

HPLC in a World Without Acetonitrile

Raw acetonitrile is a by-product of the manufacture of acrylonitrile. It is co-polymerised with butadiene and styrene to make ABS, a plastic used in cars etc, and the market for it has collapsed over the last six months. Hence so has the production of acetonitrile. There are no manufacturing plants to make acetonitrile (unlike THF or methanol) and there is little prospect of one being built. Stocks are now depleted, and hence those distilling it for use in HPLC can no longer get enough supplies. Acetonitrile is now being offered at extortionate prices. In the short term, this means that we will all have less, if any, acetonitrile available to us, and when it does come back on stream, it could be a lot more expensive.

Many HPLC methods use acetonitrile as part of the mobile phase. It is an excellent eluent. It has low viscosity, good selectivity, 100% miscibility with water, reasonable buffer solubility, and is almost transparent to UV light. HPLC methods are usually validated (checked thoroughly to ensure that correct results are obtained even if small changes to the operating conditions apply) and are sometimes registered. Validation can take up to three months, registration can take much longer, and both are expensive. So once a method is set in concrete, it is almost impossible to change. However... if the acetonitrile specified in the method ceases to be available, it is necessary at some point to bite the bullet, and make the decision to change the method.

There are two approaches. The first is to do the absolute minimum necessary to get the separation to work with another solvent, and re-register as fast as possible. This has obvious attractions in the short term, in terms of time, cost, and ease of re-registration. The alternative is to use this as a once in a lifetime opportunity to redevelop the method using modern columns etc, and to check again that the most appropriate temperature, pH, buffer concentration is being used. It takes longer, but in time to come, with the benefit of hindsight, this can seem a much wiser approach.

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THE CURRENT SITUATION

Acetonitrile is still available, but in very limited quantities, at inflated prices, and with no guarantee of continued supply. The situation is not expected to change significantly in 2009. If you have stocks of acetonitrile, keep some back. Method redevelopment will most likely be necessary, and it is much easier to show that a new method gives equivalent results to an old method if you can actually run both and compare the results. Some suppliers are reporting that stocks are trickling through. But the situation is at best precarious.

SHORT TERM SOLUTIONS IF RESIDUAL STOCKS OF ACETONITRILE ARE AVAILABLE.

If stocks of acetonitrile are still available, there are a number of steps that can be taken to make them go further:

Use a solvent recycler.

This is a device which monitors the analogue signal from the detector, and when the signal rises above (or below) a prescribed threshold value, the valve switches the eluent flow to waste. Once the signal returns within the threshold, after a time delay, the eluent flow is directed back to the eluent bottle.

Clearly this can only be used with isocratic HPLC systems, where the eluent is premixed into a single bottle. The solvent saving which can be made is proportional to the %B in the eluent (the amount of organic solvent used per litre of eluent), to the amount of flat baseline between the peaks, and to the run time. However the savings can be significant, and at today's prices for solvent, the payback period can be quite short.

Scale down to a narrower bore column.

Provided that the eluent linear velocity remains unchanged, it is possible to scale down to a smaller column fairly easily. At 1ml/min, using a 4.6mm diameter column, the eluent linear velocity is 0.14cm/s, and by using the ratio of the cross-sectional areas of the columns we can calculate the solvent savings achievable:

Column internal diameter	Flow rate (ml/min)	Solvent saving
4.6mm	1.0	-
4.0mm	0.69	31%
3.0mm	0.43	57%
2.0mm	0.19	81%
1.0mm	0.05	95%
0.5mm	0.012	98.8%
0.3mm	0.004	99.6%

Even the smallest diameter columns are available now, so this is a potentially viable solution. The problem lies in the instrumentation. We identify samples by their retention volume, which we measure as the retention time. Retention time is flow rate dependent, and so the lower the flow rate, the greater the accuracy and precision required of the pumping system. (Small flow variations represent larger and larger percentage errors, and hence retention time variation increases). Narrow bore columns offer an improved mass sensitivity, and tend to give sharper peaks, so there are other advantages if you are prepared to go here. However the injection system must be capable of injecting very small quantities, and at this level, an internal standard is a must. And the flow cell in the detector will need to have an appropriately low volume. If a current method is to be adapted to eke out existing supplies, my recommendation is not to go below 3mm unless new equipment is to be purchased.

Use a shorter column. Eg. 15cm instead of 25cm.

