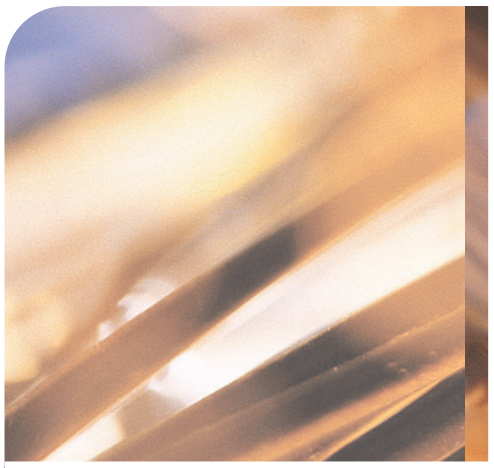

Chromatography Focus

"TO GO SUB 2µm OR NOT TO GO, THAT IS THE QUESTION." - AT LEAST FOR THE ANALYSTS IN HIGH THROUGHPUT LABS

Bernie Monaghan

A meeting held in the UK two years ago at the site of a major Pharmaceutical Company saw one of its senior managers challenge the suppliers of separation science products to bring to the table "faster, cheaper and better separations". This article looks at the options that are available to an analyst in a High Throughput Lab and discusses the pros-and cons surrounding those options. As for the characteristics that constitute a "better" separation, the author decided that this is a far too subjective criteria for this article, and anyway varies on a case-by-case basis. Manufacturers now are at a position where they have declared their hands on one of three technology options and the dividing line appears to be drawn at the use of, or not as the case may be, of columns containing sub 2µm diameter particles or not. A new generation of Instrumentation, moving on from what has been accepted as 'conventional' systems, has been developed on the back of these advances in column and particle technology. For the sake of allowing meaningful comparisons we are defining 'conventional' equipment as a system with a pump capable of delivering flow rates up to 10ml/min with maximum pressure of 6,000 psi (400 bars), columns of 150x4.6mm id containing 5µm particles and a variable wavelength UV detector with detection cell volume of 8µl.

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THEORETICAL CONSIDERATIONS

For the sake of simplicity we will consider the main equations which can illustrate the inter dependency of various parameters involved when we attempt to speed up analyses from those obtained with 'conventional' equipment.

Firstly taking the equation which describes the speed of analysis then we have

$$to = L/u$$

where to is the column dead volume expressed as time taken for an unretained peak to pass through the column with the solvent front, L is the columns length and u is the linear mobile phase (flow rate). To speed up the analysis we can use a shorter column length or a higher flow rate, or a combination of both. However changing either will affect the number of theoretical plates (Column efficiency, N) which can be described by

$$N=L/H$$

Where H, height equivalent to a theoretical plate, is roughly proportional to the particle size (smaller being better) and the influence it has with the u term is shown in the Knox equation

$$H=Au^{1/3} + B/u + Cu$$

Where A, B and C are related to various parameters of a particular column. It is easier to view this equation simplified and its practical implications in *Figure 1*.

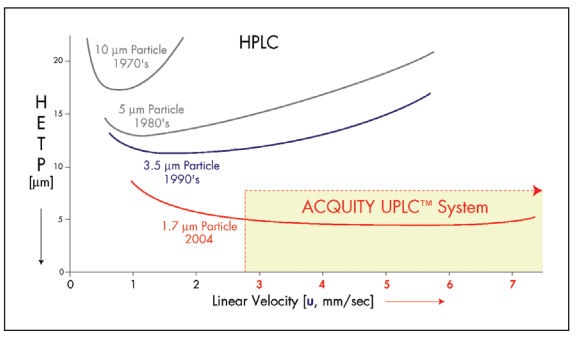


Figure 1. Van Deemter plot of HETP vs. linear velocity

Here we see the effect of increasing flow rates (linear velocity) on column efficiency (HETP, µm, lowest value is highest efficiency) for different particle diameters and an indication of when they were considered 'conventional'.

Each particle diameter has an optimum efficiency/flow rate point that reduces as the flow rate is increased. Smaller particles loose less efficiency as the flow rate increases away from that optimum point compared with larger particles.

Probably easier to relate to is the situation which shows the pressure drop across a column and how it depends on various elements of the system. We see that the pressure is directly proportional to the flow, viscosity and length and inversely proportional to the particle diameter, column i.d. and the media permeability.

$$\Delta P \propto \frac{\text{(mobile phase viscosity) x (Flow rate) x (Length of the column)}}{\text{(Permeability) x (mean particle diameter) x (column id)}}$$

From this relationship and by changing one parameter at a time it can be seen what effect for example doubling the flow rate would be in an attempt to reduce the analysis time, all other factors remaining constant. It would half the analysis time BUT also double the pressure across the column. If one wanted to reduce an analysis time even more, e.g. from 6 to one minute then increasing the flow to achieve this would certainly take the pump beyond its cut out point in a 'conventional' system so some other parameter would have to be changed to accommodate this situation and achieve the desired analysis time.

