SEARCHING FOR IMPURITIES BY LIQUID CHOMATOGRAPHY

The need for column switching

Introduction

According to current regulations we must be able to detect and quantitate impurities in drug substances and some other fine chemicals, at (apparent) concentrations down to a few hundred parts per million by weight. Experience shows that many of the impurities we do detect would not have been easily predictable. It's a standing joke that when you present the structure of a new one, the development chemist immediately cries: "but that's impossible!" There is always some concern that a new impurity will be detected by chance at an uncomfortably late stage of development. When only one separation method (nearly always liquid chromatography) has been adopted for routine use, an unsuspected impurity can be missed for the following reasons:

- It may be unretained or very strongly retained.
- It may not respond to the detection method.
    - Conversely, time may be wasted identifying an impurity with a high relative response. That's one reason why CMC pharmaceutical analysts hardly ever use fluorescence detection and should be wary of atmospheric pressure ionisation mass spectrometry.
- It may be unresolved from the main peak.

We discuss the last point on another page; with LC it can be difficult to tell whether or not a minor disturbance in the shape of the main peak may be due to a low-level impurity. We also note that when the chromatographic process isn't linear, an impurity can be spread out (tagged along) underneath the main peak.

While the range of analytical methods has changed over the years, there doesn't seem to have been much discussion of whether these changes might have increased or decreased the risk of an unexpected impurity being missed.

Should we be worried about failing to detect impurities? - some history

The assay and the determination of organic impurities are the two most important tests in the analysis of organic fine chemicals such as drug substances. In the old days, traditional wet chemical methods were used for both, and not all of these (such as volumetric assays) have been replaced entirely by chromatographic methods. Long before instrumental LC became routine, thin layer chromatography (TLC) was introduced for impurity determinations. Impurities can usually be detected down to about 1% by weight if the separation is good enough, and TLC serves as an identification method which can be quite specific if the colour or fluorescence are characteristic, or suitable spray reagents are used. As far as impurities were concerned, TLC was a major advance, bearing in mind in particular that the range of

mobile phases that can be tested during development is much wider than that available for instrumental LC.

A major advantage of TLC and other planar methods over column chromatography is that impurities that both unretained and very strongly retained are detectable. TLC can, in principle, detect anything organic; universal methods include fluorescence quenching of a fluorescent stationary phase, and spraying with various reagents including sulfuric acid. Unfortunately the latter ceased to work properly with the introduction of pre-coated plates; the organic binder interferes with colour development. It has also been proposed that the binder seriously degrades the chromatographic properties, leading analysts who are unfamiliar with the tedium of coating their own plates to underestimate the value of TLC.

Although TLC methods are still extant in some old monographs, the technique was gradually replaced by LC. The key word here is 'gradual'; I remember a meeting the early 1980s, where pharmaceutical analysts complained that the old fogeys of the pharmacopeial authorities were, as usual, doing their best to resist scientific progress. In fact, the obstacle to progress was the proprietary nature and irreproducible properties (at the time) of LC column packings; an official analytical method is for ever, and at that time you were not allowed to specify the make of materials or equipment. By contrast, in Metabolism and Pharmacokinetics the methods need survive only for the duration of the projet and they are not juridically opposable to the same extent.

An aspect of the argument above that has unfortunately faded from memory is that with GC, the other major separation technique, the stationary phases are almost entirely defined by their chemistry. When you describe a GC method you use chemical nomenclature for the stationary phase (see the Pharmacopeias); for an LC method you give the make of the column, together with an indication of the general chemical nature of the stationary phase.

As the quality of LC columns, instrumentation and automation progressed, the technique became dominant. TLC is clearly inferior when you want to combine an accurate automated assay with impurity determinations well below 1%. But what have we lost in the change?

LC meets modern performance requirements, but only with UV absorbance detection, which is not at all universal. That isn't unexpected because compendial performance requirements are established on the basis of what is feasible. Nowadays we use LC/MS during method development to check for non-absorbing impurities, but there's no guarantee that they will ionise, give a linear response, or be detectable if co-eluted with the main component. All the other detection techniques have serious disadvantages in this field; it's fortunate that most drug substances absorb in the UV. As mentioned above, unretained or strongly retained compounds are unlikely to be detected.

Checking for impurities hidden under the main peak

Perhaps the easiest thing to do when there is a doubt about an impurity in the tail of the main peak is to connect two identical columns in series. Generally, you reduce the flow rate to keep the pressure within limits; analysis time is not an issue here. Sometimes, this results in a pleasant surprise – the chromatographic efficiency and peak shape both improve more than would be expected from the increased length of column. This should not really be surprising because we routinely operate at flow rates that are a bit higher than the optimal value for

5 µm particles. Also, while the elution volume is doubled, dead volumes and other imperfections in the flow path stay the same and they may be flow dependent. I note, as an aside, that if the limit of quantitation for a small peak is marginal under standard conditions, the signal-to-noise ratio can be improved by reducing the flow rate and adjusting the acquisition and integration parameters accordingly.

