

## Selectivity: A Liquid Chromatographic ‘Weapon of Mass Resolution’

By Mike Giles & David Keay

Complete resolution is the ultimate goal when attempting separation of the components of a mixture. Resolution is directly proportional to efficiency, selectivity and retention. If the value of any one of these three determinants decreases then the only way that resolution can be maintained is by increasing the magnitude of at least one of the other two.



For the last 40 years solid-liquid chromatographers have mainly considered efficiency as the determinant of good resolution and that has led to the conclusion that all separations must have high efficiency to achieve good resolution, and that without high efficiency, the best resolution is unlikely to be attained.

Some scientists have long been aware of the potential advantages that liquid-liquid chromatography, where resolution is determined selectivity rather than efficiency, could offer to complement existing solid-liquid chromatography. Due to the generally poor performance with separation times measured in hours, and unreliability of instrumentation which was primarily supplied by enthusiasts rather than engineering companies, liquid-liquid chromatography has only been used in a small number of niche applications during the last 30 to 40 years. The wider chromatographic community has been reluctant to use the technique with the complementary benefits it offers. Apart from any other benefits that the technique offers, it shows significant advantage when used with poorly soluble samples which can be problematic when using RP-HPLC.

Now, a new generation of instrumentation for liquid-liquid chromatography – High Performance Counter current Chromatography (HPCCC) - is available and is allowing scientists to realise fully the benefits of this orthogonal liquid chromatographic technique.

### How can successful resolution be based on selectivity?

Every chemist’s separation-science education begins by performing separation experiments on mixtures using two immiscible solvents in a separating funnel and utilising the process of differential distribution (partitioning) to achieve a separation. Liquid-liquid chromatography depends on such distribution as the mechanism of separation. Selectivity is the consequence of the relative distributions of two or more solutes with a pair of immiscible solvents.

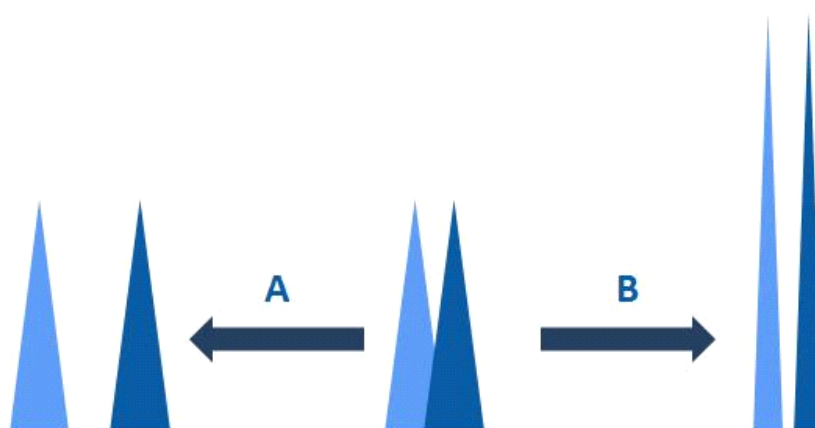


Figure n. A hypothetical situation to show the effect of increasing (A) the Selectivity term while N is held constant and (B) the Efficiency term while Selectivity is held constant, on the Resolution of two unresolved components (centre)

Fig n shows diagrammatically the complementary nature of liquid-liquid and solid-liquid chromatography when a separation is performed. The centre of the figure shows a frequently encountered chromatographic problem: a partially resolved pair of components. The availability of ever more efficient adsorbents (path B in the figure) has provided a technological solution to the problem. However, the use of adsorbents comprising sub-5 $\mu$  particles is not generally an option for preparative scale chromatographers since columns of an appropriate size would be prohibitively expensive.

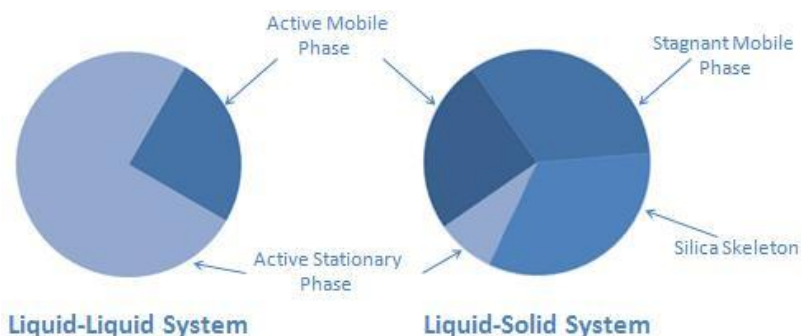
Achieving the desired resolution by improving selectivity (Path A in figure n) usually offers only limited opportunities for the solid-liquid chromatographer. By contrast, a technique such as liquid-liquid chromatography which depends on and can fully exploit differential selectivity, can achieve the desired resolution with ease despite the fact that it is a low efficiency separation technique.

