

FIGURE 12. Solvent system 1-butanol/acetic acid/water, an example of solvent system useful for peptide purification, but which cannot be used to build up gradients

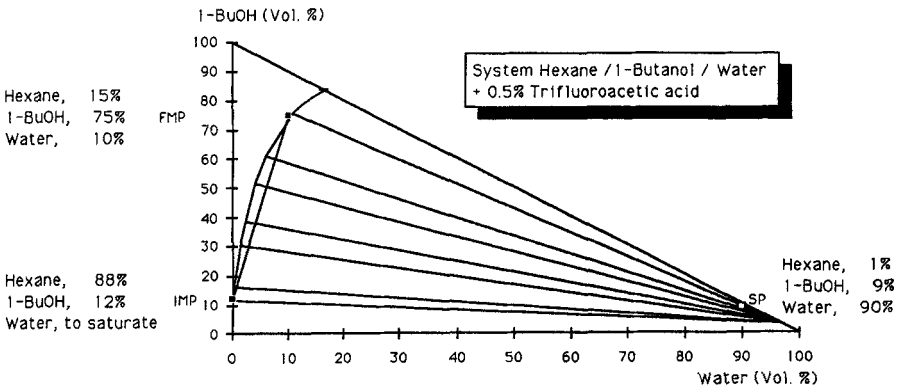

FIGURE 13. Solvent system hexane/1-butanol/water, an example of solvent system useful for peptide purification, and which can be used to build up gradients

and from the variation of the ratio of the two phases in the CPC instrument; the descending mode also leads to an unstable baseline, because the final mobile phase, FMP, is eluted with the initial stationary phase ISP (which was built up in one of the reservoirs of the pump), and they are not on the same tie-line. This produces a loss in both "stationary" phase, FMP, and methanol, which appear as droplets in the "mobile" phase ISP, and allow the composition of the internal mixture to return to the initial condition.

Another example of gradient elution is shown in Figure 10, where the authors, Vanhaelen *et al.*[6], obtained good fractionation of a commercial extract of Ginkgo biloba leaves with the system ethyl acetate/2-butanol/water, the water rich phase being the stationary phase. Vanhaelen used the systems corresponding to S_1 and S_2 to generate A, B and C; the ternary diagram found in the compilation by Sørensen and Arlt shows favorable conditions for a gradient run in the normal phase mode, because the tie lines converge towards A, and the straight line BC, indicating the composition of the mobile phase during the gradient, is very close to the left part of the binodial.

Figure 11 is a comparison between isocratic and gradient elution for the separation of some chromomycins. The fractionation was done with success by R. Bruening *et al.*[7], with a complex solvent system consisting of toluene, 2-butanone, methanol and water, selected after some trial and error TLC tests. Separation required 4 hours. The same kind of fractionation can be accomplished in about one hour, with a gradient involving the ternary system hexane/2-butanol/water.

A very promising field of application for CPC is its use for peptide purification. It would be useful to find some ternary systems for which gradients can be run.

Most of the examples found in the literature have been reported by M. Knight *et al.*[8], using the ternary system butanol/acetic acid/water shown in Figure 12.

This solvent system is an example of one we cannot use to design a gradient, because the tie lines are parallel, and no phase can be kept constant.

In Figure 13 is shown the ternary system hexane/butanol/water (with a constant addition of 0.5% trifluoroacetic acid (v/v)), which looks very favorable to run gradients in the normal phase mode, using the water rich phase as the stationary phase. We have tested this gradient, from IMP to FMP and we found it was extremely stable and reproducible, performing more