This should have been considered when the method was originally written, but if the method is isocratic, and the peaks are better than baseline resolved, this may work.

REDEVELOPING THE METHOD TO USE ANOTHER SOLVENT

The long term solution almost invariably involves redeveloping the method!

In order for a solvent to be usable as a replacement, there are a few boxes it has to tick:

- Is it transparent to UV light, preferably down to 200nm?
- Does it have a low viscosity?
- Does it mix totally with water, and dissolve the buffers we normally use with reversed phase HPLC?
- Is it relatively inexpensive (no more than about £40 for a 2.5 litre bottle)?

Only two solvents appear to be suitable, and those are methanol and tetrahydrofuran. Don't be tempted to try propionitrile! It only mixes with water up to 9%, and costs about £160 a bottle.

Before going further, it is useful to note some properties of these solvents and compare with acetonitrile.

Parameter	Acetonitrile	Methanol	THF	Water
UV Cut-off (nm)	220nm Std, 190nm Far UV	205nm	210-230nm	<190nm
Viscosity	0.37	0.60	0.55	1.00
Flash point (°C)	6	11	-18	n/a
Evap. Rate	5.8	5.2	8.0	n/a
Detection by Smell	200ppm	2000ppm	20ppm	n/a
LD50 Rat	3800	5600	3000	n/a
Refr. Index	1.344	1.328	1.407	1.333

Both THF and methanol fit the UV cut-off requirement. Note that THF can have UV-absorbing impurities, so the quality varies considerably from one supplier to another. It is hard to distil because of the presence of peroxides, which can cause explosion at around 1% levels. Since that represents 25ml in a 2.5litre Winchester, we are most unlikely to find such levels even in a very old bottle. However, under distillation conditions it's different. Hence some HPLC solvent suppliers are much better at producing THF than others. If peroxides are present in the HPLC grade solvent we use, it can cause sample degradation on the column. So sourcing a 'good' distilled HPLC grade is very desirable.

Methanol and THF have a higher viscosity than acetonitrile, so back-pressures will be higher. Note that as methanol mixes with water it forms an adduct which has a viscosity even higher than that of water. So the maximum back-pressure will be observed for mixtures of water and methanol, rather than with the pure solvents.

The flash point is important only if a leak arises. Note that THF is very volatile, and has a very low flash point. So a leak in a column heater will evaporate fast, and could cause a fire (column heater manufacturers take note. Spark free please.) THF has a smell half way between petrol and ether, and we have a much higher sensitivity to it than for methanol or acetonitrile. But that sensitivity decreases rapidly with exposure, so a visitor entering a lab will notice a leak before anyone else.

Acetonitrile is the most toxic. The rat LD50 figures are a little misleading. Cat LD50 for acetonitrile is just 60! The lower the number, the nastier it is. So moving away from acetonitrile does have advantages. Over exposure to THF vapour typically gives a nasty headache as the early warning signal.

Refractive index is especially important if using an RI detector. The higher the eluent RI, the greater the likelihood of negative peaks, and possibly the lower the actual sensitivity. Note that an RI detector measures a change in RI, so the lower the RI of the eluent, potentially the greater the sensitivity.

Before starting method development:

- Keep back enough acetonitrile for the method development. To compare methods you may need to run the old method a few times.
- Consider changing the column. Many methods use old, out-dated columns. Modern columns are available which can offer sharper peaks, lower back pressure, wider pH stability, and better selectivity. Ignoring this opportunity will haunt you and you will have no friends.
- Bear in mind that more selectivity is usually derived from the mobile phase than the stationary phase. So when optimising the mobile phase, optimise temperature, pH, buffer type, buffer concentration as well as %B.
- When choosing a column, remember that reversed phase is almost always more selective than normal phase, and always more selective than ion-exchange. C18 is more retentive than C8, which is usually more retentive than Phenyl. And develop methods with a 15cm column. You save 40% of solvent and 40% of time compared with a 25cm column, and you can always increase the column length at the end if you need to.

A systematic approach

- Select a suitable column, usually an end-capped C18 column with type B (ultrapure) silica, and a wide pH range (eg Reprosil Pur Basic C18 – pH1-11, from Dr Maisch, Germany)
- Find a suitable eluent composition for isocratic elution if possible. Isocratic is much better than gradient if you can do it.
- Optimise Methanol/Acetonitrile/THF using the Snyder triangle (see later). If you take out MeCN, this gets a lot quicker, because four of the seven runs are unnecessary.
- Try a selection of columns to see which gives best selectivity. Remember to consider polymer-based columns if working at high pH (eg Shodex ODP2 HP).
- If necessary, repeat steps 2-3.
- Optimise for Temperature, pH, buffer type, buffer concentration.
- Consider if a shorter column would give enough resolution. And consider increasing the flow to speed up analysis.
- Set up the integration and calibration.
- Validate the method.

