EXAMPLES OF ISOCRATIC, LINEAR AND STEP GRADIENT HSCCC SOLUTIONS TO ACTUAL EXPERIMENTAL PROBLEMS

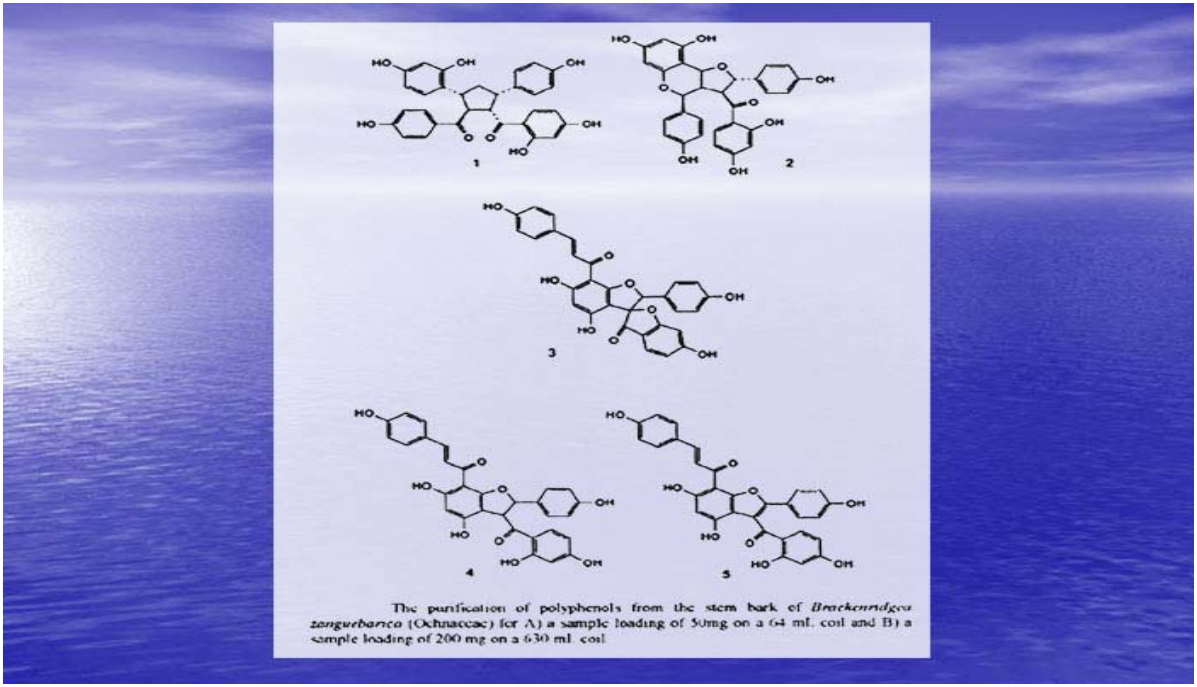
Introduction to use of isocratic biphasic solvent systems in HSCCC

In HSCCC a biphasic liquid / liquid solvent system is used (See CCC Introduction on Home Page). The mobile phase may be either the more polar solvent (reverse phase) or less polar solvent (normal phase) with their respective partner phases as the stationary phase. Indeed it is possible to change from one mode to the other at any stage during a run.

It is often best to do a HSCCC literature review (See Literature References on Home Page) to try to find solvent systems, which have been used for similar components to your sample prior to commencing optimization of any HSCCC method.

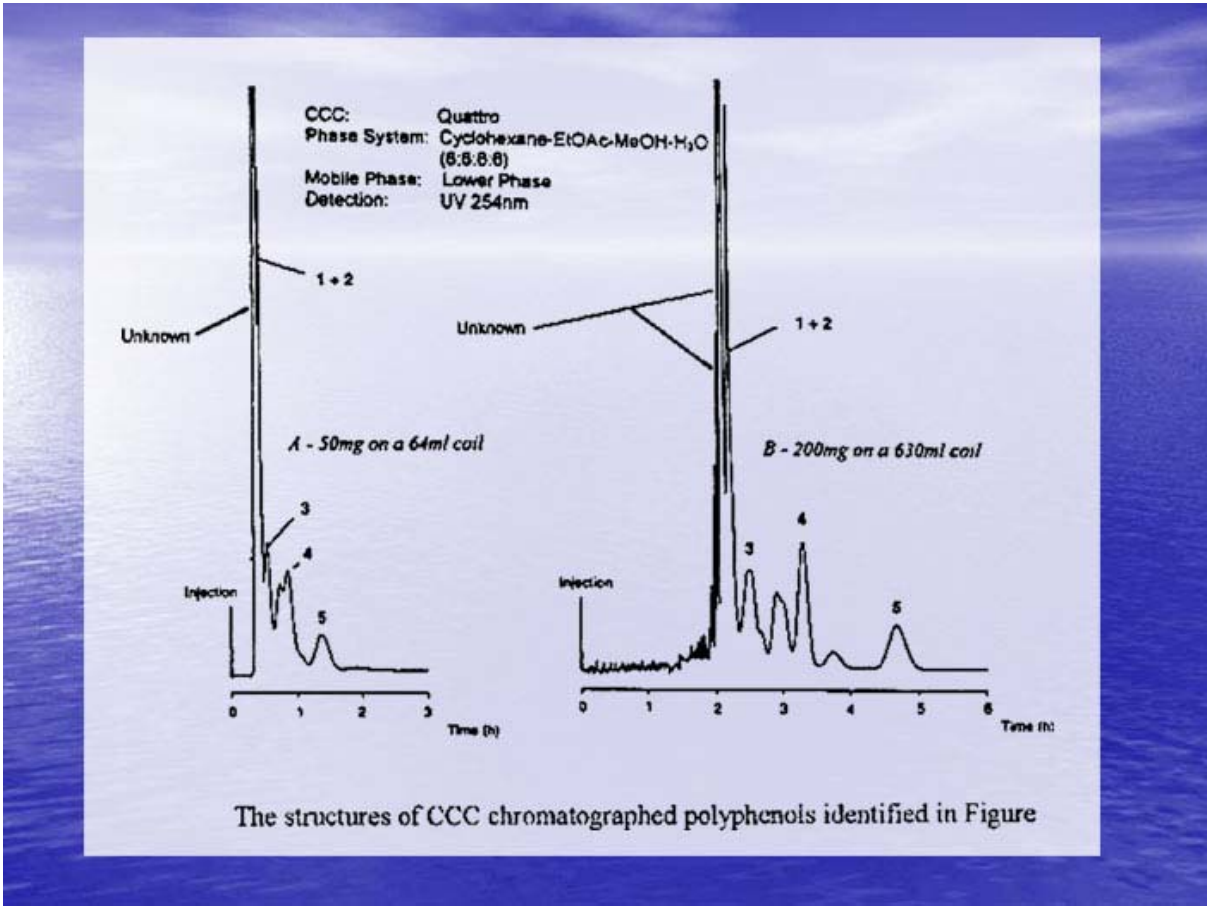
If none exists a strategy similar to that discussed in the following gradient elution sections should be utilized.

Slide 1 gives the structure of a variety of bioactive natural product polyphenols, which are then shown in Slide 2 resolved in a preparative isocratic reverse phase application using a Quattro CCC

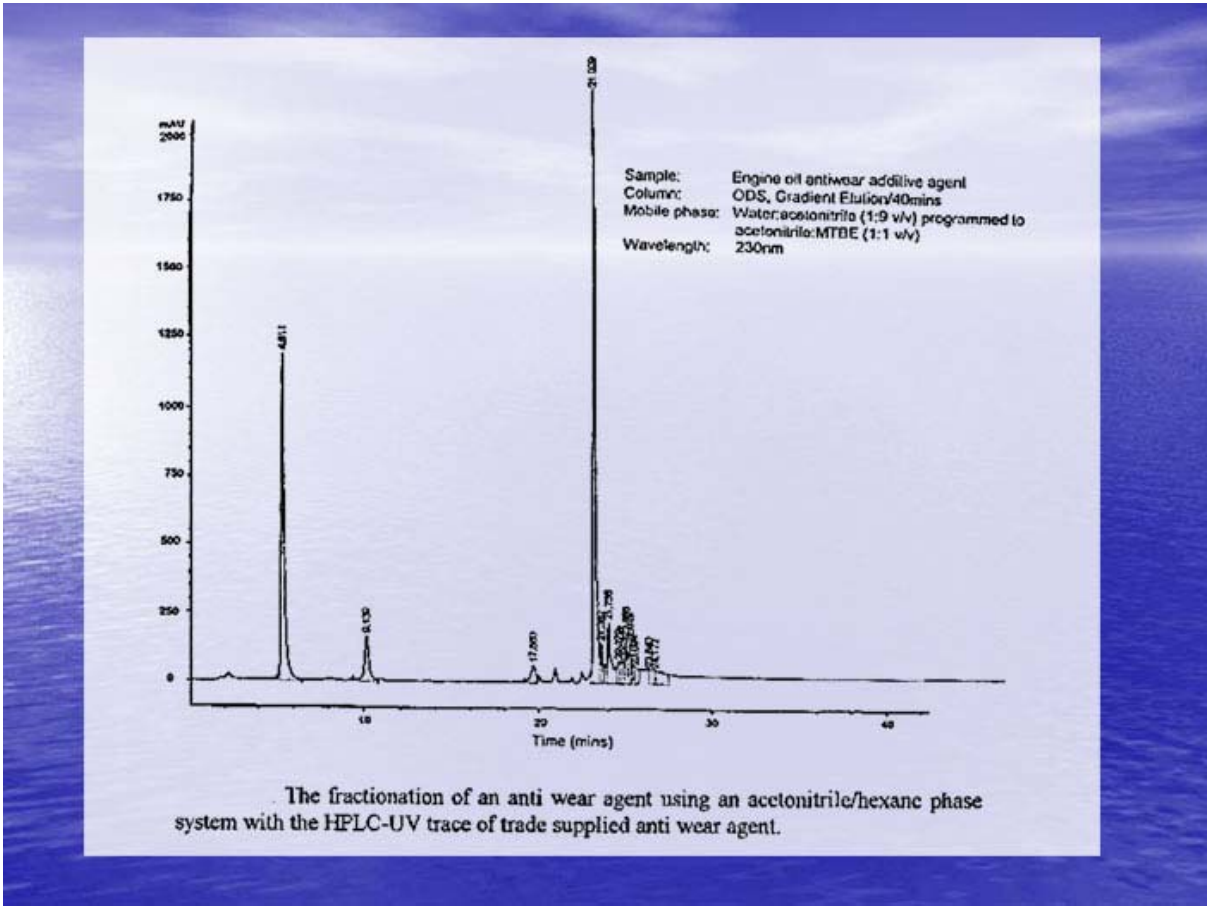


Slide 2 resolved in a preparative isocratic reverse phase application using a Quattro CCC

Slide 2 compares resolution on a 64 ml, 1.6 mm ID coil to that on a 630 ml, 1.6 mm ID coil fitted to a Quattro CCC. The additional resolution noted, however, caused a considerable increase in time of the preparation and could have been more rapidly been accomplished by the use of gradient HSCCC.

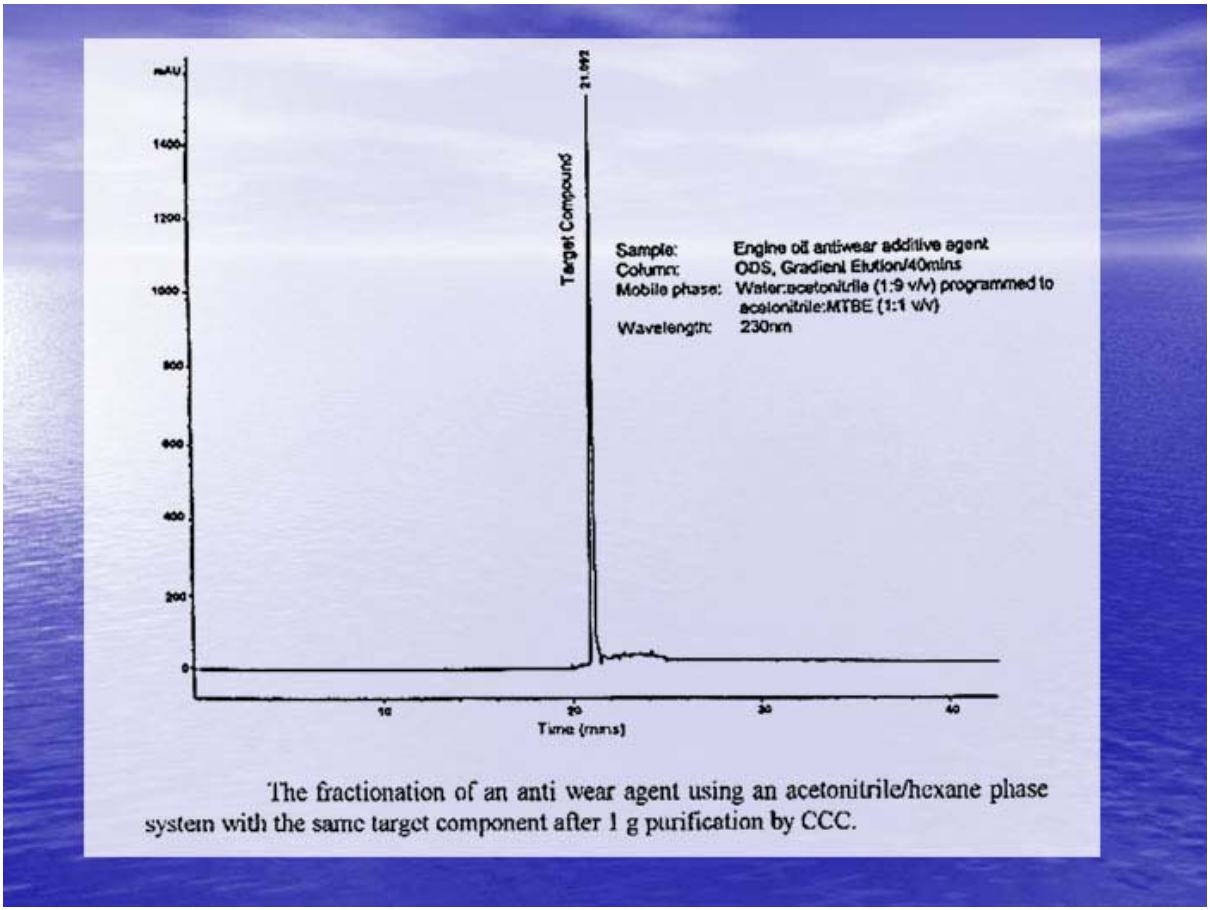


Slide 3 shows the hplc gradient chromatograph of a crude engine oil anti-wear agent formulation before purification