OVERVIEW OF OPTIONS

By working through the variables and effects that changing parameters have with each other there become several options (or combinations) that are available to the chromatographer where a reduction in analysis time may be obtained by an order of magnitude without compromising overly the chromatographic efficiency of the separation and subsequent loss of resolution.

In a nutshell the parameters that could be most commonly varied to obtain increased throughput (i.e. reduced analysis times), and the implications of changing these parameters are shown below:

- Reduce Column Length – may reduce the chromatographic efficiency required for the separation since this means having fewer plates 'on column'.
- Increase Flow rates – may take pressure drop beyond system limits and also may cause loss of efficiency as the movement away from the optimum flow rate is observed.
- Reduced Particle diameter – impacts upon the pressure drop massively since pressure is inversely proportional to the $(d_p)^2$ and too small a particle may limit the pumping system that can be used.
- Increasing the permeability of the media – interesting alternative as sufficient increase can allow the use of very high flow rates without undue pressure drop increase.
- Reducing the mobile phase viscosity – most easily achieved by increasing temperature, but stationary phases have finite upper temperature limits of stability.

SUB 2µm PARTICLES

On the face of things, reducing the particle diameter to give improved efficiency at high flow rates would appear to stall at the pressure drop question with 'conventional' equipment. In 2004 Waters introduced their ACQUITY UPLC® system which allowed for the first time columns containing 1.7µm porous particles to be run at flow rates which allow theoretical maximum efficiency to be closely approached in a system which could tolerate pressures in excess of 1000 bar. Dramatic reductions in times of analysis, improved resolution, sensitivity and peak capacity, leading to increased throughput, have been reported by the use of these columns containing 1.7µm particles (see *Figure 2*) in conjunction with shorter column length. LC hardware specifically designed to cope with the extreme pressures and reduced system volumes involved must also be used.

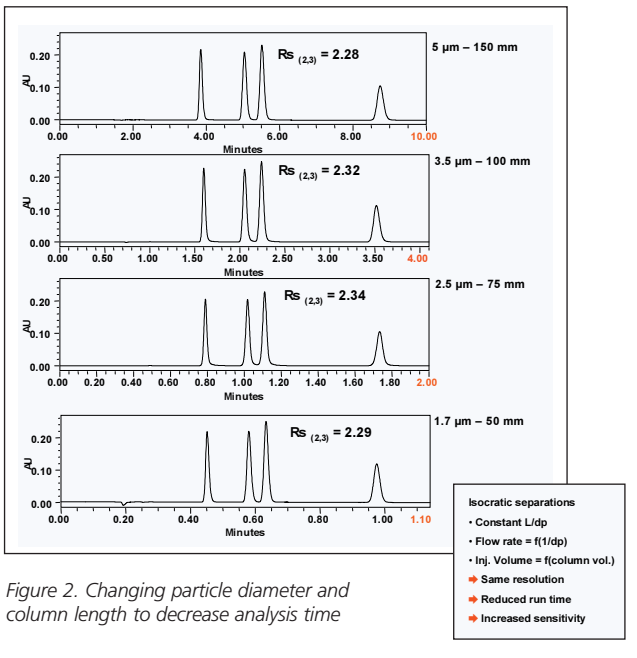


Figure 2. Changing particle diameter and column length to decrease analysis time

The competitors waited to judge if the market would accept the premise that improvements on analysis reduction times could only be realised if the columns were used on the ACQUITY UPLC systems or if the concept was a bridge too far for most scientists. Not all laboratories could afford to upgrade to a complete new system yet there was more to realising this improvement than just sourcing a pump from another supplier and putting it on the front end. Detector acquisition rates were important, as was the total dispersion volume of the system in terms of absolute volume and materials of construction.

Nevertheless, 3 years on and as can be seen from *Table 1*, other companies are now supplying systems which are capable of accepting pressures in the 1000 bars region. Interestingly enough also there are now appearing, from an increasing number of suppliers, columns containing sub 2µm particles as the concept becomes more accepted. Solvent savings are obviously an added bonus but there is extra onus on ensuring that the samples and all regents are filtered very stringently since 'plugging' can be a problem at column inlets at this scale.

Table 1. Suppliers of columns containing sub 2µm particles.