Liquid-Liquid chromatography can offer an alternative strategy which is to improve the selectivity parameter to achieve the desired resolution. Figure n shows that baseline resolution is possible by both approaches, however the chromatograms will be rather different: the increased efficiency option produces tall, narrow peaks compared with those obtained by improving selectivity, which are short and wide.

N required to give R for given $\alpha$ values		
$\alpha$	R = 1.0	R = 1.5
	N	N
1.000	$\infty$	$\infty$
1.005	650000	1450000
1.010	163000	367000
1.020	42000	94000
1.050	7100	16000
1.100	1900	4400
1.250	400	900
1.500	140	320
2.000	65	145

Solid-liquid chromatographers associate short and wide peaks with low efficiency and poor resolution. However, because there has been such a focus in the past on increasing efficiency, the fact that the resolution equation has selectivity and retention terms as well as the efficiency term has been overlooked as a development opportunity. Resolution is directly proportional to efficiency, selectivity and retention. As stated at the beginning of this article, if the value of any one of these three determinants decreases then resolution can be maintained by increasing the magnitude of either or both of the other two. This is demonstrated in table n which shows that if the efficiency of a separation is reduced then resolution is maintained if there is a sufficient increase in  $\alpha$ , the selectivity. In fact, it shows that baseline resolution ( $R = 1.5$ ) can be achieved with only a few hundred theoretical plates as long as  $\alpha$  is sufficient. HPLC exploits both selectivity and retention which offsets the low efficiency.

In low efficiency scenarios, the short and wide chromatographic peaks are not necessarily indicative of low solute mass. The resolution equation can also be expressed with a phase volume ratio term. Resolution increases as the volume ratio of stationary phase to mobile phase increases: in the resolution equation the actual term used is  $V_M/V_S$  (where  $V_M$  is the volume of mobile phase and  $V_S$  the volume of stationary phase) and is part of the denominator, i.e. high ratios of  $V_M$  to  $V_S$  are favourable. In RP-HPLC, the value of the term may approach 10 or more whereas in HPLC the value can be as low as 0.05. This means that in HPLC, the stationary phase is not only able to retain solutes better but consequently has a far higher capacity than is possible in RP-HPLC. It is for this reason that in HPLC solvent used per gram of solute processed is typically 10-20% of that used in solid-liquid chromatography.



These concepts are illustrated in the accompanying diagram where three important and highly relevant factors are shown in graphical comparison.

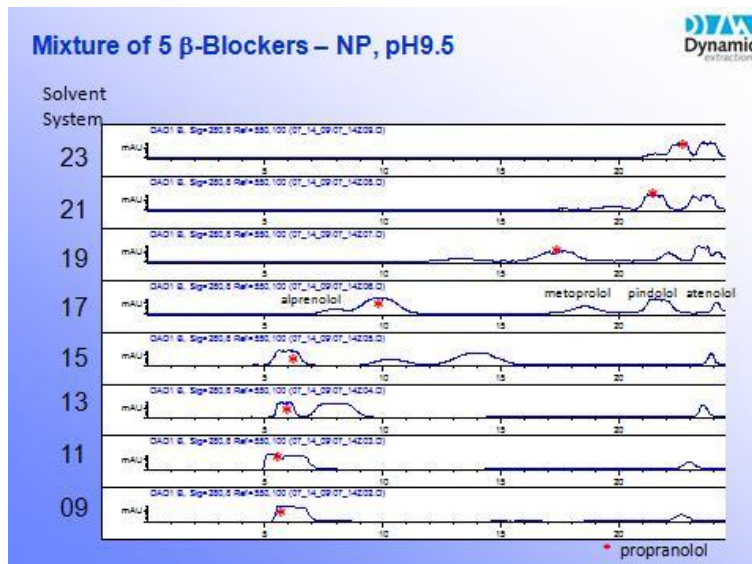
Firstly, the relative volume ratios of combined stationary and mobile phase are very different in the two columns; secondly, the phase volume ratios\* are inverse; thirdly, the whole column contents of the liquid-liquid column are actively and beneficially

involved in separations. The stationary phase support matrix of solid-liquid systems is responsible for the complications of secondary interactions. Liquid-liquid chromatography is subject only to primary interaction.