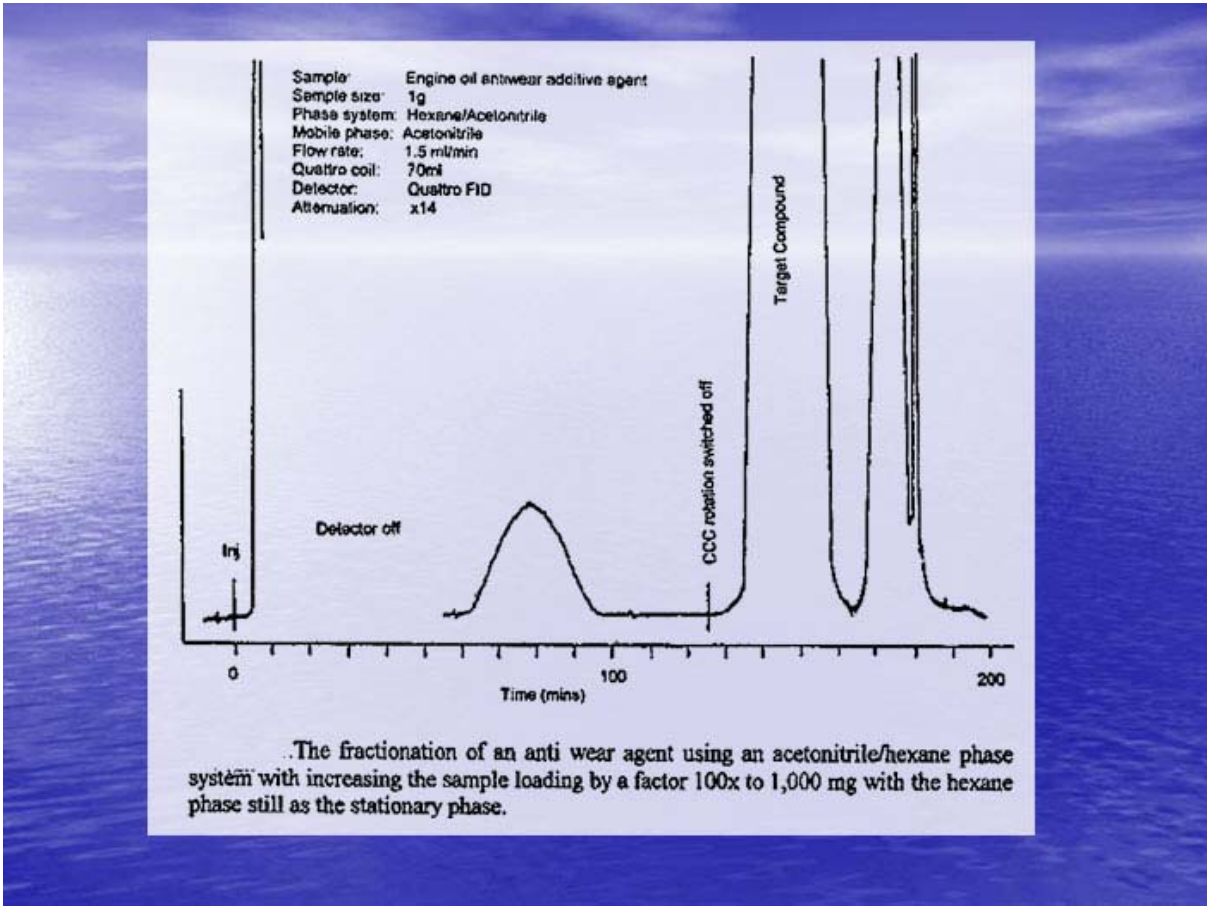
The fractionation of an anti wear agent using an acetonitrile/hexane phase system with the HPLC-UV trace of trade supplied anti wear agent.

Slide 4 shows the active ingredient after Quattro CCC purification using a non-aqueous hexane : acetonitrile biphasic solvent system with the HSCCC chromatograph shown in Slide 5



The fractionation of an anti wear agent using an acetonitrile/hexane phase system with the same target component after 1 g purification by CCC.

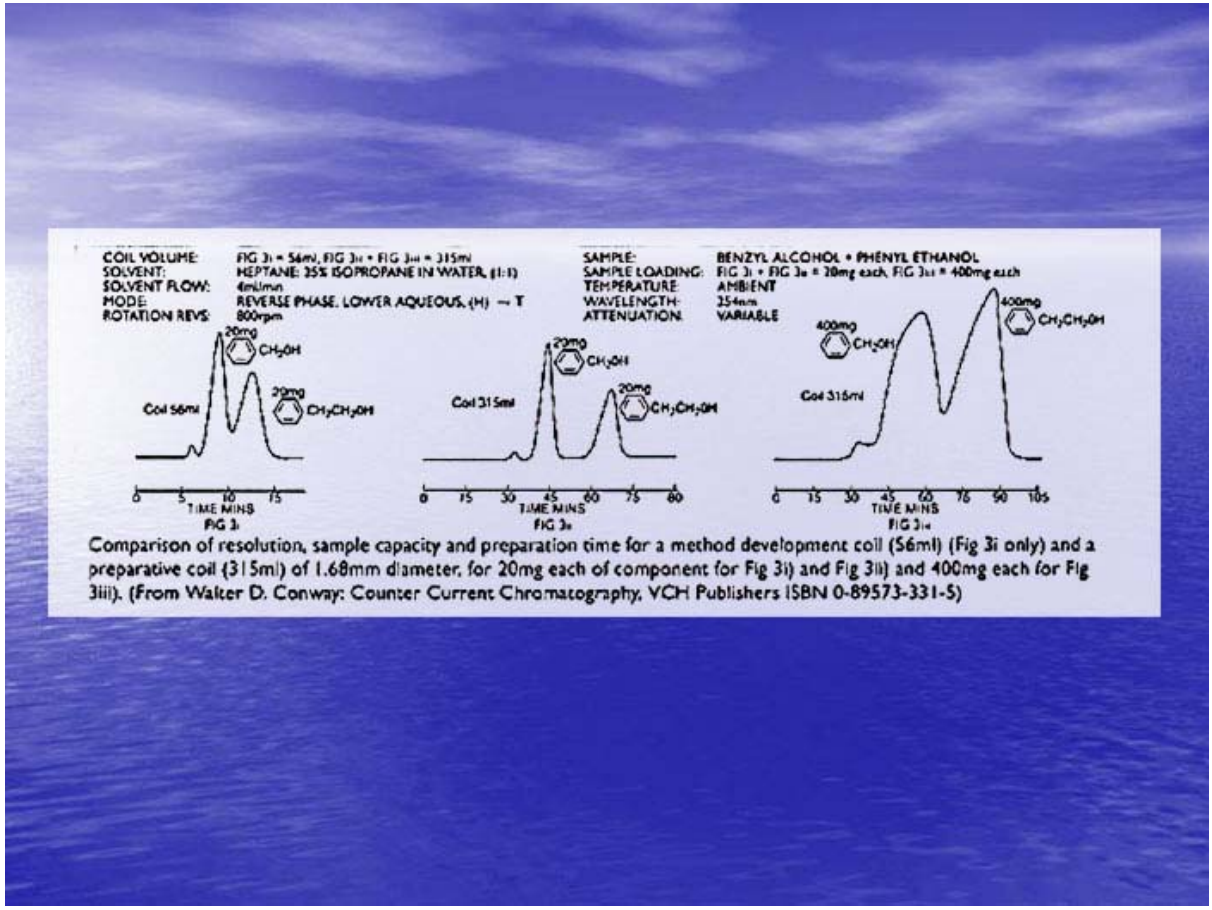
acetonitrile biphasic solvent system with the HSCCC chromatograph shown in Slide 5



The fractionation of an anti wear agent using an acetonitrile/hexane phase system with increasing the sample loading by a factor 100x to 1,000 mg with the hexane phase still as the stationary phase.

Slide 6 shows the loading / resolution of isocratic preparations on a PC Inc CCC (now obsolete) with 1.68 mm ID coils of 56 and 315 ml volume which suggests a limit of 400 mg per 315 ml volume in isocratic mode.

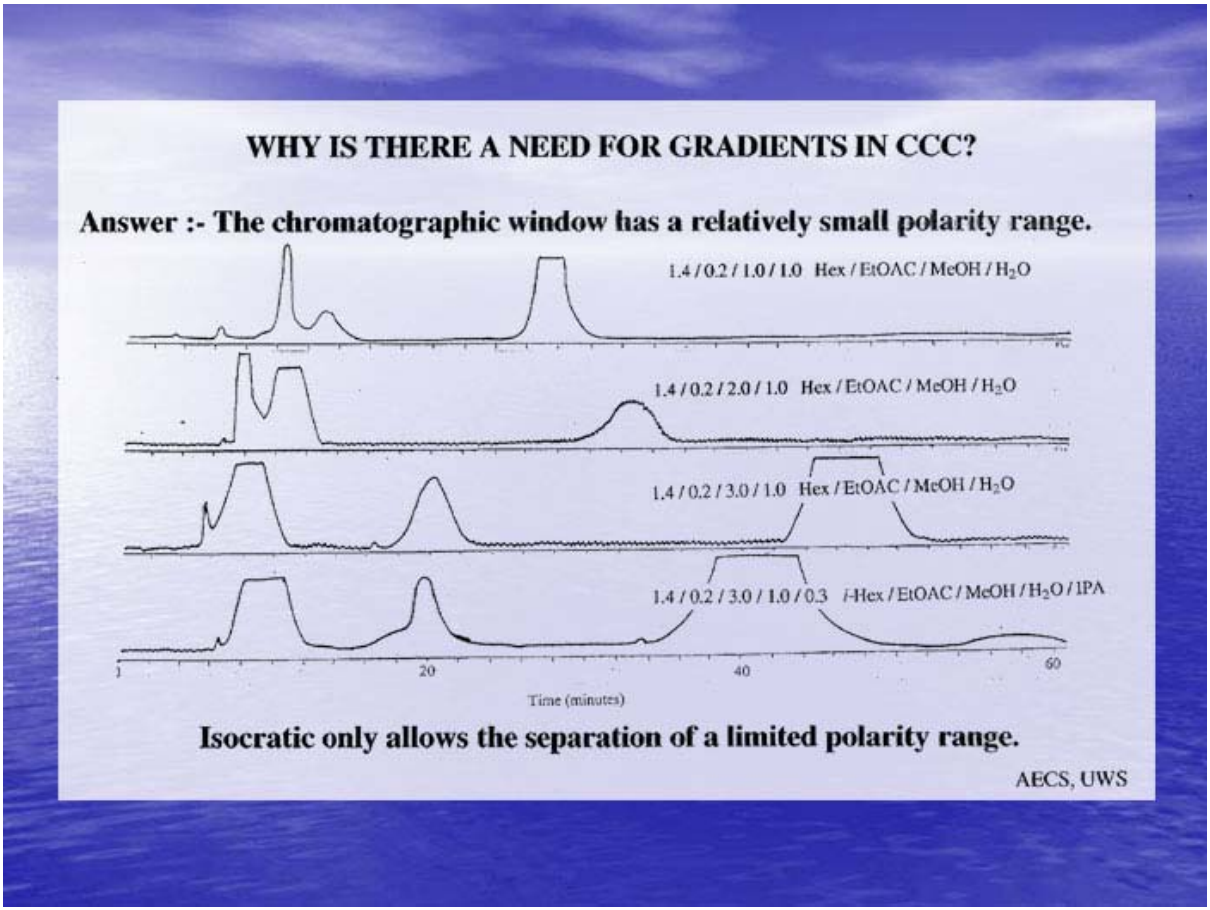
Dr Andrew Marston of the University of Lausanne (Lecture at Rio 2001, Home Page) has achieve compound loadings of as high as 20 grams on a 600 ml Quattro CCC. The author has found that approximate loadings of 1 gram per 100 ml are typical for Quattro CCC fitted with 1.6 mm ID tubing when used in gradient mode. The actual loading resolvable by HSCCC is both sample and solvent dependent and can hence vary considerably.



