# Applications of Liquid-Liquid Chromatography Instrumentation for Laboratory Preparative & Process Chemistry

*Brown, Leslie & Luu, Trinh Anh of AECS-QuikPrep Ltd, PO Box 80, Bridgend, S. Wales, UK. CF31 4XZ ( aecs@gmx.com )*
*Author for correspondence*
*Hill, Colin & Flockhart, Ian of Botanical Developments Ltd, of UK*
*Garret, Pierre-Henri & Margraff, Rodolph of ETS-Couturier, of France*
*Skouroumounis, George of University of Adelaide, of Australia*

Liquid-Liquid Chromatography instrumentation (L-LC), also referred to as Counter Current Chromatography (CCC), and Centrifugal Partition Chromatography (CPC), design has been instigated and, championed largely by Academics at University and Research Institutes in USA, Japan, Europe and China. To date instrumental L-LC has not been universally adopted by chromatographers in many commercial sectors. Discussed is a scientific treatise from a commercial viewpoint, of usage and design advantages and disadvantages of instrumental L-LC.

Keywords: Liquid-Liquid Chromatography, Counter Current / Countercurrent Chromatography, Centrifugal Partition Chromatography, Laboratory / Process Chromatography, High Throughput / Combinatorial Preparation.

## Introduction

There are very many fundamentally different modes of L-LC instrumentation design. The two major design modes in present use are planetary centrifuges (usually referred to as CCC, HSCCC or HPCCC) and sun centrifuges (often referred to as sun or droplet CPC), Recently the International CCC Committee voted to define planetary CCC as hydrodynamic CCC, and sun or droplet CPC as hydrostatic CCC, whilst acknowledging that both are Centrifugal Partition Chromatographs (CPC).

The industry wide use of CCC nomenclature has lead to much confusion in the mode of operation, as to non L-LC chromatographers; Counter Current modes would involve two liquids moving in different directions. With L-LC/CCC despite the fact they can readily be used with liquids moving in two opposing directions, in reality over 99.9+% of usage cases, only one phase is stationary and one phase is mobile. For this reason we have chosen to refer to this science as L-LC rather than CCC or CPC. In this publication we will refer to planetary CCC, HSCCC and HPCCC as hydrodynamic L-LC and sun or droplet CPC as hydrostatic L-LC.

Solid Liquid Chromatography (S-LC) techniques would include Open Tubular, Flash, Medium Pressure Liquid Chromatography (MPLC) and High Pressure (Performance) Liquid Chromatography (HPLC).

In S-LC one phase is stationary and one phase mobile. The stationary phase is often an immobilised liquid, which has been immobilised by bonding to a solid substrate. Liquid-Liquid Chromatography is therefore in many ways directly analogous to Solid Liquid Chromatography.

The main difference between L-LC and S-LC in many cases is L-LC maintains one of the pair of immiscible liquids stationary, through its physical mechanical/electrical instrument design, rather than adsorption onto a solid particle. Why then is not L-LC the equal scientifically/commercially of S-LC?

## A Review of CCC/CPC Historic Confusions, To Enable L-LC Become Mainstream Chromatography

### Confusion 1. Nomenclature.

In the Introduction the major confusions associated with the nomenclature and mode of operation of CCC/CPC is discussed. We proposed in this paper, as we have for several years to the International CCC Committee, that consideration be given to more appropriate nomenclature.

### Confusion 2. L-LC is a laboratory scale curiosity. Historically yes, now no.

Modern L-LC instrumentation can range from 7 ml for L-LC MS studies of trace amounts, to single units of 10 to 25 litres, or modular process units in any number/configuration of multiples of 3 litres capable of multiple tonnes/annum production. Research applications include natural products, nutriceuticals, agrochemicals, pharmaceuticals, and food/beverage etc. Large-scale production (20 litre + units) is mainly limited to natural product based products.

### Confusion 3. Different concepts of L-LC can interchange methodology. True on occasions, but still a misleading statement.