\*Typically in solid-liquid, supported phase, e.g. reverse phase, chromatography the ratio ( $V_S/V_M$ ) is 1:10 or less. In normal phase solid-liquid chromatography, the ratio may be up to 1:1 or 2:1. By contrast, in HPLCC, typical values for this ratio are from 2:1 to 9:1 and are often as high as 20:1 i.e. typically 70 - 90% of the total column volume is occupied by stationary phase and the value is often as much as 95%.

### The impact of selectivity on resolution

The diagram to the right shows the ammonia-modified, pH9.5, liquid-liquid chromatograms of a mixture of 5  $\beta$ -blockers which are small pharmaceutically important substances. The figure demonstrates the impact of changing the selectivity on the relative retentions and hence resolution of the compounds. The dramatic retention and selectivity changes reflect the fact that, across the range, every other HEMWat series solvent combination was used to develop the chromatograms. If adjacent steps of the series are used, the selectivity changes are more subtle. The selectivity changes can be made even more subtle by using intermediate combinations to effect very small changes in relative retention and selectivity and thereby obtain optimum resolution. The figure demonstrates what amounts to the method development process of liquid-liquid chromatography i.e. examining different solvent combinations to determine which is optimum for the separation to be effected.



The plate efficiency of the liquid-liquid chromatography system used for the separation is approximately 600 and the chromatography amply demonstrates that resolution by means of altered retention and selectivity is achievable in low efficiency systems.

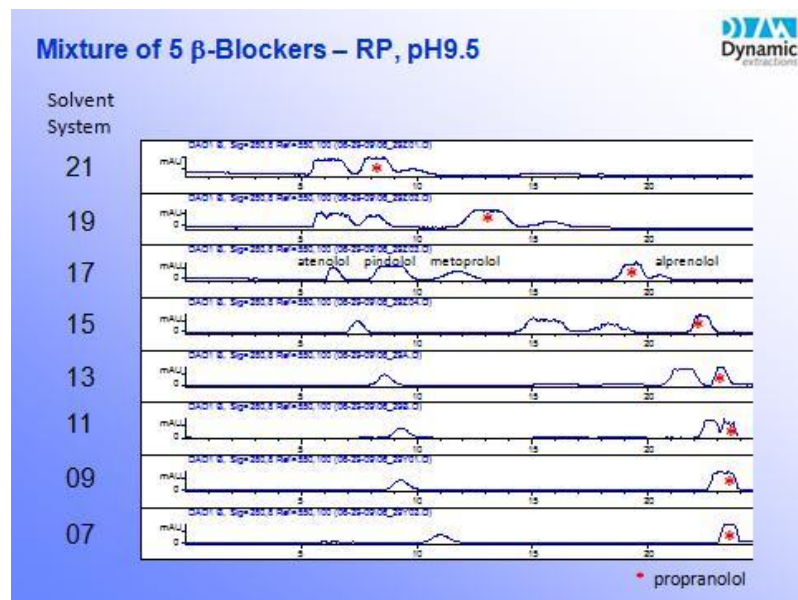
Several factors influence the selection of which HEMWat table combination of solvents is eventually used for a separation: objectives of the separation of the five  $\beta$ -blockers are discussed below but overriding the selection are decisions about whether the overall objective is maximum purity, maximum yield, or minimum run time.

With reference to the diagram above, if the objective was resolution of all five components, the optimum combination would be near 17. For the isolation of pure alprenolol, a combination near 18 would be used. To isolate the maximum yield of very pure propranolol, the optimum would be near 19. 19 would also allow isolation of atenolol. To isolate the maximum yields of pure metoprolol and pindolol, 15 would be best. There is little to choose between any of the systems from 09 to 17 as far as atenolol is concerned and any of these combinations could be used.