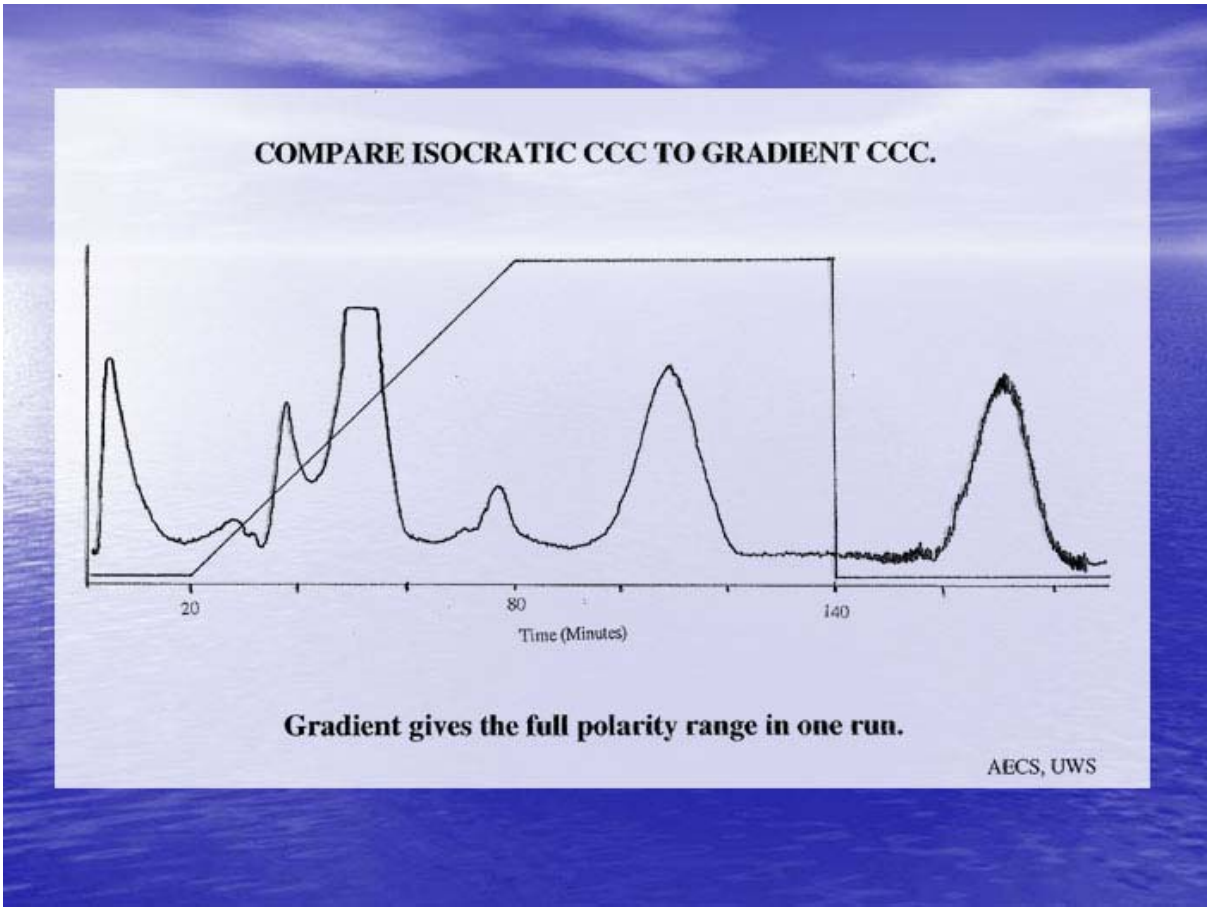
Comparison of resolution, sample capacity and preparation time for a method development coil (56ml) (Fig 3i only) and a preparative coil (315ml) of 1.68mm diameter, for 20mg each of component for Fig 3i) and Fig 3ii) and 400mg each for Fig 3iii). (From Walter D. Conway: Counter Current Chromatography, VCH Publishers ISBN 0-89573-331-5)

In HSCCC as in HPLC isocratic analysis only elutes a limited polarity window of compounds for each constant composition solvent choice, as shown in Slide 7. From this figure it will be noted that in isocratic analysis, increasing the eluent strength (in this reverse phase application, a higher amount of methanol initially and then finally both methanol and isopropanol is added to the aqueous mobile phase) to elute late eluting compounds causes early eluting compounds to co-elute and ultimately elute on the solvent front.

Progressively increasing the elution strength of the mobile phase relative to the stationary phase will in both gradient HSCCC and HPLC allow a much broader range of polarities to be examined in one chromatogram without the above problem. This is demonstrated in Slide 8, which compares the gradient elution of the same standards initially shown by isocratic elution with different strength mobile phases in Slide 7



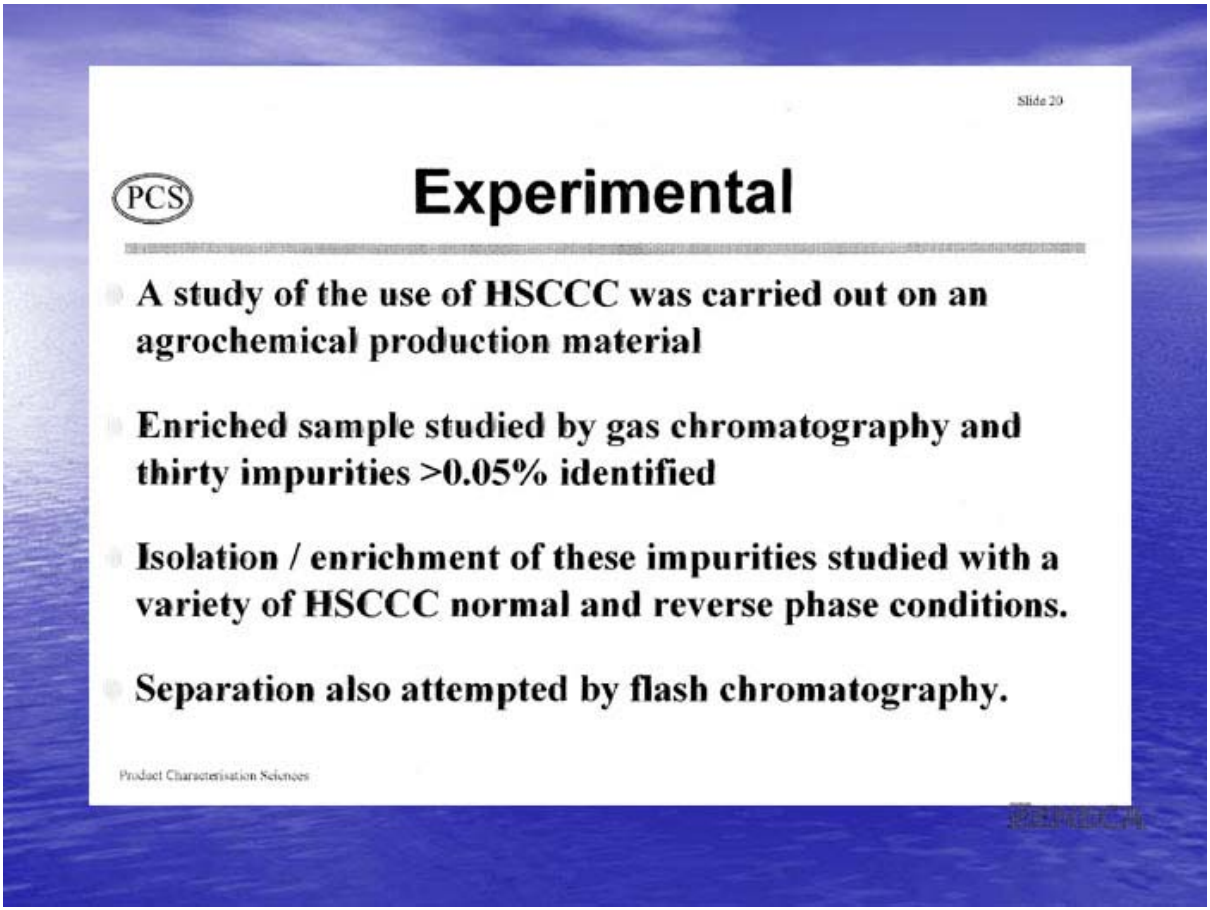
Slide 8, which compares the gradient elution of the same standards initially shown by isocratic elution with different strength mobile phases in Slide 7



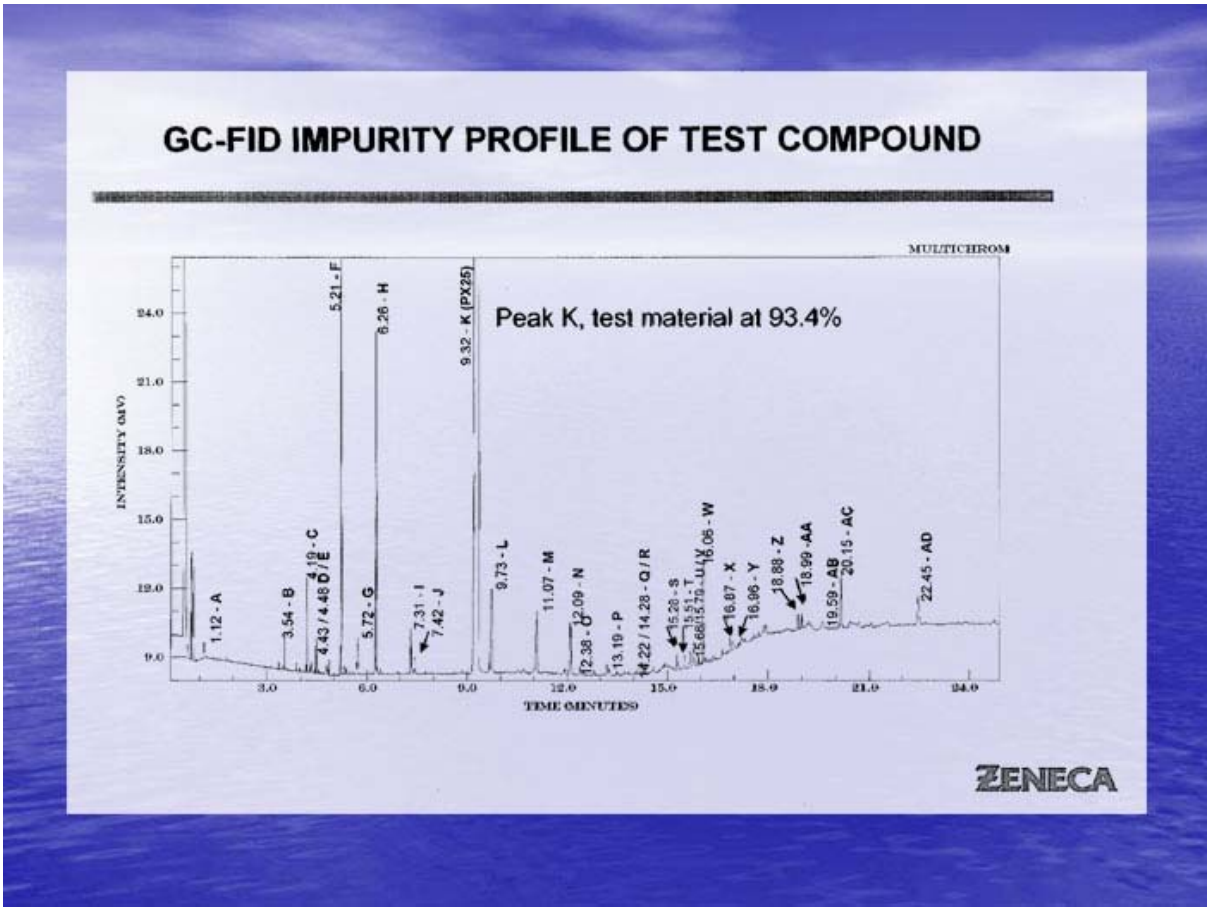
The choice of gradient solvent in reverse phase HPLC is relatively easy, most chromatographers using either a water : methanol or a water : acetonitrile gradient with or without buffering. It would be relatively rare in HPLC to use multiple solvent gradients such as water : methanol : acetonitrile : THF and variation of the relative percentages of the solvents (other than the water to organic ratio) is almost unheard of in HPLC. The situation is not so simplistic in HSCCC gradients as almost any immiscible solvent combinations can be used in the gradient, and 3, 4 or 5 + different solvents may be utilized, with on some occasions an additional solvent(s) being phased in part way through the gradient.

The effectiveness of gradient HSCCC with a Quattro CCC is shown in Slides 9, Slide 10, Slide 11 and Slide 12.

Slides 9 describes the context of the inter-comparison of gradient HSCCC to Flash Chromatography.



Slide 10 shows a GC-FID trace of the commercial agrochemical being tested for trace impurities and the relative percentages of the major component and impurities.

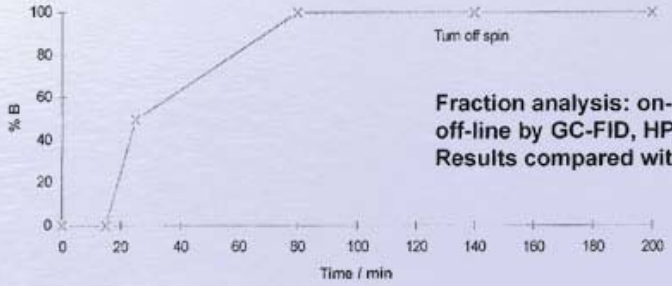


Slide 11 shows the gradient used for the HSCCC. It should be noted that a variable gradient both with regards to slope and solvent (fifth solvent added to enhance selectivity).