The reality is that the equivalence of different L-LC modes to other L-LC modes is not comparable in SLC to one manufacturer's C18 or silica column to another's C18 or silica respectively (although we all know significant differences can occur even in S-LC). It may not even be equivalent to comparing in chiral S-LC, a cellulose to an amylose carbamate. It could be more like comparing these to a Pirkle chiral column. The reality is, L-LC's with fundamentally different design principles may do the same separation, but each might do in a radically different way. Transfer of method between two different L-LC design concepts with the same solvent, target and matrix, is as much luck as science.

The above can even be applied within a single manufacturer's product range, if the manufacturer varies key L-LC design factors. The more factors they vary, the greater the likelihood of failure during Process Scale-up. Rationalisation of design by using modularity of design in both modern forms of L-LC instrumentation can reduce the problem of scale-up to make them no more difficult than in reverse phase S-LC.

The key design factors in hydrodynamic L-LC are sun & planet radii, beta values (even if compensation of changing rotation speed is made), coil winding technique, tubing bore etc. For hydrostatic L-LC the key design parameters are chamber shape/design/insertions, chamber volume, sun radii etc. For both L-LC principles as more factors are changed, the percentage success of Process Scale-up and cross compatibility between different L-LC fundamentally different design modes will reduce.

**Confusion 4. Solvent selection in L-LC is not scientific. Incorrect, it is governed by same principles as S-LC**

From discussions, non L-LC chromatographers have repeatedly said that they see L-LC biphasic solvent choice in published methods, as a variety of abstract solvent mixes or quote, "Witches Brews". At best, they see the Arizona etc methods as "semi-defined scales of set ratios, in supposed sequences, for which L-LC researchers chose convenient, unrealistic standards to argue their validity".

Our biphasic solvent selection research (presently being prepared for publication) shows this does not need to be so. Biphasic solvent selection in L-LC is governed by exactly the same principles as S-LC, with target & matrix solubility, functionalities, polarities, molecular weights, dipole moments, shape configurations, complex and micelle formation, pH etc all taken into account and utilised in comparison to known solvent properties, as defined by the Synder Triangle and solvent polarity series.

**Confusion 5. At Big Prep 5 it was confirmed, a Company has over one million bioactive compounds in its library, and all only required S-LC. One could ask do we need L-LC? The answer is definitely yes. To assist this statement, we would add that a Quattro CCC was recently custom designed for a USA Pharmaceutical Company for High Throughput Preparation application. This custom build shows that not only is L-LC's maintenance of compound integrity applicable in searching for unknown bio-actives/taste/colour/nutriceuticals etc in natural products, but it may possibly one day have a place in mainstream Pharmaceuticals as well (see Results & Discussion).**

Why is the answer above yes? The answer above is yes in part because the above initial statement is self-fulfilling prophecy. If research only ever used S-LC to define a library, by definition any compound that would be absorbed by the phase, or would have been degraded by the phase would not be in the library. Perhaps it would be well for us all to remember that silica is used as a catalyst for certain hydrolysis reactions. The question that should be asked is how many bioactive targets or cytotoxic contaminants may be missed owing to use of only S-LC? (Ref Results & Discussion). The polarity limit of RP-C18 / NP-silica etc in HPLC etc is alarmingly small, compared to L-LC. With stop rotation-wash-off or elution-extrusion each L-LC run can go from infinitely polar, to infinitely non-polar (or vice versa). If using a switching valve, Head to Tail may be reversed during a run, and even change from a reverse phase to normal phase run (or vice versa) at any time of the users choosing.