Supplier	Product Name
Waters	ACQUITY® UPLC® 1.7µm
Thermo Scientific	Hypersil GOLD® 1.9µm
Machery Nagel	Nucleodur® – 1.8µm
Agilent	Zorbax®RRHT 1.8µm
Dr Maisch GmbH	Reprosil® Gold-Turbo 1.8µm
Grace Davison	Platinum® LC 1.5µm

PARTICLES >2µm BUT <3µm

Laboratories who still need to cope with an increasing workload and whose recourse is to reduce the analysis times, but have no budgets to upgrade to the UPLC or equivalent systems, still are catered for from suppliers. Their respite comes in the form of fast LC columns which are sometimes nothing more than the 3µm offerings that have been available for many years relabelled for use as high throughput columns or something similar, often in 20, 30 or 50mm long format. It is true that these columns may offer a performance advantage in that they should not plug so easily but an increasing number of companies are offering columns with particles less that 3µm but greater than 2µm (*Table 2*) which offer performance more towards the sub 2µm columns without the need to upgrade to a 1000 bars system. This is so because the pressure is proportional to the particle diameter squared so if all else is equal then a column containing 2.5µm particles will produce less than half of the pressure associated with a column containing 1.7µm particles. This could then allow 'conventional' 400 bar pumps to be used especially with short column lengths and reasonably fast flow rates.

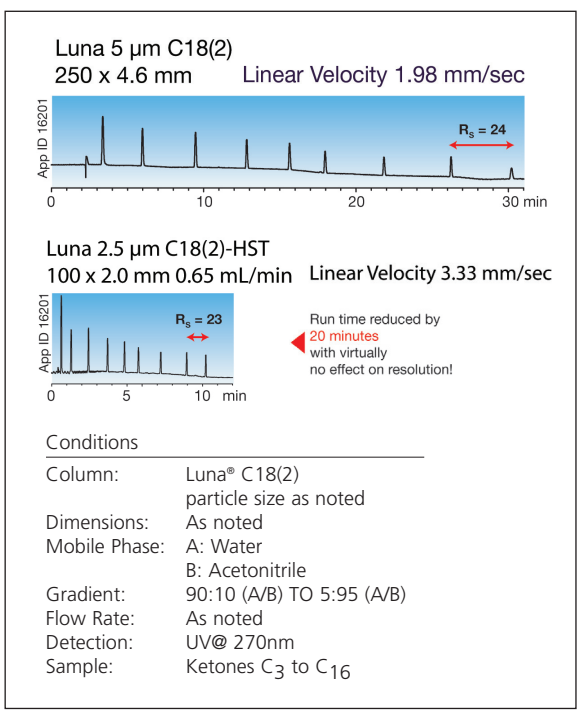


Figure 3. Effect of reducing column length, and particle diameter whilst increasing flow, on resolution

Increasing linear velocity would be expected to reduce system resolution but this can be minimised substantially.

Figure 3 shows that a 250x4.6mm column containing 5µm particles with a total analysis time of 30 mins. and resolution factor for the last two peaks of 24 can be reduced to less than 10 mins. whilst maintaining resolution and staying within 'conventional' instrumentation pumping values. By using a combination of shorter column, higher linear flow rates and reducing the particle diameter to 2.5µm the last two peaks reduce their resolution hardly at all and the analysis time could be further reduced by shorter column length and increased flow rates.

Resolution (Rs) is proportional to the square root of the number of plates described as;

$$Rs \propto \sqrt{N}$$

Table 2. Suppliers of columns containing particles greater than 2µm (but less than 3µm) aimed at high throughput analyses needs.

Supplier	Product Name
Phenomenex	Luna® 2.5µm C18 (2)-HST
Mac-Mod Scientific Inc.	HALO C18 Fused Core ™ 2.7µm
Jasco	X-PressPack® C18 S 2µm
Shimadzu	Shim-Pak® XR-ODS 2.2µm
Sigma-Aldrich/Supelco	Ascentis Express Fused Core ™ –2.7µm,

MONOLITHS

As mentioned above another option to allow fast analysis times is to use monolith technology where the permeability of the column is drastically increased thus allowing columns of 50-150mm length containing monoliths of either modified silicas or polymer to be used with high efficiencies. Although not intended as alternatives for high throughput labs, monoliths due to their higher permeability's (>80%) may be used up to 9 ml/min on a 100x4.6mm column to a maximum of 200 bars, without causing pressure problems on conventional systems. The pressures generated can be easily accommodated with 400 bars pumping systems.