If resolved components are eluted from a liquid-liquid column, they must logically have been resolved at some point whilst still within the column: another regularly used technique in liquid-liquid chromatography is extrusion of the column contents. This is accomplished by displacement of the column contents whilst maintaining the resolution of the solutes within. The technique, which is essentially impossible in solid-liquid chromatography, can be used to dramatically reduce run times. In the example of the  $\beta$ -blocker mixture above, normal elution using solvent combination 17 could be stopped at or even a little before the complete elution of propranolol and the column contents extruded to produce pure metoprolol, pindolol and atenolol. If isolation of only atenolol was required then the fastest isolation would be by using HEMWat 09 where alprenolol, propranolol metoprolol and pindolol are rapidly eluted together. Following their elution, the column contents would be extruded to obtain pure atenolol.

However there is a far more elegant and yet faster way to isolate the atenolol if this was the objective: in liquid-liquid chromatography, interchange of the roles of the stationary and mobile phases is possible and is used as part of the normal chromatographic process. This technique is of course impossible in solid-liquid chromatography.

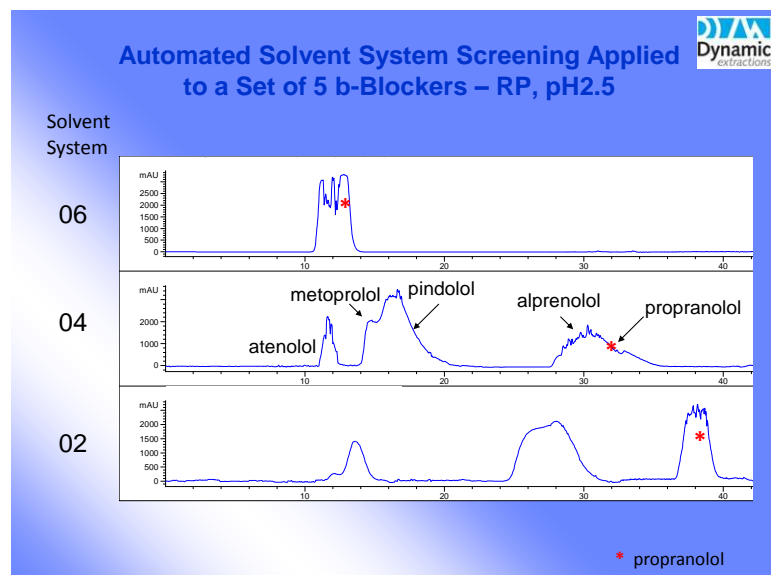
The previous figure shows chromatograms that were developed using Normal Phase elution where, in respect of the HEMWat table, the lower, more polar phase is used as the stationary phase and the upper less polar phase is used as the mobile phase for the chromatography.



The figure to the left shows the similar changes in relative retentions and selectivity that were obtained when the chromatograms of the  $\beta$ -blocker mixture were developed using Reverse Phase elution. Apart from the fact that the elution order is reversed, there are key differences in the selectivities and resolutions. Using near HEMWat 17 would certainly produce the fastest possible liquid-liquid chromatographic isolation of atenolol and would also allow fast and complete resolution of pindolol and metoprolol and could be used for the isolation of propranolol and alprenolol but as shown in the previous figure, the fastest and best resolution of this last pair is by use of normal phase

elution.

The above examples demonstrate the enormous and facile (all of the techniques discussed above are very easily accomplished) range of ways that liquid-liquid chromatographic techniques can be used.



The figure to the left shows the impact of changing the modifier from ammonia to HCl. In this particular case resolution has been lost but the changes in selectivity relative to the high pH elution are dramatic: any retention and resolution requires the use of HEMWat 05 or less.

The impact of changing pH is significant in both solid- and liquid-liquid systems: advantageously in liquid-liquid systems, the full range (0 – 14) of pH can be applied to enhance selectivity and resolution.

## Achieving changes in selectivity

The changes in retention shown in the previous figures are obtained by the use of a stepped-polarity series of biphasic solvent mixtures. The members of this series, known as HEMWat, are prepared from readily available solvents: heptane (or hexane), ethyl acetate, methanol and water, which are used in a range of defined combinations which allows the preparation of solvent mixtures that cover a wide range of polarity from non-polar to polar. The compositions of the 28 standard mixtures in the table are shown to the right.

The HEMWat series covers a wide range of solvent mixture polarities and is therefore suitable for use with solutes which have a wide range of polarities and structural types. The HEMWat table allows the retention and resolution of solutes such as amino acids, sugars and alkaloids at one end of the polarity range, to lipophilic substances such as terpenes, at the other.