Preparing Solvent Mixtures of Equivalent Eluent Strengths

When changing solvents, there are three ways to work out the composition of an eluent with equivalent eluent strength. The first is to use a table:

Acetonitrile %	Methanol %	Tetrahydrofuran %
10	13	8
20	27	15
30	39	22
40	50	30
50	60	37
60	70	45
70	79	52
80	87	59
90	93	67
100	100	72

These values vary a little depending upon who prepared the table, but they serve as a guide.

Using the table it can be seen that for a 50:50 acetonitrile: water mixture, the equivalent using methanol: water is 60:40, and for THF: water, the equivalent is 37:63.

The second method is to calculate using the reversed phase polarity values supplied by Snyder et al. P' is the solvent polarity value, and the values for our solvents are:

P' Acetonitrile	=	3.1
P' Methanol	=	3.0
P' Tetrahydrofuran	=	4.4
P' Water	=	0

The equation to use is: $\Phi_1 P'_1 = \Phi_2 P'_2$

Where Φ is the volume fraction of the solvent.

So if you have a mixture of 50:50 acetonitrile: water, to calculate how much methanol to use:

$$\Phi \text{ MeOH} = \frac{0.5 \times 3.1}{3.0} = 0.52$$

So 52% methanol: 48% water should be used.

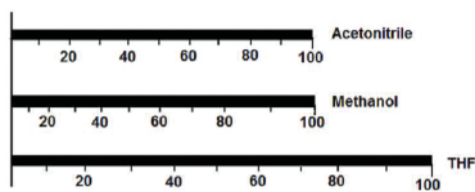
Similarly for THF:

$$\Phi \text{ THF} = \frac{0.5 \times 3.1}{4.4} = 0.35$$

So 35% THF: 65% water should be used.

Note that a different result has been obtained than using the table above. This is because we are using a fixed P' value for methanol, but methanol: water mixtures do not follow a linear increase in eluent strength with composition. See the nomogram below.

Finally, a nomogram (or nomograph) can be used:



Using this method to find an equivalent to 50:50 acetonitrile: water:

For methanol we get 60:40 methanol: water

For THF we get about 37:63 THF: water

This result is the same as with the table, but slightly different from the calculation above.

SELECTIVITY DIFFERENCES BETWEEN ACETONITRILE, METHANOL AND THF

Resolution is defined by the equation below.

$$R_s = \frac{N^{1/2} (\alpha - 1) (k')}{4 \alpha (1+k')}$$

N = Efficiency

α = Selectivity

k' = Relative retention time

Assuming that we do not change the eluent strength, using the information in the previous section, and there is no change to efficiency, the only parameter likely to cause problems is selectivity.

Selectivity is a function of the interaction between the eluent and the column with the sample components. Hence by changing the solvent used as eluent, we may change the selectivity. Essentially this means that although the components of the sample will elute in approximately the same time as before, the position of the peaks relative to each other can change. This can help, and give us a better separation, and it can cause peaks to merge or even co-elute.

There are three main solvent properties which affect selectivity:

1. Proton donor (acidity)
2. Proton acceptor (basicity)
3. Dipole

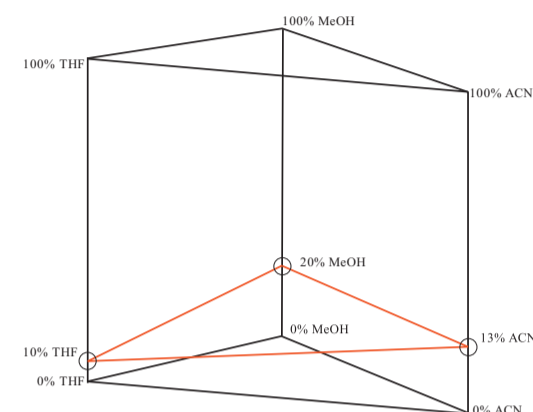
There is no suggestion that the solvents we use are acids or bases. But a sample component which has a slightly acidic hydrogen, will be attracted more to a solvent which has proton acceptor properties (eg THF) than to the others. A sample component which has an atom with a lone pair of electrons will be more attracted to a solvent which has proton donor properties (eg methanol). And a sample component which has a dipole moment will align itself more readily with a solvent that also has a dipole (eg acetonitrile). These attractions are in addition to the natural interactions due to the sample polarity, and hence if a new attraction is added, the component will spend more time in the eluent, and be eluted faster, relative to the other sample components. Similarly, if an 'extra' attraction is lost due to the solvent change, that component will spend less time in the eluent than before, and elute later, relative to the other sample components.