Results

Best separation was achieved with reverse phase elution:

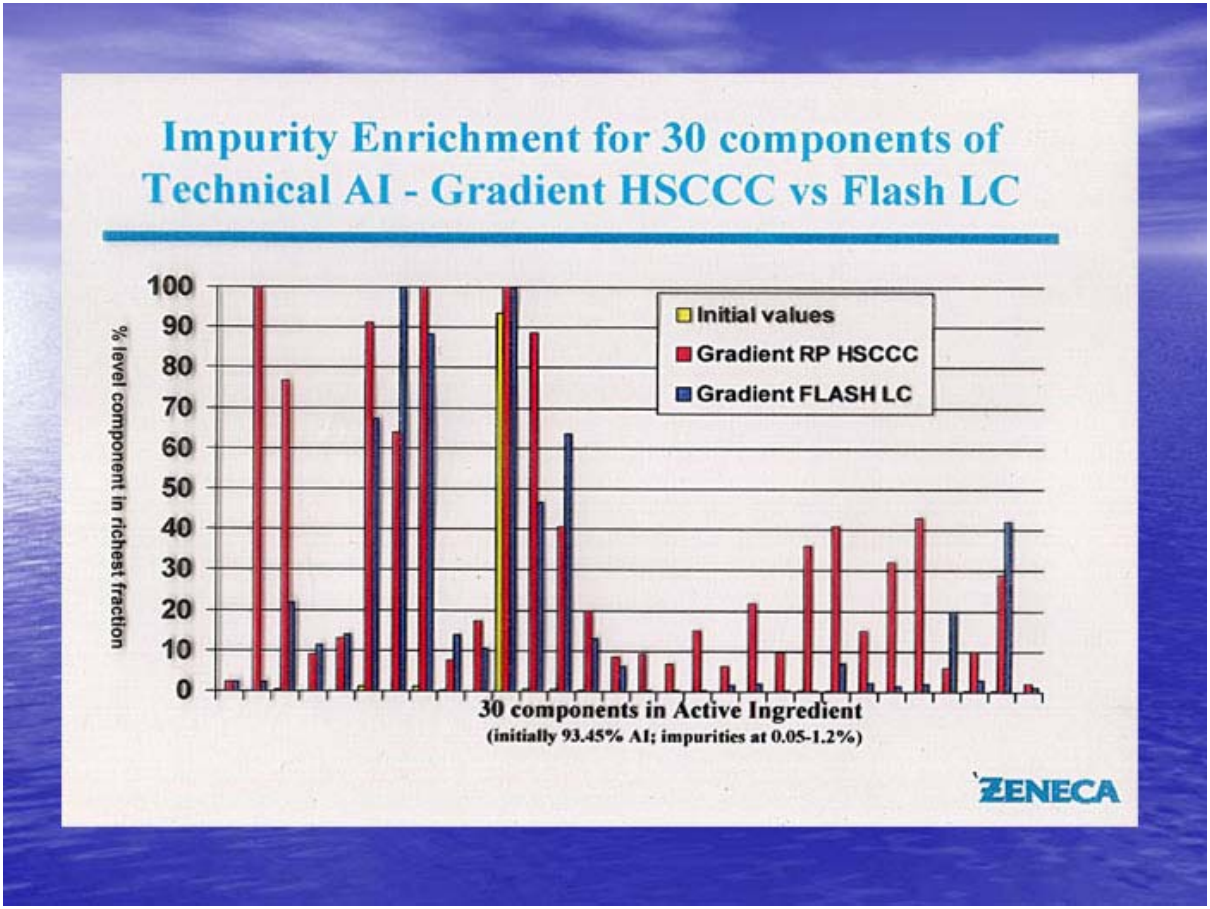
	EtOAc	Hexane	MeOH	EtOH	H_2O
A	0.8	1.4	0.1	0	1
B	0.8	1.4	2.5	0.5	1



Fraction analysis: on- line UV +ELSD
off-line by GC-FID, HPLC +ELSD
Results compared with Flash separation

ZENECA

Slide 12 Shows the relative percentage of each the compound in the start mixture (shown in yellow, note that many of the percentages are so low as to be not noticeable), plus the % of the compound in the isolated/purified Flash Chromatography separation (shown in blue) and the same for the Quattro gradient HSCCC separation (shown in red). It will be noted that in the vast majority of cases Quattro CCC purification yielded both higher recovery and higher purity than Flash Chromatography. This result is even more remarkable if one considers that the chromatographers doing the research had over 40 years experience with Flash Chromatography with this and similar samples and only a few months experience with the Quattro CCC after a training period with the author.



Gradients need not only be limited to changes in organic solvents but also can in addition or by themselves be pH gradients.

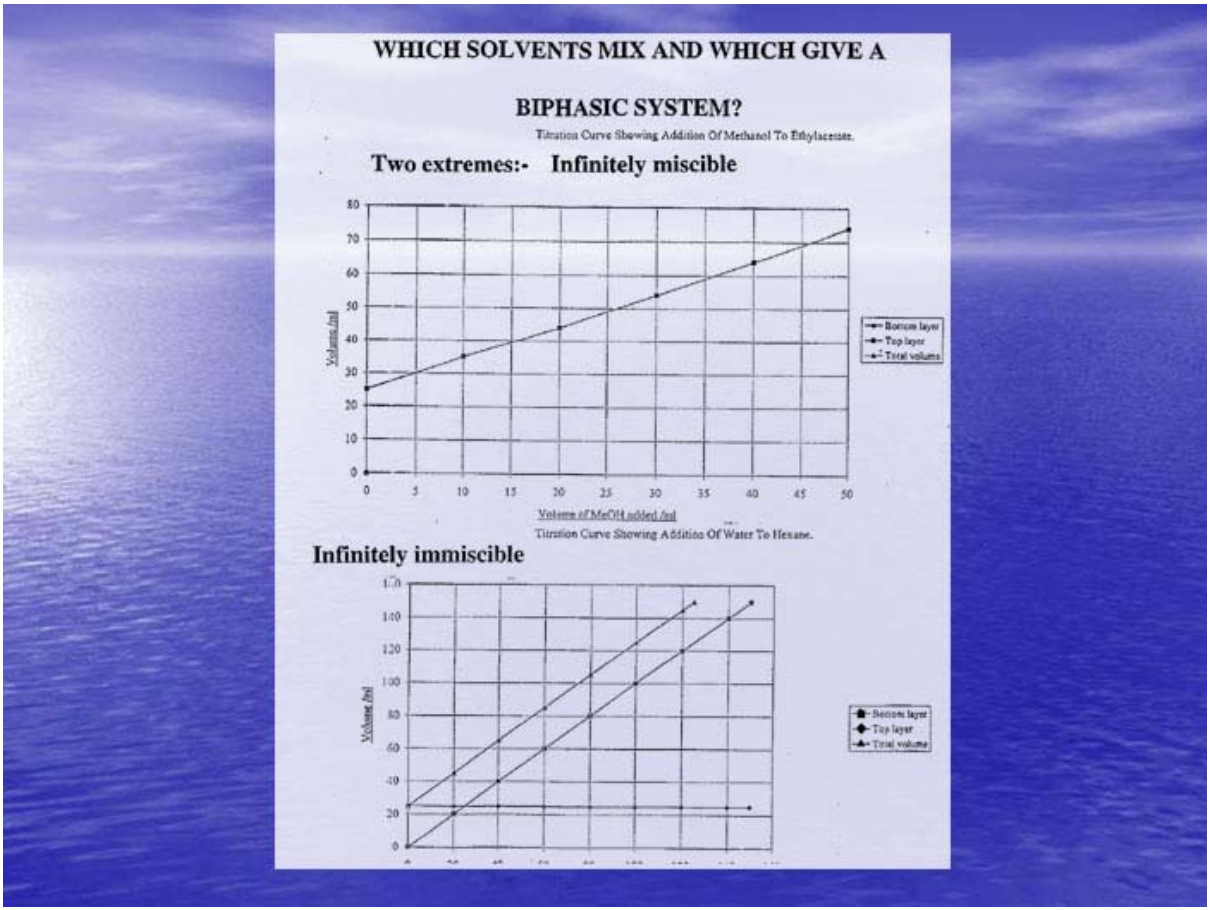
There is therefore a need for a generic strategy for choice of gradient solvents in HSCCC.

Which miscibility of solvents can be used for gradients ?

The variations possible in biphasic solvent immiscibility is shown below.

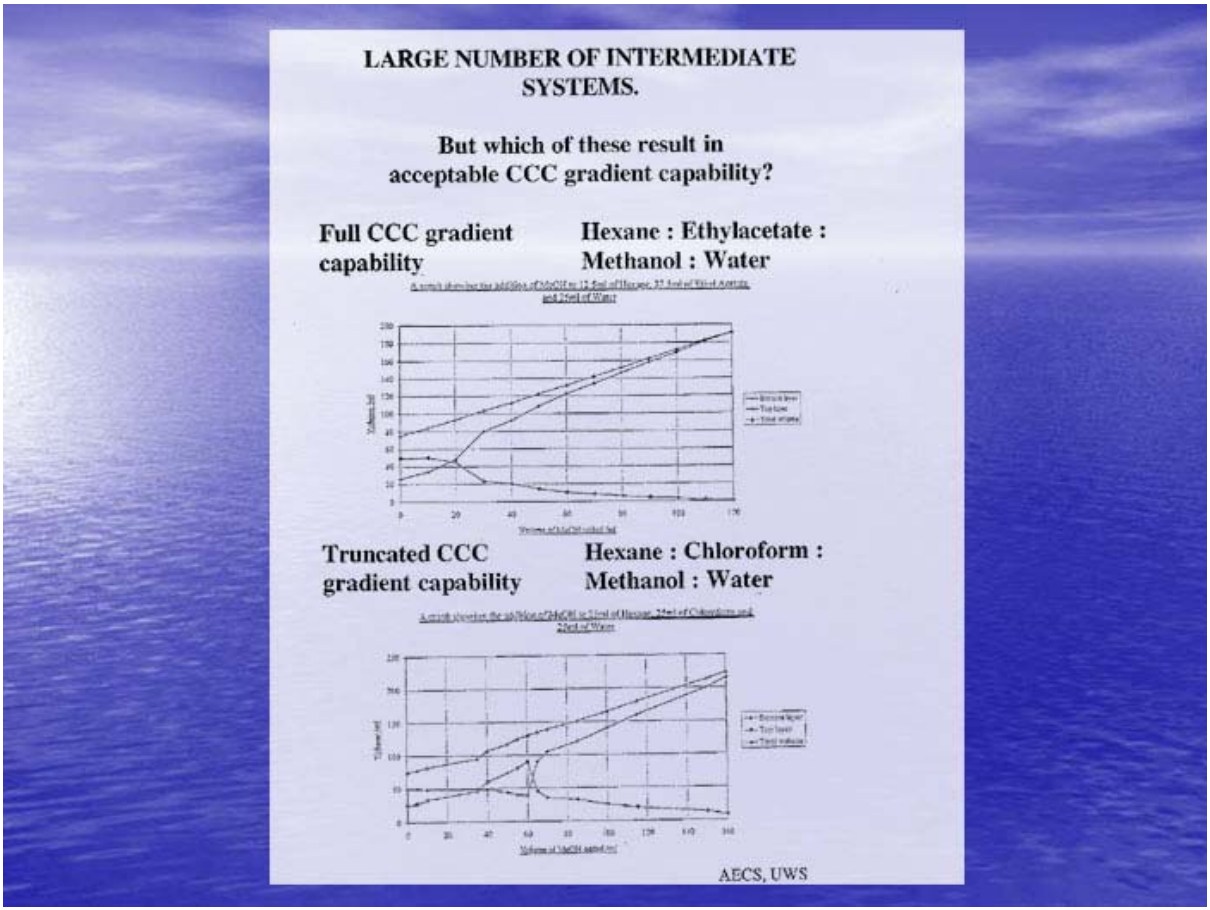
Slide 13 shows totally miscible solvents and totally immiscible solvents. Slide 14 shows partially immiscible solvents where no relative density differences between upper and lower layer occurs as gradient solvent modifier is added and an example of a chloroform gradient where an inversion of an upper aqueous layer to a lower aqueous layer occurs as the organic gradient modifier is progressively added.

Examples on Slide 13 have no application in CCC whilst the first example on



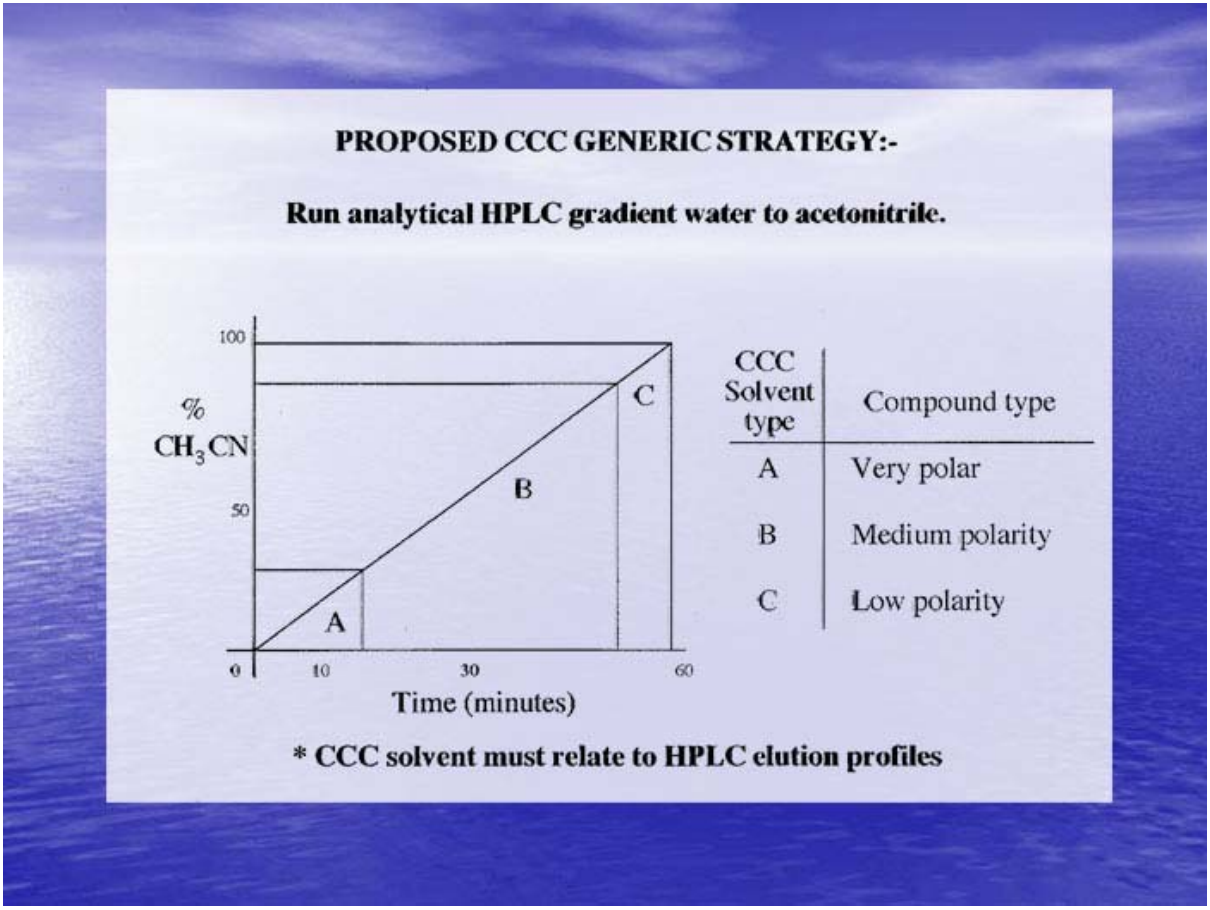
Slide 14 shows partially immiscible solvents where no relative density differences between upper and lower layer occurs as gradient solvent modifier is added and an example of a chloroform gradient where an inversion of an upper aqueous layer to a lower aqueous layer occurs as the organic gradient modifier is progressively added.