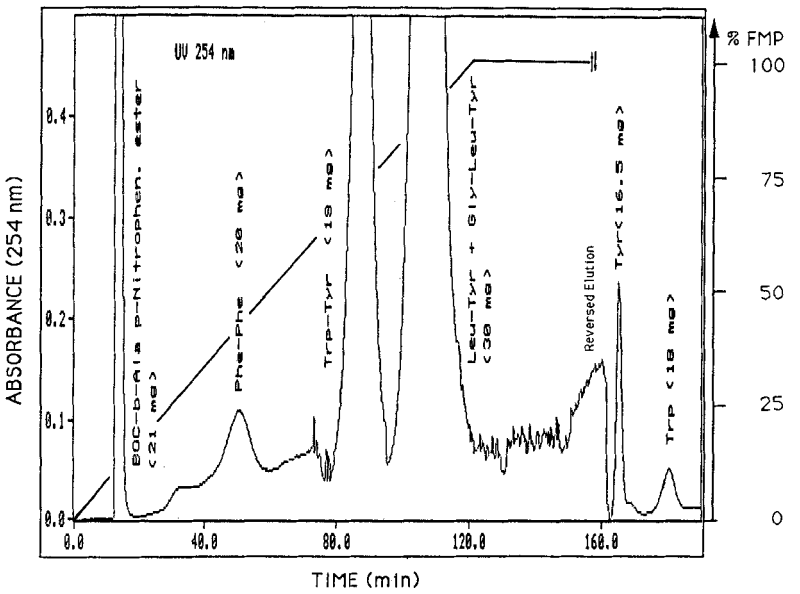

FIGURE 14. Gradient elution with the hexane/1-butanol/Water system Compositions initial (IMP) and final (FMP) mobile phases, and of stationary (ISP) phase are calculated from the diagram of Fig. 13.

	Hexane	1-Butanol	H_2O
IMP	88	12	sat.
FMP	15	75	10
SP	1	9	90

(+0.5% TFA everywhere)
Vo (mobile phase volume) : 52 ml
Mobile phase : hexane/butanol-rich phase, ascending mode
Reversed elution at T≈160 min
Rotational speed : 700 to 800 rpm
Flow rate : 4 ml/min
Pressure drop : 47 to 40 bars, room temperature
Detection UV at 254 nm, 0.5 AUFS

Sample in 4 ml	BOC-β-Ala, p-Nitrophenyl ester	21 mg
	Phe-Phe	20 mg
	Trp-Tyr	19 mg
	Gly-Leu-Tyr (trace of Leu-Tyr)	30 mg
	Tyr	16.5 mg
	Trp	18 mg

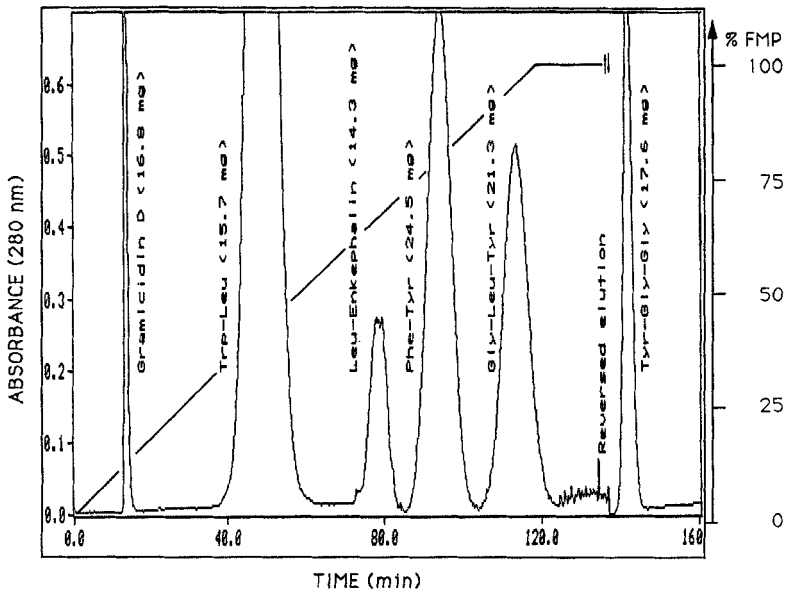

FIGURE 15. Gradient elution with the hexane/1-butanol/Water system Compositions initial (IMP) and final (FMP) mobile phases, and of stationary (ISP) phase are calculated from the diagram of Fig. 13.