So when redeveloping a method, we may be able to predict which solvent to use. It is possible to put numbers on these parameters. They are given as a percentage of the total polarity of the solvent, and so always sum to 1.0. The following table may help:

Solvent	Dipole	Proton Donor	Proton Acceptor
Methanol	0.28	0.43	0.29
Acetonitrile	0.60	0.15	0.25
THF	0.51	0.00	0.49
Water	0.39	0.43	0.18
Acetic Acid	0.31	0.54	0.15

EVALUATING THESE SELECTIVITY DIFFERENCES IN JUST SEVEN EXPERIMENTS

Consider the triangular diagram below first developed by Snyder et al. Each of the three axes represents 0-100% of that solvent in water, i.e. binary mixtures. In between the axes but along the defining edges of the triangle are ternary mixtures made up of the two solvents plus water, and anywhere inside the triangular boundaries represents quaternary mixtures of all three solvents plus water.



As an example, assume 13:87 acetonitrile: water was found to be satisfactory, and we then calculated using the nomogram that 20% methanol in water and 10% THF in water would give similar elution times. We can then construct a triangular plane as shown above, where all mobile phase compositions in that triangle have the same eluent strength, and hence will give acceptable elution times but different selectivity.

Now try seven experiments:

First the three corners of the triangular plane. i.e. the binary mixtures:

Run 1: 13:87 acetonitrile: water

Run 2: 20:80 methanol: water

Run 3: 10:90 THF: water

Then the three mid-points on the edges. i.e. ternary mixtures

Run 4: 10:7:83 methanol: acetonitrile: water

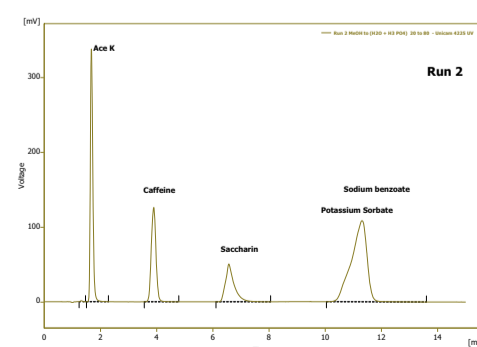
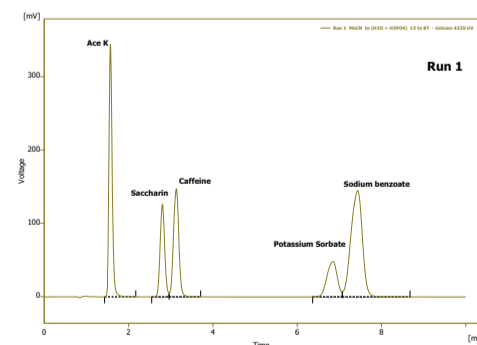
Run 5: 10:5:85 methanol: THF: water