Examples on Slide 13 have no application in CCC whilst the first example on Slide 14 is an usable, predictable gradient throughout the gradient. The second chloroform gradient of Slide 14 which has an inversion of its upper aqueous layer to a lower aqueous layer as the methanol gradient modifier is added, can only be used either before or after the inversion point, as the inversion would void the stationary phase in HSCC



How to generically optimize a HSCCC gradient.

Experimentation has shown that there is a workable correlation between the elution characteristics of a 0 to 100 % B Water (A) to Acetonitrile (B) reverse phase HPLC gradient to the polarity of components and hence potential generic HSCCC solvent systems as shown in Slide 15.



Section A :- Highly-polar components can usually be accommodated by :-

PEG : Water or Butanol : Methanol : Water gradients.

Section B :- Mid-polar compounds by :-

Hexane : Ethyl Acetate : Methanol : Water gradients.

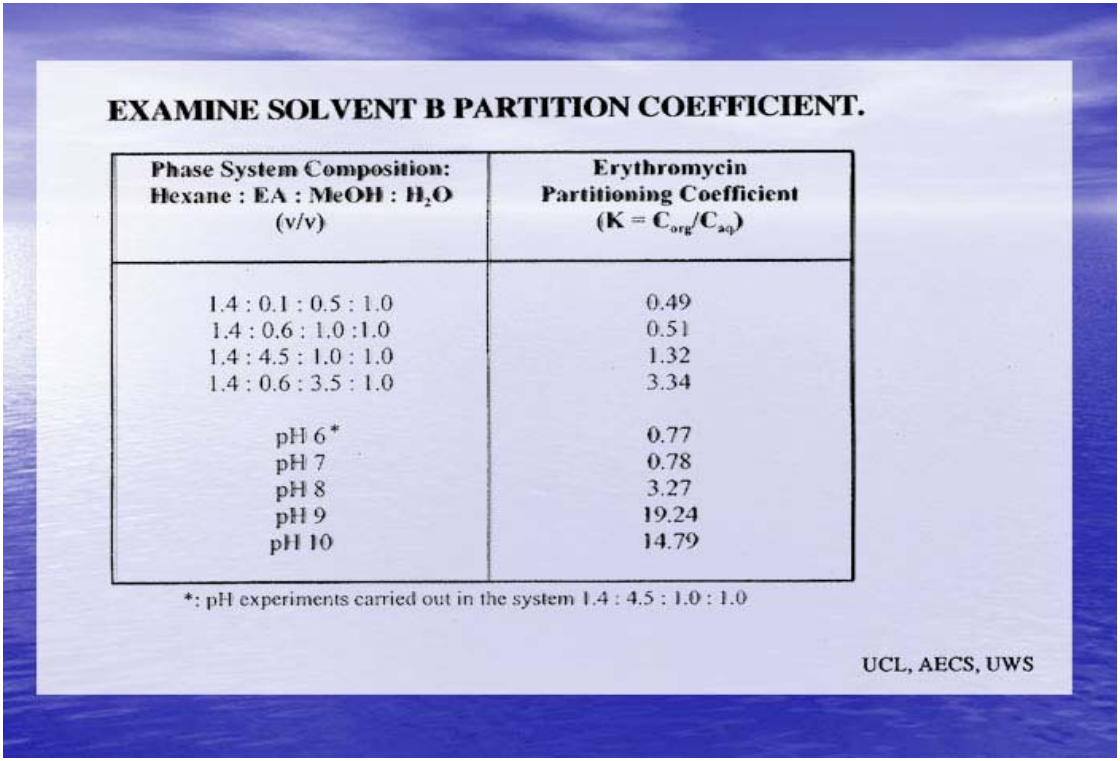
Section C :- Non-polar components by non aqueous mixes such as :-

Hexane : Modifier : Methanol (and / or Acetonitrile) gradients.

Optimization within a Section can often be facilitated by using simple test tube shake partition tests (with suitable analysis as appropriate, the partition / the relative concentrations in the upper and lower phase can be assessed) to model potential CCC response to various solvent mixes.

The user should aim to achieve a partition coefficient of between 0.5 and 1.5 and should then choose an appropriate mix and examine the affect of pH on this partitioning.

The effect of pH on partitioning can on occasions be very considerable Slide 16.



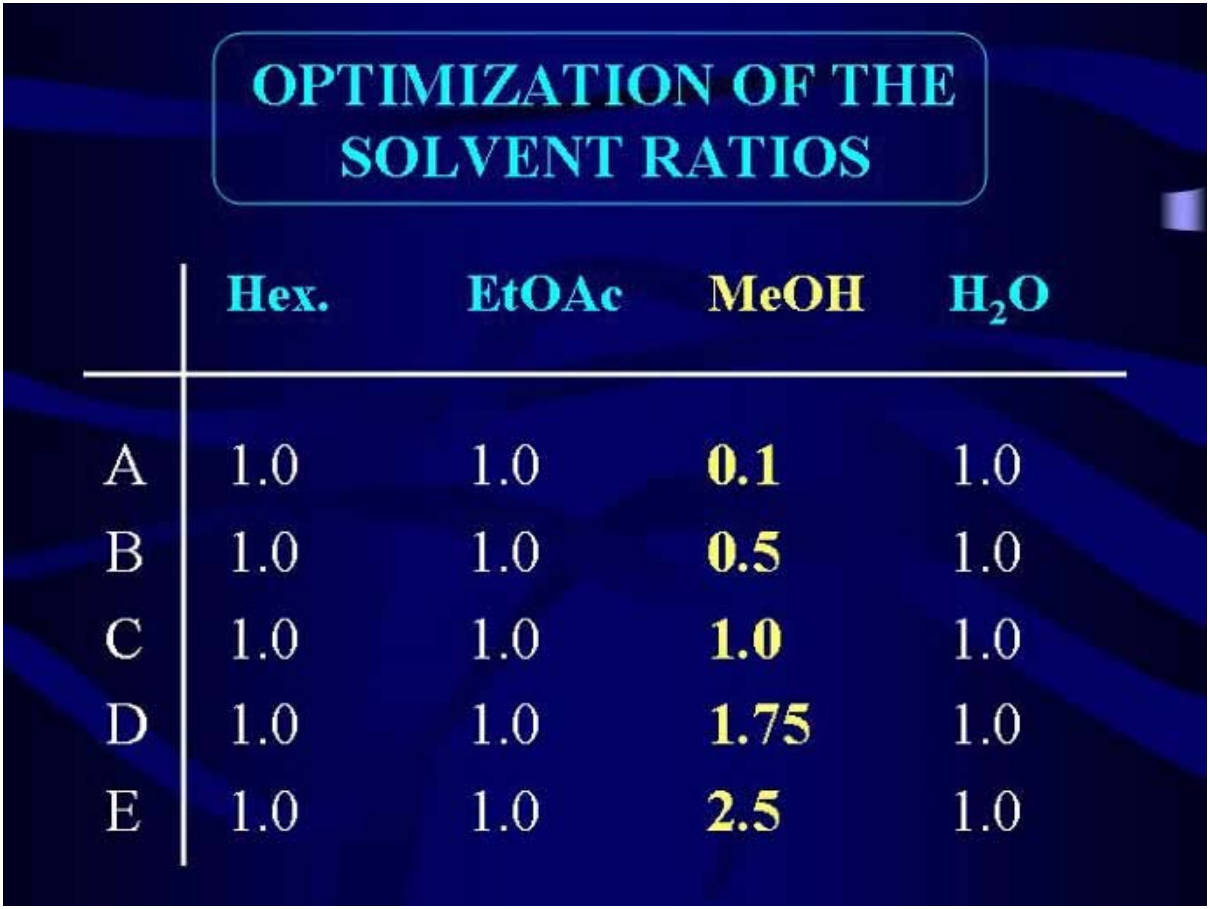
EXAMINE SOLVENT B PARTITION COEFFICIENT.

Phase System Composition: Hexane : EA : MeOH : H_2O (v/v)	Erythromycin Partitioning Coefficient ($K = C_{org}/C_{aq}$)
1.4 : 0.1 : 0.5 : 1.0	0.49
1.4 : 0.6 : 1.0 :1.0	0.51
1.4 : 4.5 : 1.0 : 1.0	1.32
1.4 : 0.6 : 3.5 : 1.0	3.34
pH 6*	0.77
pH 7	0.78
pH 8	3.27
pH 9	19.24
pH 10	14.79

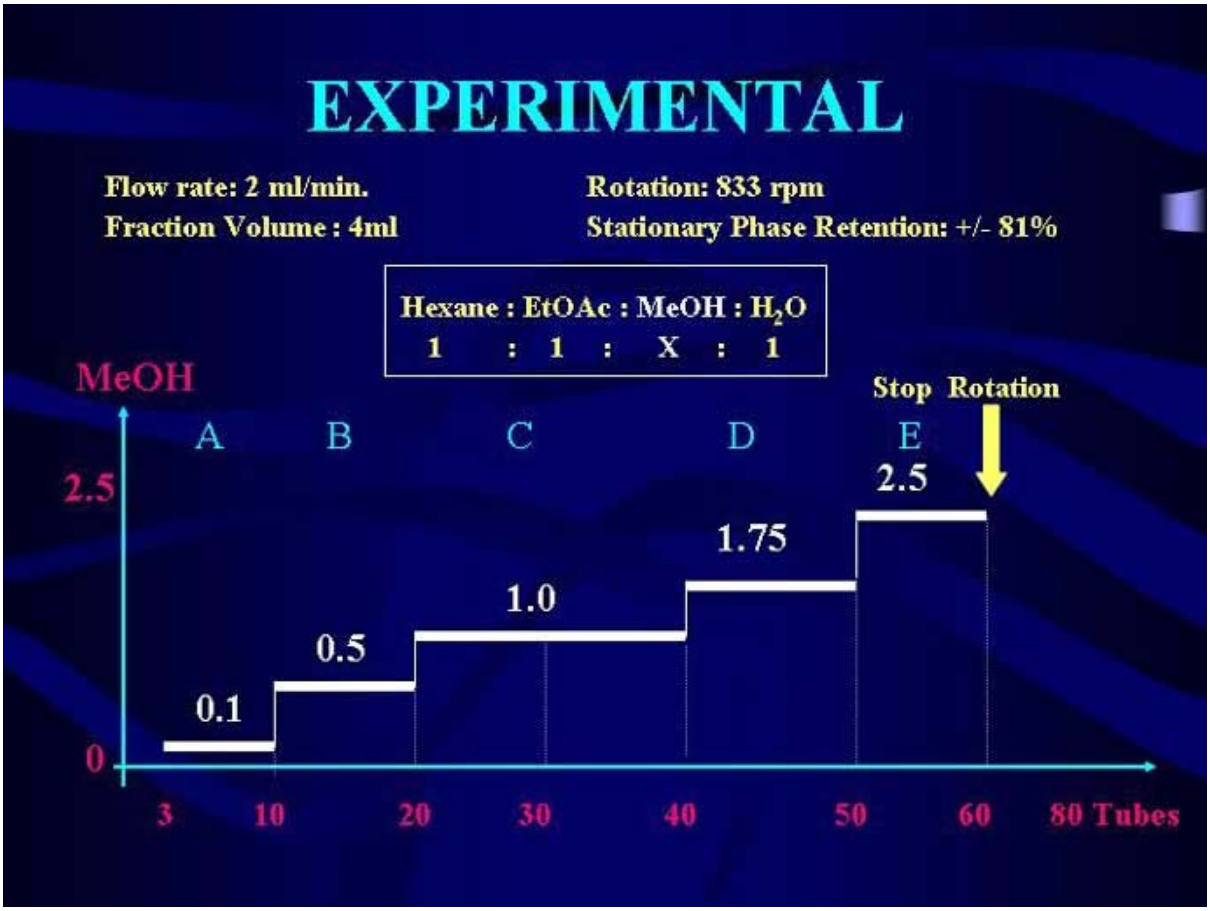
*: pH experiments carried out in the system 1.4 : 4.5 : 1.0 : 1.0

UCL, AECS, UWS

For this reason it is often easier to ignore test tube simulating a CCC response and instead use a mid-polarity generic CCC gradient as shown in the first lecture presentation :- that is a reverse phase gradient with stepped increases of the methanol content of the aqueous mobile phase used to first define the components polarity, and then begin optimization from the result, as required :-See Slide 17 and Slide 18.



For this reason it is often easier to ignore test tube simulating a CCC response and instead use a mid-polarity generic CCC gradient as shown in the first lecture presentation :- that is a reverse phase gradient with stepped increases of the methanol content of the aqueous mobile phase used to first define the components polarity, and then begin optimization from the result, as required :-See Slide 17 and Slide 18.



Alternatively a literature search could be utilized if looking for known components to see if published HSCCC methods are available for separations with a similar matrix to your sample. By choosing solvent initial and final gradient strengths to bracket the strength of the organic mobile phase for normal phase gradients and the aqueous phase in reverse phase gradients it is highly likely that a successful separation will be achieved.

Affect of HSCCC design on isocratic or gradient choice.

In commercially available HSCCC there are many variations in design :

i) preferred direction of coil winding same as rotation (PC Inc, CentriChrom, Quattro) or wound opposite to rotation (PharmaTech)
ii) the gravity force differential can be obtained by a small sun and planet radius but high speed (PharmTech Analytical), or larger sun and planet radius but slower speed (PC Inc, CentriChrom, Quattro, PharmaTech Prep)
iii) the internal diameter of tubing / rotation speed.
iv) temperature control (Quattro) no temperature control (P C inc, CentriChrom, PharmaTech)

All of the above differences can affect isocratic solvent choice, but can become critical when trying to do repeated analyses with same gradient on different days, or trying to transfer a method used on one design of HSCCC to another design.

Experimentation even within a single instrument with all parameters set, including temperature (Quattro), have shown that gradient and even isocratic methods cannot always tolerate differences in internal bore for certain solvent systems.

Users must therefore specify all parameters in HSCCC and acknowledge that even change of a seemingly minor parameter may prevent reproduction of results, but if all parameters are controlled, no difficulty in reproducing gradient results have been experienced in the author's five years experience of gradient HSCCC applications.

Why step gradients preferable to linear gradients in HSCCC

In HSCCC the maintenance of stationary phase in the coil is facilitated by rotation.