**Confusion 6. L-LC always has low plate counts. Why bother with L-LC? The reply is, L-LC has massively higher stationary phase retention than S-LC, thus achieves resolution with low plate count, plus L-LC can achieve selectivity through its vast stationary phase options. L-LC can use half to less than a tenth of solvent to prepare the same mass of target in same matrix when cross-compared to SLC. L-LC has a very high loading capacity (5 to 15% of coil volume), plus only requires low cost, low-pressure liquid pumps. All these factors make further consideration of L-LC important as cheaper more "Green" techniques are researched.**

Regarding low plate per metre (p/m) counts, it should be noted that the percentage of stationary phase is fundamental to full resolution equation, though this factor is often deleted in HPLC texts. The reason HPLC etc requires high plate counts is in part that it has a very low percentage of stationary phase relative to the total content of the containing vessel.

In Flash and HPLC the numbers of stationary phases are extremely limited compared to L-LC. Therefore resolution by massive changes in selectivity is limited in S-LC.

L-LC traditionally uses 70 to 98% stationary phase. In a reverse phase C18 column, the C18 might be 10 to 21% of the stationary phase, which is itself a small percentage of total container void volume/void mass. In L-LC logical biphasic solvent selection, with options of isocratic (1,2), linear or step gradients (3), ionic liquids, pH based frontal chromatography (called pH Zone refining in CCC), micelles, reverse micelles, aqueous/aqueous, aqueous/organic, organic/organic, stop rotation-wash-off, elution-extrusion are all usable using the same L-LC system. All these possibilities are created only by choice of different biphasic or even triphasic solvents. Inorganic cations (inclusive precious metals, transition metals, radioactive isotopes) and anions, plus organic compounds, even certain shape orientations/chiral compounds, can be resolved with a single L-LC instrument.

**Confusion 7. L-LC is mainly a stand-alone chromatography technique. Incorrect, Sequential L-LC and HPLC has been for the last 10 years our preferred operation mode in Contract Preparations of targets.**

Almost every difficult application at the AECS-QuikPrep Ltd laboratory uses L-LC to polarity fractionate highly complex matrices (that would poison an HPLC column with one injection), into narrow polarity bands. We would stress the mutual benefit of Sequential L-LC and HPLC, and never view L-LC and HPLC as mutually exclusive. The L-LC fractions are so extremely restricted in polarity, that they only require isocratic HPLC to complete our standard Sequential L-LC to HPLC runs. 95 to 99+% pure target can be obtained after a single Generic Gradient L-LC run and Sequential HPLC Prep column (see below). This Sequential L-LC and HPLC principle can be repeatedly successful, even when starting from unknown, and totally uncharacterised, raw natural product or crude synthesis materials. Sequential L-LC to L-LC, utilising different biphasic solvents for the second L-LC run, is only used if targets irreversibly adsorb or degrade on 5um, end capped C18 etc HPLC phases.

Context


*Figure 1*

**Quattro OT HPL-LC™ Modular Lab to Process**

| Model | Width In mm | Depth In mm | Height In mm | Weight In kilo | Volume Range In ml | Max No Of Coils | Coil Type |
|---|---|---|---|---|---|---|---|
| IntroPrep™ cccMSPrep™ | 310 | 580 | 440 | 47 to 50 | 7 to 140 | 2 | PTFE or S/S |
| QuikPrep™ | 350 | 580 | 440 | 60 to 65 | 7 to 750 | 4 | PTFE or S/S |
| LabPrep™ | 430 | 580 | 440 | 88 to 98 | 30 to 1706 | 4 | PTFE or S/S |
| HTPrep™ | 430 | 580 | 440 | 65 to 98 | 30 to 1706 | 4 | PTFE or S/S |
| PilotPrep™ | 750 | 580 | 440 | Variable | 100 to 3000 | 3 | PTFE or S/S |
| ProcessPrep™ | 750 | 580 | 440 | Variable | Any number Modules of 3000 | Variable | PTFE Or S/S |