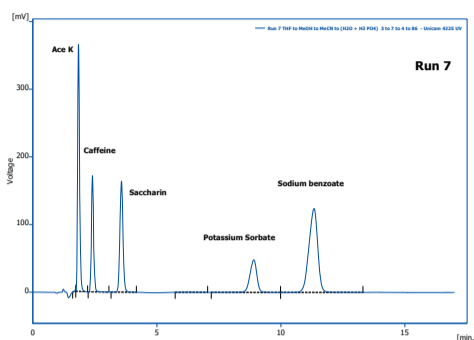
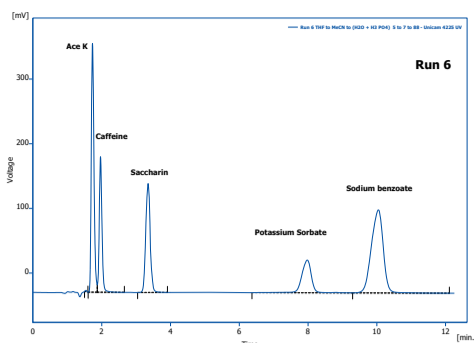
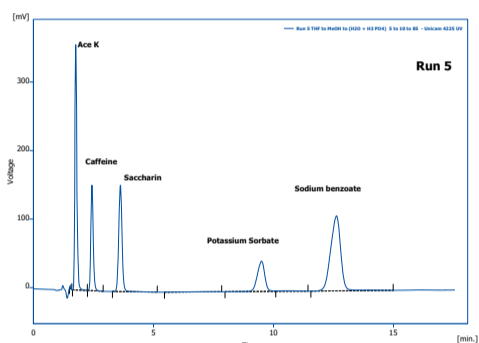
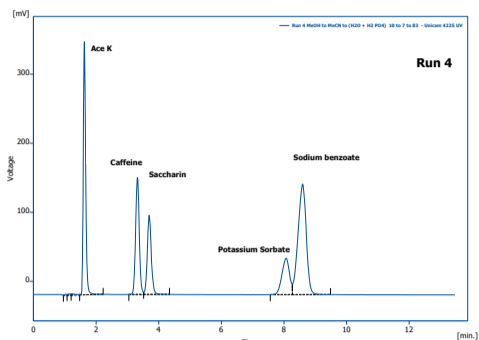
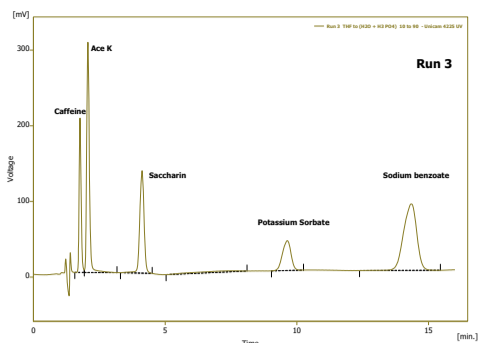
Run 6: 7:5:88 acetonitrile: THF: water

Finally the centre. i.e. a quaternary mixture

Run 7: 7:4:3:86 methanol: acetonitrile: THF: water

The chromatograms below show the results using the above mobile phases for acesulfame K, caffeine, saccharin, potassium sorbate and sodium benzoate.





It is clear that the acetonitrile (Run1) gives an excellent and quick separation. Methanol struggles to resolve the last two peaks (sorbate and benzoate) and gives longer run times.

THF gives excellent selectivity for sorbate and benzoate (almost too good), but its selectivity for caffeine causes potential resolution problems. So the THF/Methanol combination appears to be the best choice (Run 5).

A FEW FINAL NOTES:

1. Simple is best. A binary mixture is much easier to make up accurately so the errors are less. Unless the optimum is required to get resolution, a compromise will be better in the interest of robustness and reproducibility.
2. THF is the strongest of our three eluents. So at low concentrations it is more critical to get the eluent composition exactly right than it is with methanol or acetonitrile. A small change makes a bigger difference in retention times than it would with the weaker solvents. So it may be preferable to choose methanol over THF when the eluent is weak.
3. If a gradient is being used, remember that only 72% THF is equivalent to 100% acetonitrile. If a higher concentration of THF is used, apart from being unnecessary, it may cause components to wash off a guard column which would have been unmoved by acetonitrile. However this can be used to our advantage. We had a gradient separation which required a 30 minute hold at 100% acetonitrile to clear the non-polar species from the column. Using THF, as soon as we got to 85%, the last component eluted, eliminating the need for the 30 minute hold, and in fact eliminating the need for a guard column!
4. Remember that THF is available as stabilised or unstabilised grades. The stabilised product contains butylated hydroxytoluene (BHT) which does not mix with water, and which totally absorbs UV light at most wavelengths used in HPLC. So only use the stabilised product if running GPC with an RI detector. THF is a lot more stable than most people claim, so although it is given a very short shelf life, it will actually last much longer. To extend its life, purge the bottle with nitrogen before storage.
5. THF attacks PEEK. This is especially true of PEEK tubing, where there is a high contact surface. The tubing will weaken and split within about 2 days of use, possibly in the column heater. So if working with THF, use steel connecting tubing. Finger-tight PEEK fittings are usually ok though, because they have minimal contact with the THF.

For more in-depth information or to attend a course (next is due on April 16th) on this topic please contact the author at stuart@laserchrom.co.uk or visit www.laserchrom.com

Customised method redevelopment and validation services are also available.