Switching off rotation will, if either the stationary phase or mobile phase continues to be pumped, void both the mobile and stationary phase.

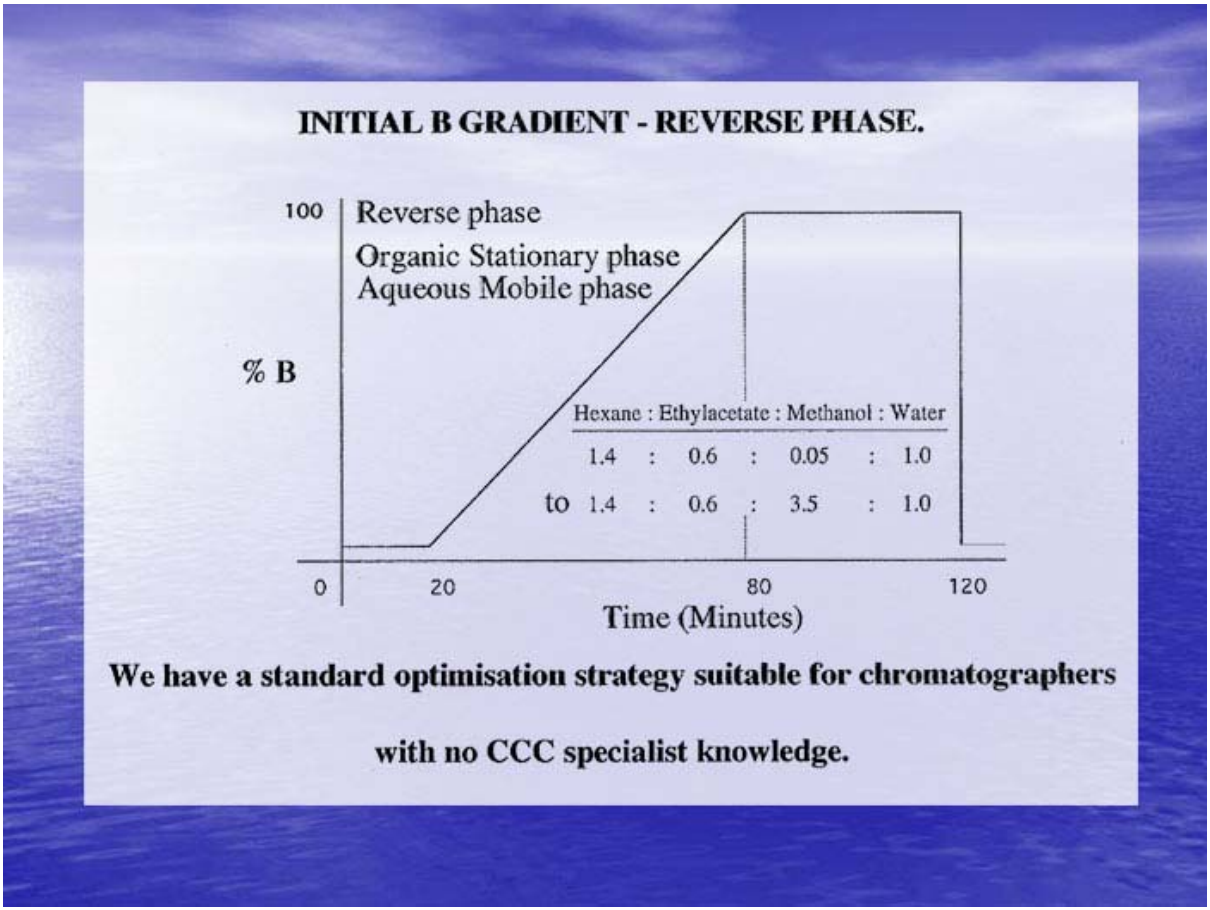
Experimentation has shown that very little band spreading occurs during this voiding of the coil, so that collection of the coil contents will allow the accurate prediction of the retention characteristics of the retained components to be obtained.

Thus :-

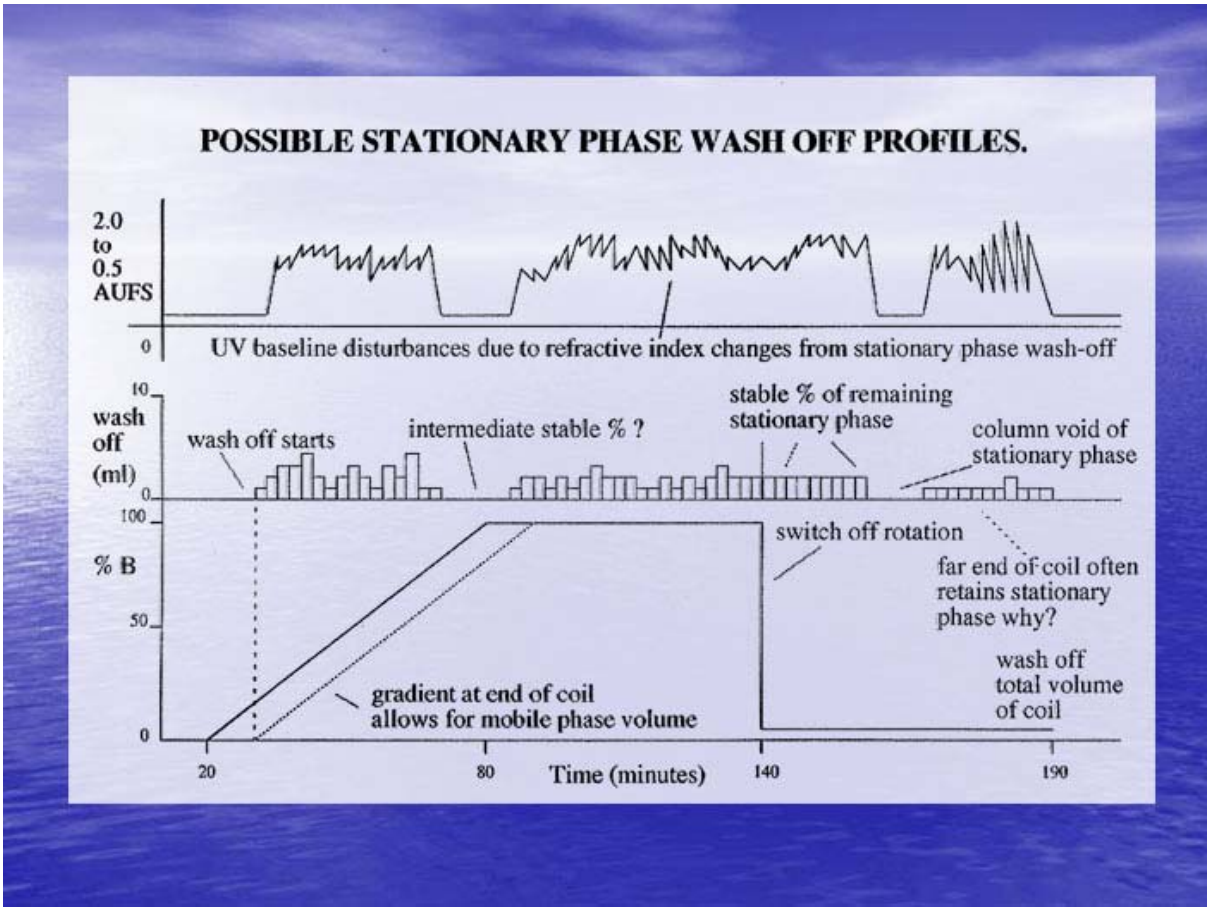
1. If no retention of a component in a specific stationary phase occurs then this component will elute at solvent front.
2. Intermediate retentions can be correlated to elution position on the gradient.
3. Infinitely retained components void in the last drop of flushed out stationary phase.

Once the elution profile is established optimization of the best HSCCC system for any or all components becomes logical.

A typical linear gradient in HSCCC is shown in Slide 19 plus the typical affect of such a gradient on an ultra violet detector response and on the stationary phase bleed, is shown in Slide 20.



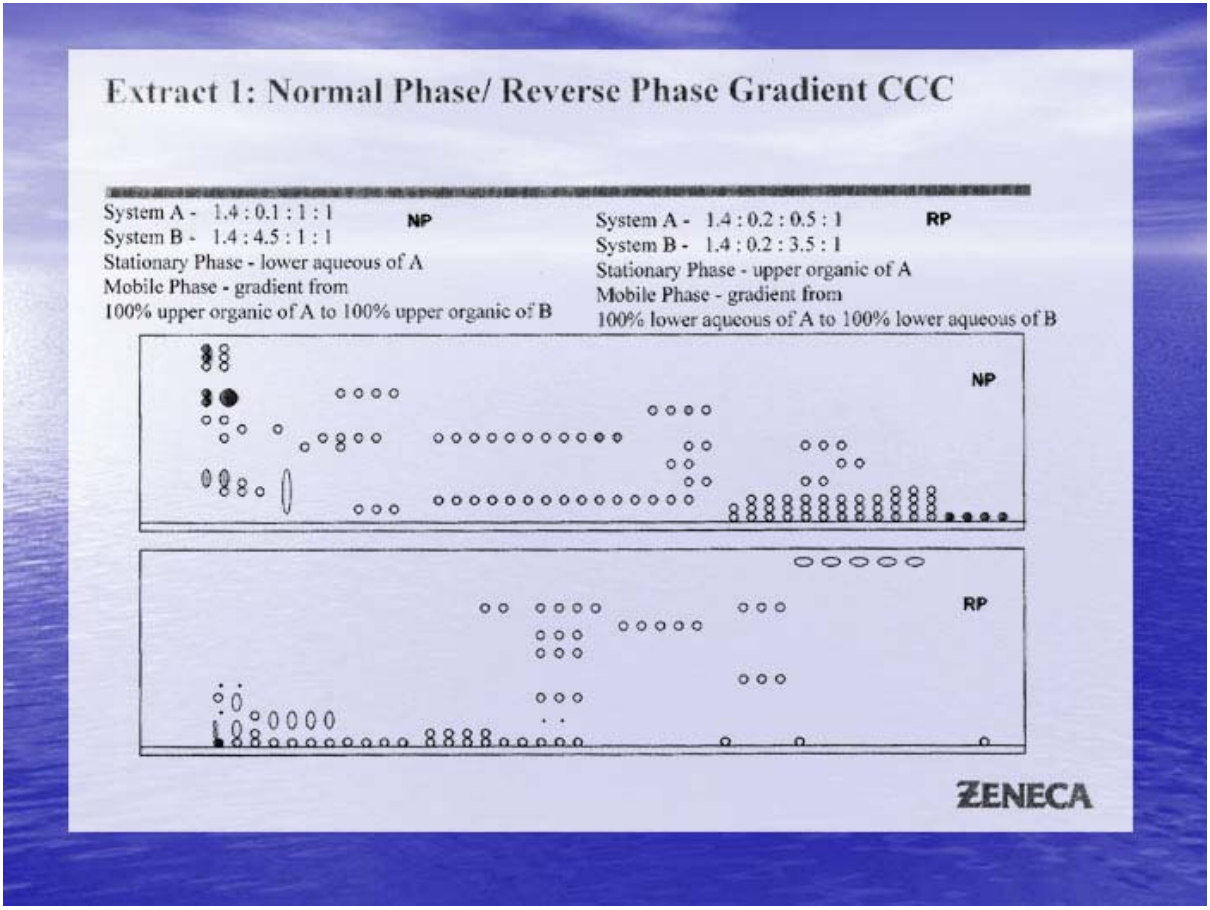
A typical linear gradient in HSCCC is shown in Slide 19 plus the typical affect of such a gradient on an ultra violet detector response and on the stationary phase bleed, is shown in Slide 20.



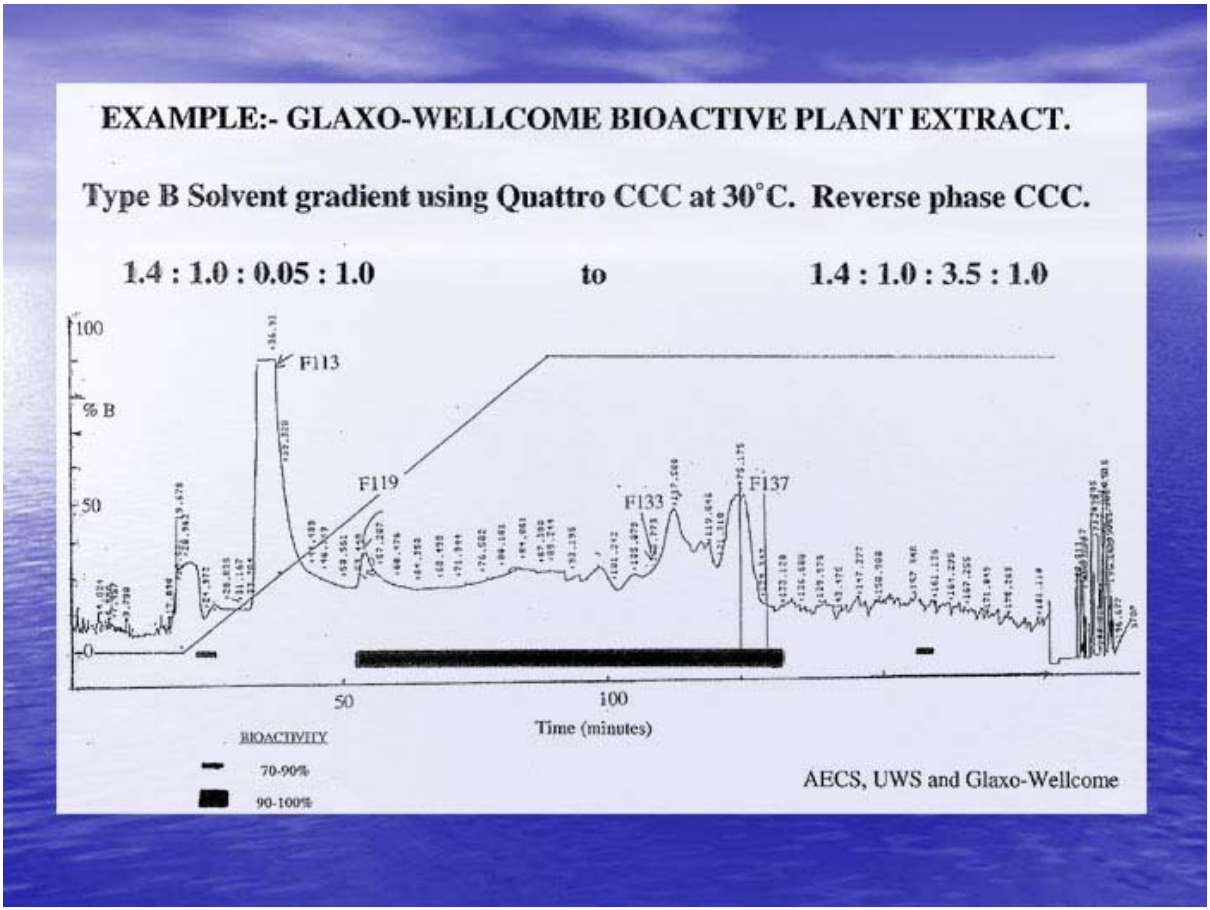
In gradient HSCCC it is possible to vary either the upper or lower phase modifier solvent in either a normal or reverse phase gradient.