Although mounting two columns in series gives twice the number of theoretical plates, we know from textbooks that the peak capacity is less than doubled. The peak capacity is the maximum hypothetical number of peaks that could be cleanly separated from each other, without the peaks becoming so broad that they are no longer detectable. It can't be precisely defined, particularly in our situation where the main peak can mask the impurities we are searching for, over a large part of the chromatogram. When we connect two columns in series, while the centroid of the partly-separated impurity peak becomes more distant from the centroid of the main peak, the main peak continues to broaden and overlap with it. A natural reaction of a chromatographer working in our field may be to further optimise the separation method, but this has its limits if we don't know in advance whether there really is an impurity under the main peak. A solution that dates from the early days of gas chromatography is to switch the two columns in series only for the time-slot during which the suspected impurity would be eluting from the first column; most but not all of the main component is diverted to waste. The rest of the time, the second column is eluted with fresh mobile phase. The technique is called 'heart-cutting', and you have to be careful with terminology when doing literature searches. Note that switching is for only one or a few time-slots; there is no attempt to perform comprehensive 2-D chromatography.

I didn't find an instructive LC example, but here is a GC one "borrowed" from the CHROMacademy:

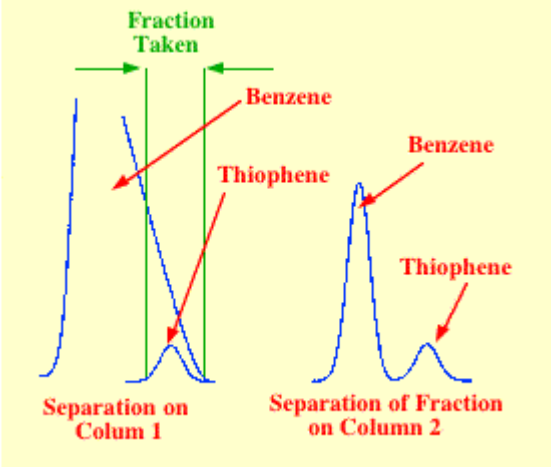


Illustration of heartcutting in gas chromatography (CHROMacademy)

The main peak (benzene) is shown chromatographically overloaded, but the principle applies also to linear chromatography. Other examples can be found in papers by D. R. Deans (see Deans, 1981).

> For readers unfamiliar with the theory, the advantage of heart-cutting over columns simply connected in series can be explained from first principles. We can think of

peak dispersion as the result of random stochastic events that move a solute molecule backwards or forwards along the axial direction. The direction of 10 successive impacts of solvent molecules on a benzene molecule (due to their thermal motion) could be simulated by throwing a coin 10 times. The most probable outcome is 5 heads and 5 tails, whereby the benezene molecule remains in its initial position (apart from motion due to solvent flow). However, for one simulation out of 1024, the molecule moves 10 steps towards the leading edge of the peak and in 1/1024 cases it moves 10 steps towards the tail. Now, there can be nothing special about the benzene molecules that have migrated slower or faster than average, and if we pass a slice of the peak onto a second identical column (with no additional dispersion), the most probable migration rate of a given molecule is that of the centroid of the peak. Also, since there are fewer benzene molecules than on the first column, the resolution requirements are lower.

I labour this point because the vast literature on column switching tends to emphasise the use of different ("orthogonal") separation mechanisms for the two columns (Guiochon *et al*., 2008). The point to emphasise for CMC pharmaceutical analysts is that confidence in the validity of a method can be significantly increased by carrying out a heart-cutting experiment between two identical columns. No additional chromatographic development work is needed.