*Figure 2*

Fig 1 & 2 show the chassis and coil/volume options of the Quattro CCC™ model range. ("J" Type Planetary Centrifuge, open, constant id tubing, wound on a planetary bobbin, with no rotating seals). The bobbins (planetary rotating body, holds the coiled columns) can have tubing with different material choice. Options include PTFE, Stainless Steel or Titanium. Tubing bore for id can vary from 0.5 to 12.5 mm, and volumes from 7 to 3000 ml for a single rotor assembly. A single bobbin can have two coils. All models except the entry IntroPrep™ have two dynamically balanced bobbins, with up to 4 coils as an option. Each coil can be used independently for same or different preparations, or used in any combination, in series with any coil or multiple of coils of the same id. Uniquely for hydrodynamic L-LC model ranges, all models share the same key L-LC design parameters, inclusive of the same sun & planet radii, speed ranges, beta values, winding techniques and only tubing bore is varied. This model range is also the only one that allows even the largest bore to be tested on a laboratory based unit, prior to introduction to process based preparation. Hybrid coil winding, that is multiple id.'s in the same instrument or bobbin, can be manufactured produced. Multiple bobbin sets for a single chassis are available. In this way the major

difficulty of needing several different instruments, to validate scale-up is avoided.

For Process Chromatography, the base module is of 3 litres. Bobbins are interchangeable, and can be exchanged for re-winding if PTFE tubing chosen and cGMP requires virgin material. Most would use stainless steel or titanium tubing and appropriate cleaning techniques, but renewing PTFE coils is an option. If different bore sizes are required, different bobbin sets may be used. Bobbins can be used in series, in parallel or in simulated moving bed operations. Clutches and switching valves allow operating mode changes.


*Figure 3*

The Partitron CPC™ is shown in Fig 3 (sun centrifuge, with separation chambers and id restricted links between chambers, with 2 rotating seals). This model range has a single process-scale chassis unit. Most hydrostatic CPC are manufactured as chambers created by a sandwich of machined or etched chambers formed into a stainless steel disc, with layers of PTFE sealing the individual disc layers from other layers. The whole assembly is bolted together, but can be prone to leaks and blockages. Machining and in particular etching of any surface radically increases the surface area exposed. Viewed under a microscope the machined/ etched surfaces will appear as mountains and valleys relative to the same material before machining/etching. As CPC units are particularly suited to aqueous/aqueous chromatography of peptides and proteins, enzymes etc, all of which are prone to degradation, the choice of machined/etched stainless steel for most hydrostatic L-LC is suspect.

The Partitron CPC™ was specifically designed for large scale, GMP process chromatography. A totally different construction is utilised. The whole rotor assembly (Fig 3) is machined from a single titanium block. Titanium is well recognised in chromatography for its inertness. A variety of titanium rotors, with volumes from 5 to 25 litres, with either one or two volumes per rotor, may be fitted to this versatile, uniquely modular hydrostatic L-LC.

Fig 4 shows an industrial sub & super-critical extraction plant which is used in conjunction with L-LC production and research

**Results & Discission**

All experiments in Case Studies completed with a Quattro CCC™.

Discussions regarding Confusions 1 to 2
Unpublished Grant funded research ("The


*Figure 4*

Industrial Scale up of Countercurrent Chromatography". BBSRC/DTI LINK Award Ref: 100/BCE08803. Feb 98 - Jan 00 (£322,668), a collaboration of AECS, Brunel University, University College of Swansea, GSK, Astra Zeneca & Shell Research) supported comments by CCC experts, that CCC of different designs or even a single concept, if one varies key parameters this can, on occasions, prohibit scale-up. Keeping all parameters the same, only changing tubing bore, certain scale-ups failed. AECS & Brunel University interpreted the implication of these results in radically different ways in their subsequent independent commercialisation of L-LC. AECS rationalised design to minimise variability and has spent 9 years increasing its understanding scale-up failures. Brunel University and staff developed a range of CCC with radically different sun & planet radii, speed ranges etc. and formed their own spin-off company (DE Ltd) six years ago to exploit their research.

Discussions regarding Confusions 3 to 7
Non-confidential research is detailed below along with confidential research (concept only), plus on our website www.ccc4labprep.com and in publications.