Experimentation has shown in Section B gradient compositions Slide 15 that for reverse phase gradients the best results are usually obtained by varying the methanol ratio and in normal phase gradients the best results are obtained by varying the ethyl acetate ratio.

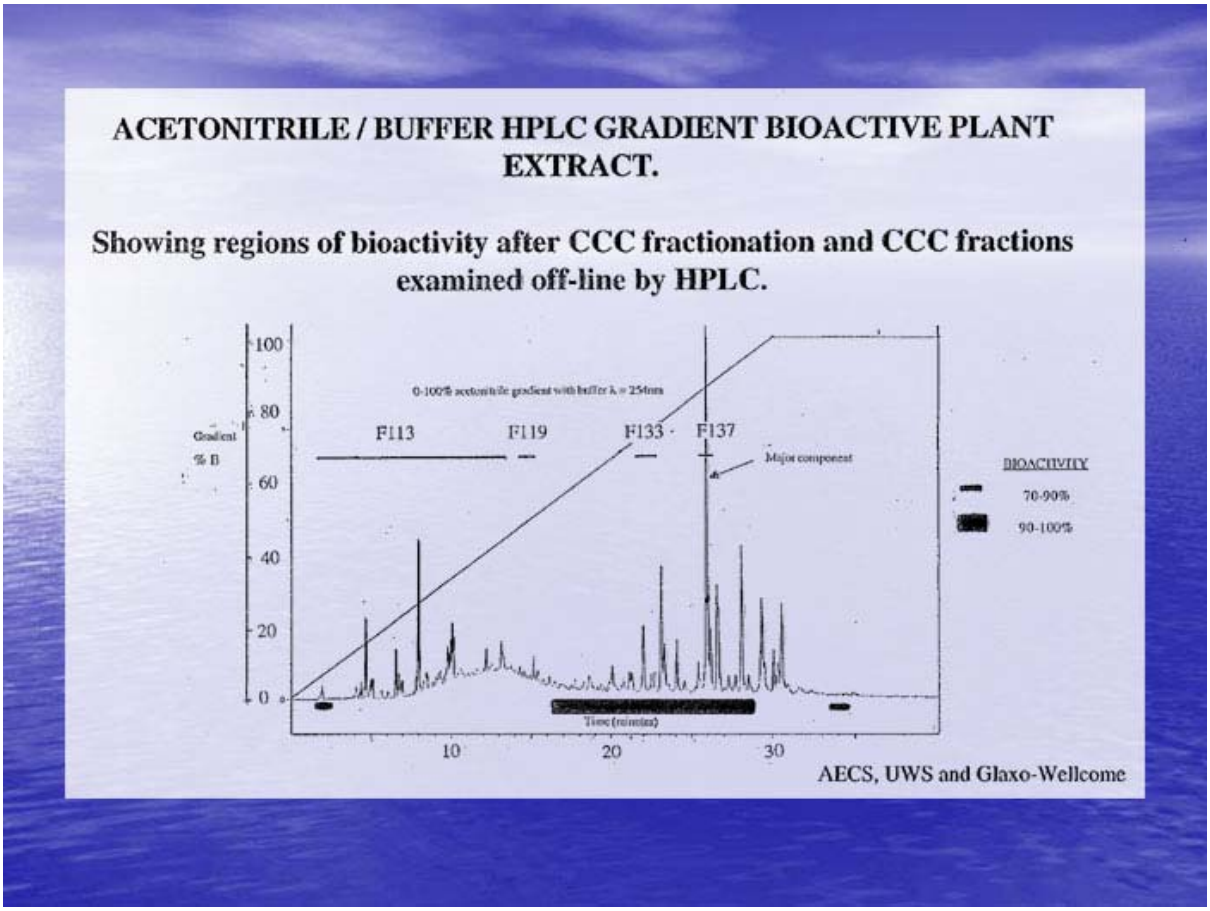
The radically different selectivity when doing the above whilst screening complex natural product samples, for novel bioactivity etc. sample is shown in Slide 21.



Whilst linear gradients have been successfully used in many applications including one step isolation of novel bio actives from untreated plant extracts as shown in Slide 22 a difficulty arises when trying to extrapolate from the linear gradient elution position to a suitable isocratic elution system.
The considerable effectiveness of an appropriate linear gradient can be see by comparing the complexity of the gradient hplc bioactive natural product extract, Slide 23 to the extremely high purity of the Quattro CCC purified bioactive ingredient shown it Slide 24. This slide also shows that 95% of this active ingredient was contained in a single 4 ml fraction which from the UV trace of the CCC run Slide 22 appeared to be identical to the previous fraction, which in fact contained only a minor trace of this bioactive component.



The considerable effectiveness of an appropriate linear gradient can be see by comparing the complexity of the gradient hplc bioactive natural product extract, Slide 23 to the extremely high purity of the Quattro CCC purified bioactive ingredient shown it Slide 24. This slide also shows that 95% of this active ingredient was contained in a single 4 ml fraction which from the UV trace of the CCC run Slide 22 appeared to be identical to the previous fraction, which in fact contained only a minor trace of this bioactive component.



The considerable effectiveness of an appropriate linear gradient can be see by comparing the complexity of the gradient hplc bioactive natural product extract, Slide 23 to the extremely high purity of the Quattro CCC purified bioactive ingredient shown it Slide 24. This slide also shows that 95% of this active ingredient was contained in a single 4 ml

fraction which from the UV trace of the CCC run Slide 22 appeared to be identical to the previous fraction, which in fact contained only a minor trace of this bioactive component. We should like to stress that online monitoring, especially by UV can be very misleading in CCC, and it is best to fraction collect and do offline tlc, hplc, hplc-ms, nmr or bioactivity assessment of these collected fractions in order to achieve a true picture of the resolving power of Quattro CCC's.

In hplc it is relatively easy to extrapolate an isocratic condition from a gradient because only the mobile phase is changing.

In HSCCC if one makes an isocratic mix based on the elution profile, then that isocratic mix will have a different stationary phase solvent ratio from that in the column. This occurs as both phases in HSCCC are interactive and inside a dynamically changing coil is a very different equilibrium environment from a static unchanging test tube.

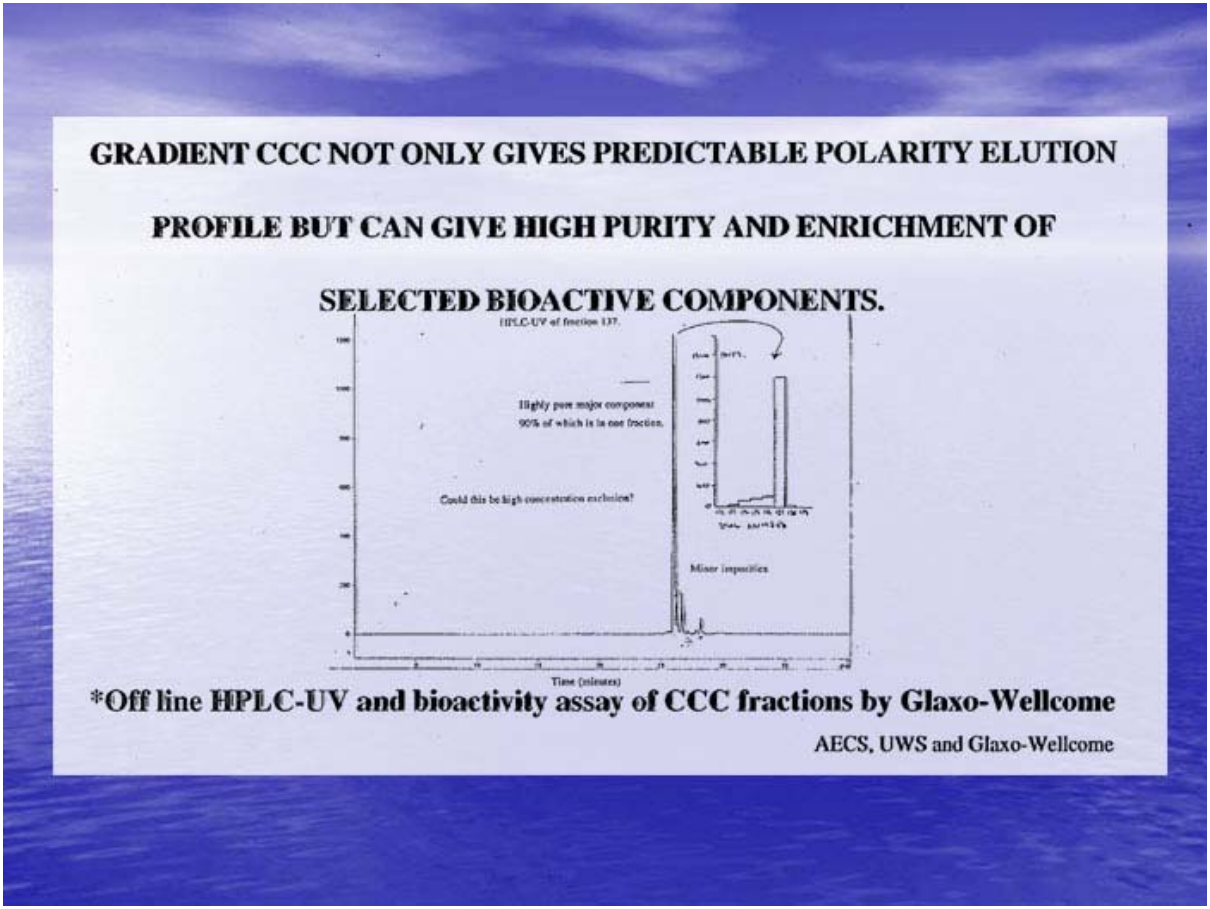
This will happen for both linear and step gradients, but the constancy of the step isocratic portion, allows easier optimization than in the linear gradient situation.

In addition, with step gradients, the additional wash-off of stationary phase once stepped initiated rapidly equilibrates and stabilizes. In Slide 20 a typical complex wash-off profile of a linear gradient is shown. These complex wash-off profiles also cause considerable extra complication when trying to optimize an isocratic separation from a linear gradient.

When method developing a novel HSCCC separation where test tube partitioning prescreening has proven unsuccessful, it is recommended to first try a reverse phase gradient and fractionate the complex matrix into predictable polarity bands. It is possible that component(s) of interest may still be in the presence of other components (sometimes a single CCC run can yield individual component purities of 98% +).

The gradient composition of the fraction of interest is noted.

The HSCCC chromatographer can then utilize the different selectivity of reverse and normal phase gradients, see Slide 21 and run a new normal phase gradient, which brackets the organic modifier elution concentration for the collected fraction of interest, which requires addition purification.



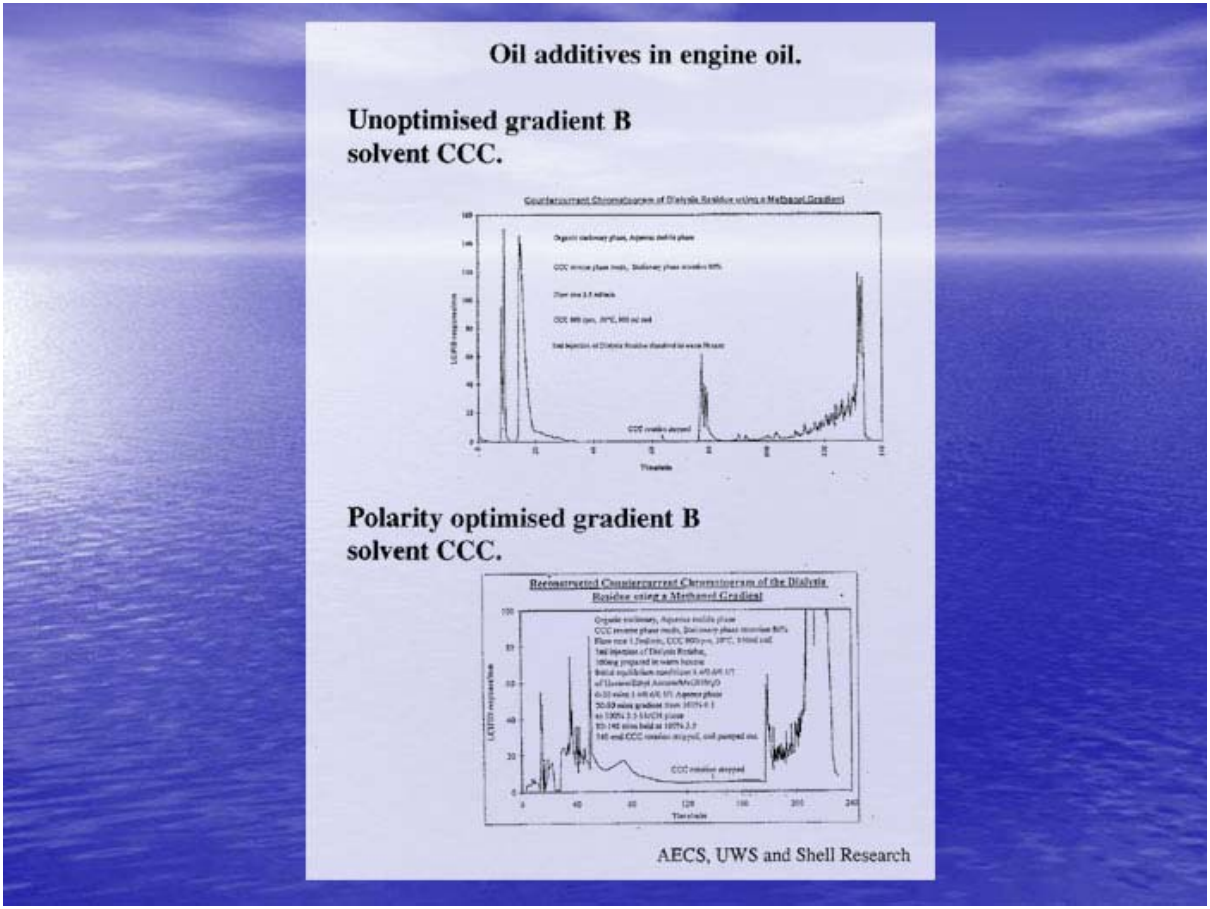
The fraction of interest is dried and dissolved in the start mobile or mobile / stationary or stationary phase as required, and the new normal phase step gradient run. Usually adequate resolution is obtained by this two dimensional CCC. This approach can easily be modified and enhanced by adding a fifth solvent for enhanced selectivity. The author is presently researching two-dimensional CCC and two-dimensional CCC / Prep HPLC.