Column switching techniques

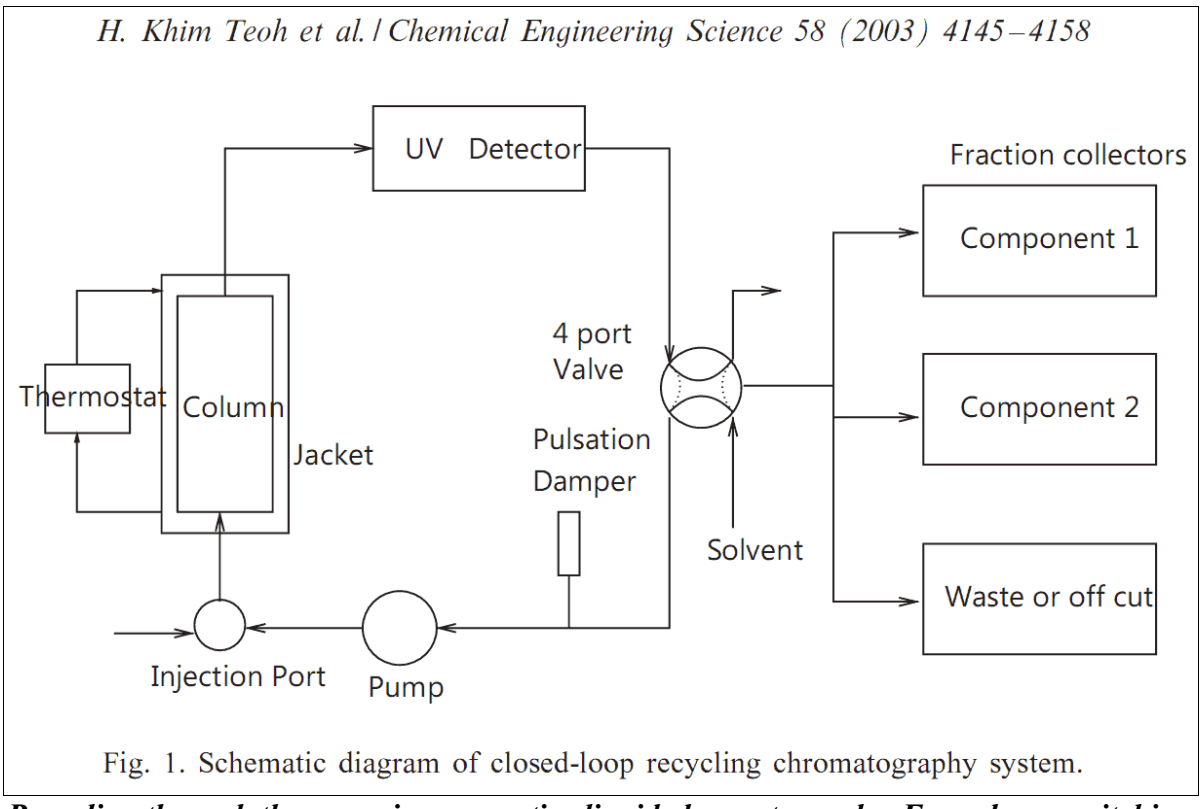
Gas chromatography
Column switching is as old as column chromatography, and has been routine for decades for numerous GC applications. CMC pharmaceutical analysts may not be familiar with this because we don't often encounter difficult GC separations. Given the chromatographic efficiency of capillary GC columns, which have been available for several decades, this is yet another a reminder that increasing the number of theoretical plates can't solve every separation problem. GC column switching is straightforward in the sense that the mobile phase is always the same and it has no effect on selectivity; consequently, you can easily use any pair of stationary phases when selectivity needs to be improved.

Since gases are highly compressible, switching a peak from one column to another must be done at constant pressure. The flow-path between the columns must be both chemically inert and operated at high temperature; in general, the analytes should not come into contact with valves or other elements with moving parts. D. R. Deans developed methods for 'valveless' column switching with pressure balancing, and although he worked in the petroleum industry, he published some of this work as early as the 1960s. An example of heart-cutting can be found in a paper published in 1981. The methods were adapted for capillary columns. SGE marketed equipment for retrofitting existing equipment in the 1980s and we published a few applications using it. Several instrument manufacturers now propose accessories and fully integrated systems using microfabricated components.

Liquid chromatography
Column switching is also widely used in analytical liquid chromatography and ion chromatography (which is still treated as a separate technique). However – hence this note – it's practically absent from compendial pharmaceutical methods. I don't know whether it's used much for pharmaceutical method development and evaluation, because that kind of work is rarely published.

A huge number of papers on other applications have been published, but (as with our own LC-MS methods), the applications tend to be specific to the analytical problem in hand. A major review was published by Guiochon *et al*. in 2008; this emphasises the "orthogonality" aspects, and also the technical difficulties which have not yet been resolved when the integrity of the preceeding separation must be maintained during high-pressure switching. Switching is often used essentially for sample clean-up, however sophisticated that may be; timing is not critical with these methods. Most of the usual analytical techniques don't seem really suitable for routinely checking out single-column methods because they don't allow you to take a slice of a peak that is both adjustable in width and finely timed.

A lot of the published analytical LC switching methods involve either trapping the analyte in a fixed-volume holding loop (which may contain a stationary phase), or switching techniques that involve severe pressure surges that disturb the chromatographic process. Starting some time in the 1980s, automated preparative equipment of varying sizes began to be used for small molecules. Because of the nature of the applications, little was published to begin with. Partly in order to optimise the amount of expensive stationary phases, column switching and (more usually) recycling, as well as more elaborate switching techniques (simulated movig bed), rapidly became routine. Pressure differences are not a problem because transfers can be done through the pumps, an approach that's impossible on the analytical scale. Here's a figure from a paper on recycling, chosen because there's no paywall, and because it illustrates the level of theoretical and engineering sophistication in the preparative field.