**Case Study 1.** Client had a complex extract, when target mix prepared by reverse phase HPLC, had desired bioactivity. When process transferred to industrial non-HPLC manufacture, target mix exhibited extreme cytotoxicity. L-LC was used in direct cross correlation to gradient prep HPLC (a single multi gram injection of same matrix onto a custom packed 50 x 250 mm, 15um C18 column, poisoned column, yet multiple L-LC preparations could be run) showed that laboratory studies with end capped, C18 HPLC prep columns, removed the then unknown cytotoxic compounds, which L-LC methods found.

**Case Study 2.** Client had complex mixture, which had taken Sequential Flash, MPLC & HPLC 3 different International Labs each 6 months to prepare target. Two contract laboratories refused to do repeat preparations. By Sequential L-LC and HPLC, target was prepared in 4 weeks for first preparation and was completed in less than one week in repeat separation. There was a massive; over ten-fold reduction in solvent usage, as well as the obvious huge time saving.

**Case Study 3.** During LINK Grant project working with GSK the results shown in Fig 5 were obtained. Two HPLC gradient traces are shown.

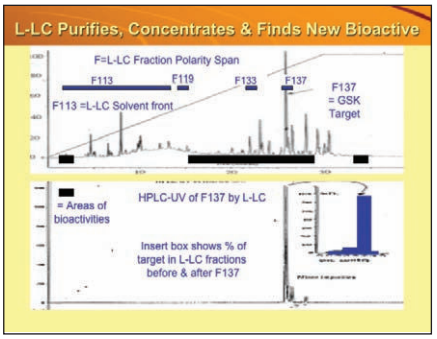


*Figure 5*

Top is original gradient HPLC. Below is the HPLC of a single 4 ml fraction from a 200+ ml gradient Quattro L-LC run. The insert shows the amount of target in fractions before and after the main fraction. Over 90% of target was in one single 4 ml fraction. The bars labelled F above top chromatograph show polarity range of L-LC fractions. Apart from solvent front, all show the very small polarity range of OT HPL-LC fractions. In addition an unknown bioactive was found.

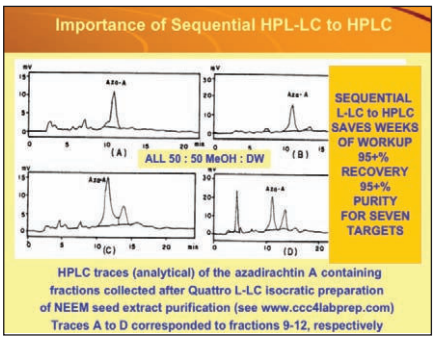


*Figure 6*

**Case Study 4.** Sequential L-LC plus HPLC. The NEEM tree is the Holy Tree of India; it produces such a variety of bioactive targets, that villages in India define it as their Pharmacy. Fig 6 shows collaborative research with the University of Vicosa, Brasil. Previous to installing the Quattro L-LC, Prof Gulab Jham took months to prepare just the required amounts of AzA, by Sequential L-LC & HPLC, AzA and six other key related compounds, never prepared in that laboratory before, were prepared in weeks with better than 95% recovery and better than 95% purity (4). An injection/recovery mass balance was conducted, by weighing the dried residue in each L-LC fraction. Within the scope of the method, a full mass balance was obtained. That would be an extreme rarity in S-LC for a raw natural product injection.