This is because monoliths are comprised of a single rod of material with relatively large through pores (ca. 2µm) – *Figure 4* top- along with smaller mesopores around 13nm to give desired surface area and hence retention and efficiency to be of use (*Figure 4*). The 2µm macropores act as throughpores and enable the analytes to be transported, under low pressures, to the activated surface for separation by the chromatographic process. The surface area is ~ 300m^2g^{-1} made possible by the mesopores.

Reductions of up to 8 fold in pressures with monolith columns have been reported compared to columns containing 3.5µm particles of comparable geometry.

An effect of this is shown in *Figure 5*, where the linear velocity is increased markedly without causing pressure drop issues within the system. Rapid re-equilibration times are possible with monoliths and this allows fast cycle times to be utilised for complex samples.

Table 3. Suppliers of Columns based on monolith technology.

Supplier	Product Name
BIA Separations d.o.o	CIM®
Dionex	Pro- and PepSwift™
Phenomenex	Onyx™
VWR International GmbH	Chromolith®

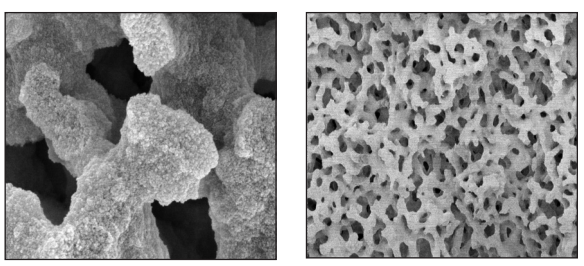
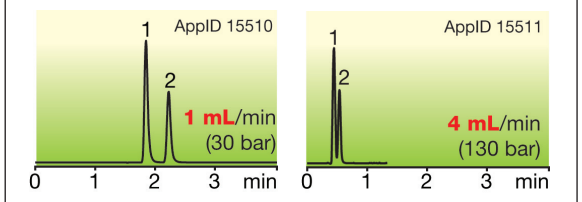


Figure 4. Electron Micrographs of macro- (left) and mesopore (right) structure of typical monolith



Conditions

Column:	Onyx™ Monolithic C18
Dimensions:	100 X 4.6 mm
Order No.:	CHO-7643
Mobile Phase:	0.1% TFA in water / Acentontrile (95/5,v/v)
Flow Rate:	1 mL/min to 4 mL/min
Gradient:	5:95 (A/B) linear to 95.5 over 8 min, Hold for 5 min
Detection:	UV@ 220 nm
Temperature:	22°c
Sample:	1. Maleic Acid 2. Fumaric Acid

Figure 5. Effect of increasing flow rate on analysis time in monolith column

CONCLUSIONS

It is obvious that all 3 options discussed above offer advantages in reducing analysis times over 'conventional' chromatography data obtained from equipment as defined in the introduction section of the article. Just which option is suitable for each laboratory will depend to a certain degree on the equipment that the scientist has available and any budgetary constraints applicable. Clearly it is not practicable to run columns containing sub 2µm particles on a system with a maximum pressure of 400 bars unless extremely short columns are used and this still will impact upon the flow that can be used. In these cases where equipment is the limiting step the monoliths and possibly columns with >2µm particles would be easier to cope with, yet still allow vastly improved throughput, compared to 'conventional' equipment. The exact application and detection system used also need to be considered since LC-MS systems are more suitable for short columns whereas monoliths offer excellent chromatographic resolution at high flow rates without compromising efficiency unduly.

A further variable that has not been discussed is the use of temperature to reduce pressure across the column thus allowing even faster flow rates to be utilised, again with a trade off of some loss of efficiency. Very fast 'ballistic' gradients when run on these short columns containing sub 2µm particles do generate a certain amount of heat due to the pressures involved and questions have been raised as to the reproducibility of gradient runs due to re-equilibration times possibly affecting total cycle times.

The final point worthy of mention is that certainly when looking to decrease analysis times by an order of magnitude, we appear to be going in a direction which requires a greater degree of theoretical and practical competences from the analysts than has been necessary when working with 'conventional' systems. Columns containing small particles are more prone to failure by plugging with particulate matter far easier than columns with 5µm particles thus the filtration of samples and mobile phases needs scaling up a notch. Similarly an understanding involving, and the implications resulting when one is changed, the relationship between flow, pressure, efficiency, detector acquisition capabilities and total system volumes becomes more important if the theoretical possibilities are to be realised in a practical manner. This appears to be in contrast to the black box, 'sample in this end, data out this end and don't worry about the bit in the middle' approach that some manufacturers are advocating. Indeed, certainly in the UK, the comments frequently heard from Industry is that the undergraduates delivered by the Universities are somewhere short of the level they need to be when it comes to Separation Sciences competences especially on a practical level.

ACKNOWLEDGEMENTS

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