Collaboration with other researchers on these novel topics is always welcome.

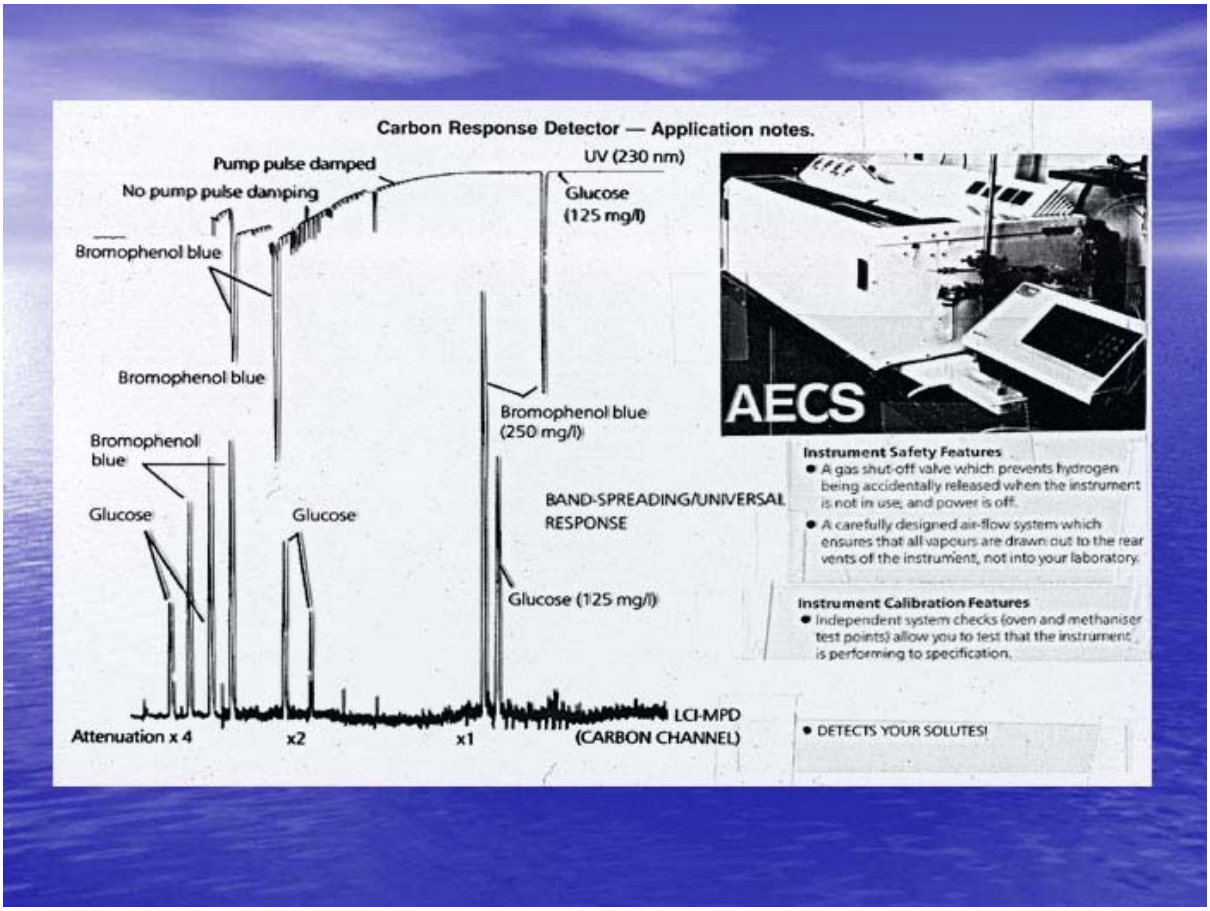
It is believed the later orthogonal techniques will have considerable merit, as generic CCC gradients can separate complex natural products etc without fear of expensive Prep HPLC column poisoning, into predictable polarity fractions. The relatively low complexity CCC fractions of known polarity can then be easily resolved into individual constituents

by chromatographers who will invariably have more experience in HPLC than CCC. The chromatographer will also have the added advantage of having far less loading to put onto the preparative HPLC column, therefore greatly reducing time and cost.

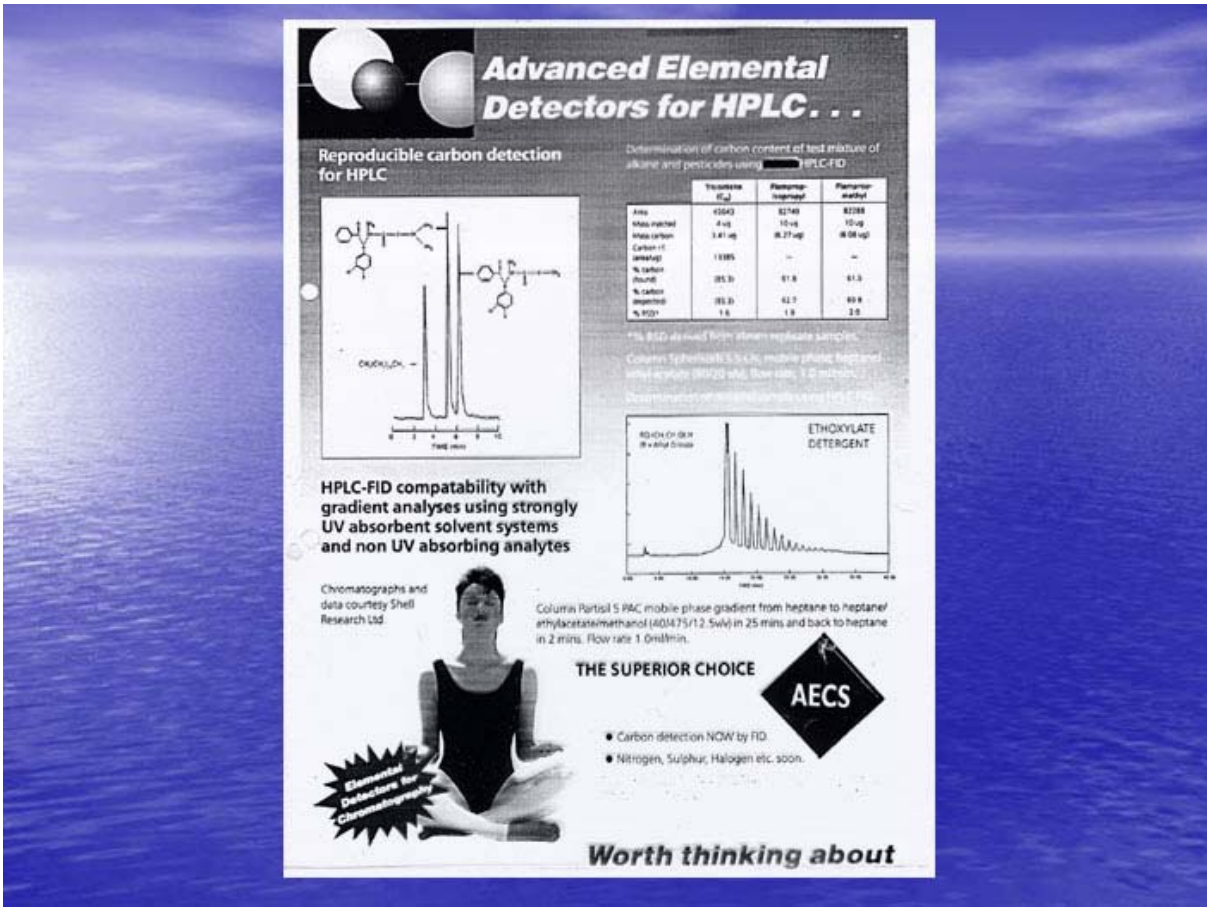
Component polarity profiles unthinkable in hplc are common in gradient HSCCC. The author has injected near boiling hexane containing engine oil additives into a Quattro CCC and achieved gradient elution polarity profiles from low molecular weight polar detergent surfactants (ionic molecules with 200 to 300 molecular weight) right through to very high molecular weight non-polar viscosity index modifiers (with molecular weights up to 500,000 +) Slide 25.



2. A comparison of the interface coupled to a Micro-Wave Plasma detector is should in Slide 27 where the elemental response from the MPD is compared to a tradition UV detector for a compound with a chromophore and one without. It will be noted that glucose is only detected with the LCI-MPD and that no discernable peak broadening occurs when using the LCI as compared to a UV detector.



3. Slide 28 gives example of the LCI-FID for gradient hplc chromatographs for pesticide / alkanes / ethoxylated detergents etc.
4. Slide 25 gives examples of the LCI-FID working with gradient elution from a Quattro CCC.



Summary of the use of isocratic, step and linear gradients in HSCCC

Brief descriptions and practical examples of the relative merits of isocratic, step and linear gradients in are given below.

1. In general isocratic HSCCC as in HPLC will allow a relatively narrow polarity window of compounds to be examined. Although in HSCC the unique option exists to stop rotation and elute the stationary phase with its retained components. This procedure is very valuable in isocratic, step and linear Brief descriptions and practical examples of the relative merits of isocratic, step and linear gradients in HSCCC / CPC are given above.
2. With isocratic analysis, it is highly recommended to do a literature search for similar compounds / matrices prior to beginning CCC. This option is more limited in gradient

CCC, as relatively few methods have been published, but gradients based around proven isocratic conditions are often successful.
3. Step and linear gradient HSCCC have an infinitely greater potential to cover a wider polarity range of compounds in a single run than HPLC.
4. Unpublished research by the UK Consortium showed that engine oil additives ranging from low molecular weight highly polar surfactants (few 100's molecular weight) to very non- polar high molecular weight (several 100,000's) viscosity index modifiers could all be examined in a single HSCCC gradient with good resolution of these and all intermediate polarities. Such a separation is not possible by any single HPLC column technology presently available.
5. Solvent choice in step and linear gradients can be facilitated by defining the polarity of components of interest by linear water to acetonitrile reverse phase hplc gradient. Based on this polarity definition appropriate generic solvent systems can be chosen.
6. Test tube and to a lesser extent tlc partition tests can help with defining the best possible gradient. Two examples of very successful additional phase optimization by test tube partition testing have been detailed in the first slide show in Applications.
7. It is the authors experience that caution has to be taken with this approach as on many occasions no correlation between these test tube / tlc partitions and the HSCCC results may result.
8. The easiest way to proceed with novel CCC method development on such occasions is to use gradient HPLC to define polarity and generic choice of gradient system.

9. A standard reverse phase step gradient (the gradient in Slide 17 + Slide 18) is an excellent trial reverse phase step gradient for Section B polarity solvents with stop rotation and collection of stationary phase will allow definition of the polarity for the total polarity range of compounds present in the sample.

10. If inadequate resolution is achieved several logical options exist :

I. Repeat the separation using a normal phase gradient and exploit the different selectivity of reverse and normal phase gradients.
II. Take the fraction of interest, which is now defined for HSCCC solvent elution, and re-inject this relatively pure (and probably far lower mass) sample into an appropriate normal phase HSCCC gradient, Section V-C. Alternatively use known polarity to choose an preparative HPLC column (if active compound not adversely affected by solid phase support). The advantage of the later, if appropriate, is the matrix will be relatively pure, and the mass injected far less, both making this fraction far more suitable for preparative HPLC than, for example a crude plant extract.
III. Examine the literature to see if any similar compounds / matrices separated by isocratic HSCCC and evolve step / linear gradients based on these.
IV. Examine the chemistry of components of interest and there relative solubility's in various solvents, and applying the standard like to like, best separates criteria common

to most forms of chromatography, devise novel gradients based on the near infinite variety of immiscible solvent systems available

In summary the Quattro HSCCC offer separation scientists unique options in analytical, laboratory, pilot plant and process scale chromatography, which can be readily assessed by gradient method development strategies.

Acknowledgements : Part of the Quattro HSCCC research above represents a summary of an initial collaborative program between Dr Les Brown (author) of AECS, exclusive worldwide distributors of the QUATTRO CCC, University College of Swansea, UK (Prof David Games, David Wheatley, Dr Rachel Ann-Whiteside, Dr Huw Kidwell) and University College of London, UK (Dr Gary Lye, Andrew Booth), BIB, UK plus Shell Research, UK (Dr Stuart Forbes and Euan McKerrel), Astra Zeneca, UK (Colin Strawson, Dr Ian McConvey, Antony Graham, Phillip Shering), Syngenta, UK (Brian Kemp, Dr Peter Massey, Linda Russel) and GlaxoSmithKline, UK (Dr Mike Dawson, Dr Martin Hayes, Stephen Jackson, Dr Tracey Shoulder, Dr Chris Preston). This project was partially funded by all the companies involved and by an UK LINK - BBSRC Grant plus UK EPSRC PhD Grant funding