**Case Study 5.** Deguelin obtained from an Amazonian plant is very valuable (~$20,000 g), the contaminant rotenone is of little value, but contaminates extracts. Researchers with decades of historic Japanese CPC 1000 ml instrument experience for this separation achieved loading of 150 mg per 1000ml CPC capacity. On upgrade to a modern manufactured 1000 ml CPC they doubled loadings to 300 mg per 1000ml CPC capacity. Their method failed on the Quattro CCC. A method developed in less than a day increased loading to 1625 mg per 1000ml Quattro L-LC capacity; over ten times that of historic CPC. The client subsequently increased

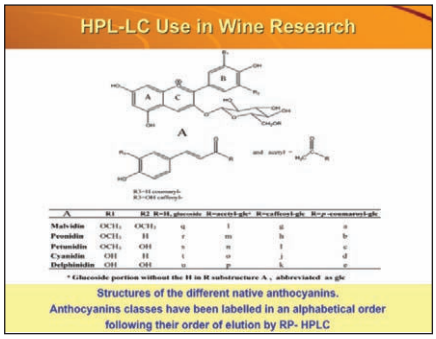


*Figure 7*

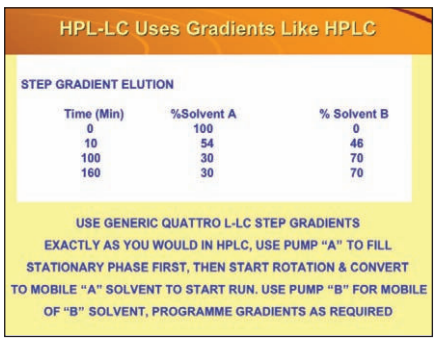


*Figure 8*

the initial loading to closer to a typical 5 to 40g loading per 1000 ml.

Case Study Wine Research Fig 7, 8, 9 & 10 by wine researchers (5) shows the worth of gradients in L-LC, and of L-LC in unravelling difficult identification issues. This research led to the targets sensory properties being determined and tentative structural elucidation of new unknown oligomeric anthocyanins. The chromatogram on HPLC revealed an absence of standard baseline hump seen once the monomers etc were

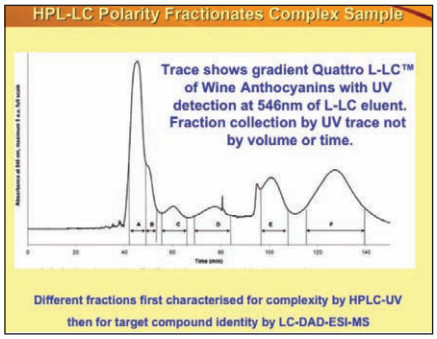


*Figure 9*

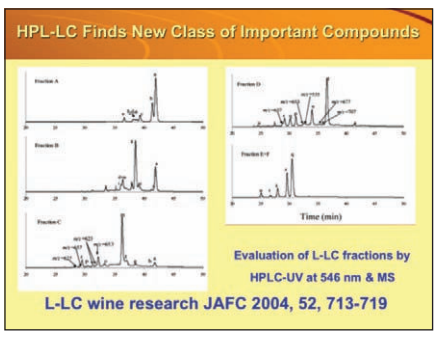


*Figure 10*

separated by L-LC. This highlights the value of doing a L-L chromatography sample polarity screening. L-LC helped these wine researchers to identify a new class of compounds (oligomeric anthocyanin species), and to study their influence to the colour and sensory properties in wine.

Case Study 7 HTPrep/Combinatorial. In 2007 we custom designed the World's first Quattro HTPrep™ for a Pharmaceutical Company in the USA. The research was presented at CCC2008 and published in the proceedings (6).

## Conclusions

Liquid-Liquid Chromatography has matured into a valid science, which after almost 60 years deserves to be integrated into mainstream laboratory and process chromatography. L-LC been shown to compliment HPLC, with narrow range polarity cutting and by helping to find peaks co-eluting in HPLC. One beauty of L-LC is that the separation is based largely on defining on the polarities of targets, therefore classes of compounds can be separated which can then be optimised without sample loss. These narrow polarity range classes can finally be passed through a HPLC, assuming sample losses can be tolerated. If not, Sequential L-LC to L-LC with different solvents may be utilised. L-LC is a low-pressure technique (typically 100 to 500 psi) thus it can use lower price ancillary equipment than HPLC. L-LC usage has the potential for considerable solvent cost and timesavings.

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