	Hexane	1-Butanol	H_2O
IMP	88	12	sat.
FMP	15	75	10
SP	1	9	90

(+0.5% TFA everywhere)
Vo (mobile phase volume) : 54 ml
Mobile phase : hexane/butanol-rich phase, ascending mode
Reversed elution at T≈135 min
Rotational speed : 700 to 800 rpm
Flow rate : 4 ml/min
Pressure drop : 47 to 40 bars, room temperature
Detection UV at 280 nm, 0.7 AUFS

Sample in 4 ml	Gramicidin D	16.8 mg
	Trp-Leu	15.7 mg
	Leu-Enkephalin (Tyr-Gly-Gly-Phe-Leu)	14.3 mg
	Phe-Tyr	24.5 mg
	Gly-Leu-Tyr	21.3 mg
	Tyr-Gly-Gly	17.6 mg

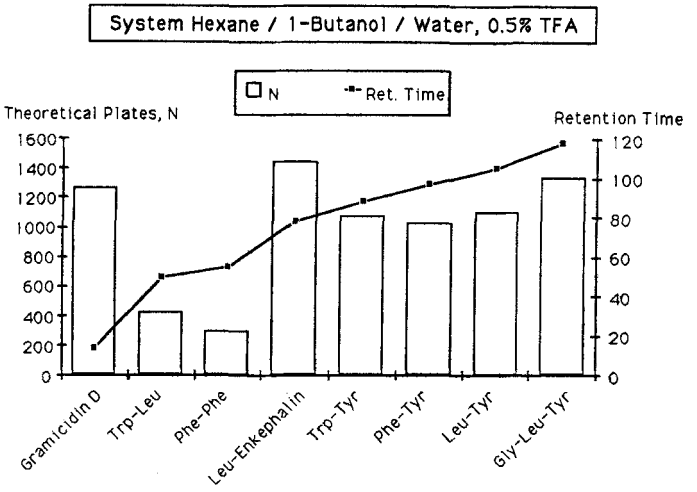

FIGURE 16. Effect of the gradient on the pseudo number of theoretical plates estimated on some peptide peaks.

than 30 runs with or without inverting the elution mode at the end of a gradient to elute the strongly retained solutes, and with the pressure being always the same with the initial conditions, reflecting the stability of the stationary phase.

Figures 14 and 15 are two examples of separation of small peptides using a two hour gradient, followed by reversed elution to elute the hydrophilic solutes. UV detection was at 254 and 280 nm, but we did some runs with UV monitoring at 220 nm without problem, except for an undulating baseline during the beginning of the gradient. This was probably generated by some impurities in the chemicals, and which should not be a problem for preparative scale CPC.

Figure 16 shows the "pseudo number of theoretical plates" and the retention times of the corresponding peaks. Due to the compression effect of the gradient for the late eluted peaks, we have more than 1000 plates for all the peaks eluting during the second hour of the two hours gradient.

We have now to find other ternary system to fractionate hydrophobic peptides, which elute at the void volume with this system (e.g., the quaternary system chloroform/acetic acid/formic acid/water, already used for purification of strongly hydrophobic peptides[9], and for which it should

be easy to develop a gradient of formic acid), and some others to fractionate the more hydrophilic peptides, which elute only after inverting the elution for this solvent system.

Conclusion

If we know the ternary phase diagram for a solvent system, we can predict stability of the stationary phase and design mobile phases to undergo gradient elution. So, we can shorten the time for an experiment and also simplify the way to find a biphasic system for a given solute mixture.

This, combined with the large capacity of the stationary phase and the ease of refreshing it, gives to CPC excellent qualities for preparative applications.

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