Method development, the process of purity and loading mass/volume optimisation of a separation is simple and is now, optionally, an automated, unattended, procedure that can be programmed into an existing analytical HPLC instrument that is fitted with a quaternary pump and in which

the HPLC instrument is simply regarded and treated as a column. The quaternary pump allows on-demand mixing of the desired members of the solvent series to be screened (scouted) and the instrument's auto-sampler is used to load the samples under investigation. The chromatographer then chooses the most suitable, unmodified or modified, with for example an appropriate pH buffer, solvent combination for optimization of the method. A complete scouting experiment using 8 HEMWat solvent combinations can be completed within 3 hours

## How chemists can benefit from use of liquid-liquid chromatography

The combination of the automated solvent system selection as described above with the direct separation scale-up capability of HPLC instrumentation provides a path to gram amounts of pure compound within a few hours. Reduced to actual practice, a few milligrams of a target sample is subjected to a standard scouting method, developed based upon the specific needs of the laboratory, covering perhaps 5-10 of the solvent systems shown in the table above. Separation condition decisions are made by simple visual inspection of the resulting chromatographic data. The solvent combination scouting process is functionally equivalent to scouting multiple RP HPLC or SFC stationary phases. Once a solvent system has been selected, preparative scale separation is purely an issue of linear, volumetric scale up using a larger

No		Heptane	EtOAc	MeOH	Butanol	Water
1		0	0	0	5	5
2		0	1	0	4	5
3		0	2	0	3	5
4		0	3	0	2	5
5		0	4	0	1	5
6		0	1	0	0	1
7	More	1	19	1	0	19
8	Polar	1	9	1	0	9
9		1	6	1	0	6
10	↑	1	5	1	0	5
11		1	4	1	0	4
12		1	3	1	0	3
13		2	5	2	0	5
14		1	2	1	0	2
15		2	3	2	0	3
16		5	6	5	0	6
17		1	1	1	0	1
18		6	5	6	0	5
19		3	2	3	0	2
20		2	1	2	0	1
21		5	2	5	0	2
22		3	1	3	0	1
23		4	1	4	0	1
24	↓	5	1	5	0	1
25	Less	6	1	6	0	1
26	Polar	9	1	9	0	1
27		19	1	19	0	1
28		1	0	1	0	0

### Features of Liquid-Liquid Chromatography

- High mass and volume injection loadings
- Improved handling of sample solubility issues
- Ease and cost of scale-up
- Orthogonal (to HPLC) purification technique
- Green Chromatography with very low solvent usage
- Total sample recovery
- Reduced sample preparation
- Tolerance to all pH and buffer additives
- Uniquely single mode interaction

volume column on the HPCCC instrument. The chemist can complete this whole process within a day.

#### Applications of Liquid-Liquid Chromatography

Small and large synthetic molecules

Peptides

Natural products

Preparative scale separations

Sample prep prior to MS in ADME applications

High viscosity samples

Low solubility samples

Chemically or physically contaminated samples

HPCCC is a 'green' chemistry technique by virtue of the reduced solvent consumption per unit mass of purified material. Typically 0.5-2 grams of material can be purified using a 135mL HPCCC column. This high, mass loading per unit volume of stationary phase is the consequence of the fact that the entire, stationary and mobile phases are actively involved in the separation. The rich cut of the target compound is approximately 25% of the total column volume, only 30-35mL for a column of this size. Using elution-extrusion would further reduce this volume. Elution at such high solute concentrations dramatically reduces sample workup time, labour and energy consumption whilst producing less waste, when compared with RP HPLC separations at this scale. Total solvent consumption for this scale of separation, assuming a one column volume elution-extrusion mode separation, would be 300mL or 150-600mL/gram.

#### Conclusions

HPCCC could be used in many cases to replace HPLC but the real place for the technique is in the armamentarium of the modern chromatographer as the 'weapon of mass resolution' : to be used, as all should be, when it is 'the best tool for the job' which it unquestionably and frequently is.

In the final analysis, there is little scope for increasing efficiency beyond that offered by sub-2 micron particles currently used for analytical HPLC or the 5-10 micron particles used for laboratory preparative scale HPLC and the 20-30 micron, or larger, particles used for plant scale preparative chromatography, due to both cost and technological limitations. However, when considering CCC, although the options for increasing efficiency are limited with current technology, those for increasing selectivity and thereby resolution are relatively unlimited and represent the next area for the technological development of chromatographic resolution.