Recycling through the pump in preparative liquid chromatography. For column switching two systems are used in tandem.

In the configurations I will describe, 4-port valves can be replaced by 6-port ones with a jumper that doesn't interfere with the chromatography. Some published designs have stoppers for unused ports, but this prevents the system from being purged after use.

From the early 1990s, when analytical LC had become well established in CMC pharmaceutical analysis, the authorities began to pay more attention to impurities down to the 1 part per thousand level. This was driven by largely undisclosed safety concerns and, presumably, by the fact that such impurities could now be detected. Everyone conveniently forgot that that in general that only applied to UV/vis absorbance detection. Coincidentally, we encountered a couple of new chemical entities that had, as they say, challenging impurity profiles. We recall that at that time it was nearly always necessary to isolate enough of an impurity to obtain a clean NMR spectrum, because atmospheric pressure ionisation LC/MS with accurate mass measurement had not yet hit the CMC pharmaceutical scene. The usual approach was to collect partially-separated fractions, remove buffer salts as best we could, and then concentrate the fractions for re-injection. It rapidly became obvious that column switching would be quicker and easier.

Dominique Tessier set up a Gilson so-called semi-preparative system, with 25 mm o.d. x 25 cm columns and 1/16 inch plumbing. The single-piston pumps were designed for such work; dead volumes were small, and narrow-bore inlet tubing could be used because the intake stroke was not too rapid. We bought columns (10 μm particles) matching the most commonly-used analytical ones, and packed some others using an axial compression apparatus. The detectors need to resist pressure surges; fortunately, we had analytical cells designed for supercritical chromatography. We'll mention control and data recording systems later; for the moment it's enough to say that I don't have any usable records and can't illustrate separations of impurities that appeared as a small bumps in the tail of the main peak.

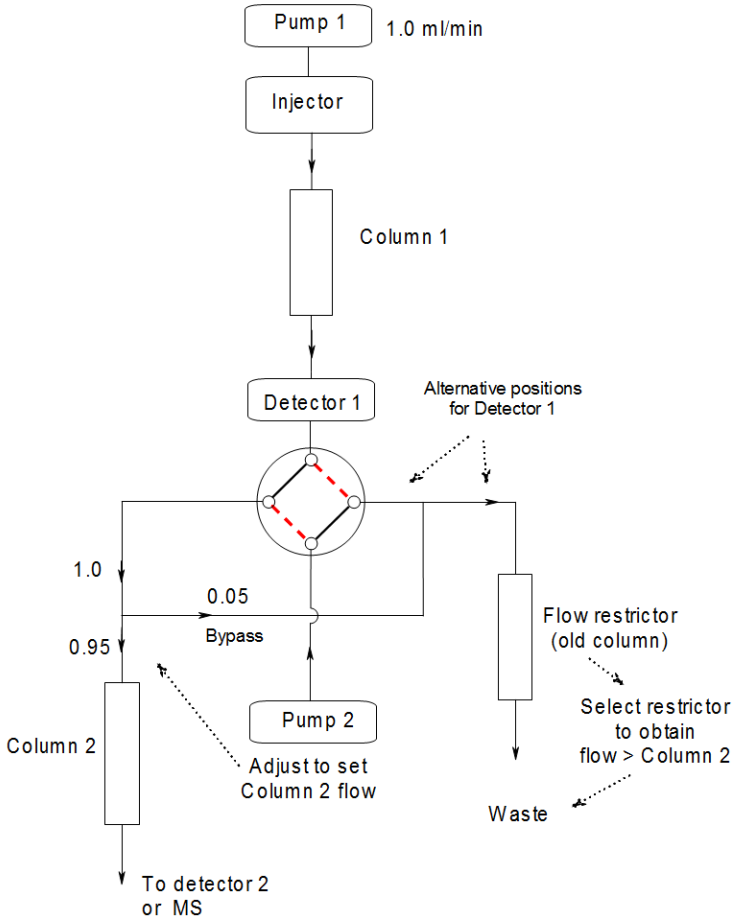
Recycling (as in the article above by Teoh *et al.*) has its limitations when isolating impurities, because tailing residues of the main peak quickly obscure the chromatogram. We connected two systems together, so as to heart-cut onto a second identical column while the first one was being purged. We then recycled the impurity through the two columns in series. In practice, this became a bit of a game because, for example, if the impurity we were chasing was going round for the fourth time, it could get confused with some other impurity on its third lap. Genuinely real-time control and data acquision are almost essential but were not yet available.

An interesting aspect of this work was that the preparative equipment had better separating power than we could achieve with analytical chromatography. Detection limits were the same because we used analytical UV flow cells. I strongly recommend using small-scale preparative equipment for evaluating analytical methods by means of column switching. However, the experience led us to explore, very briefly, the state of the art of column switching on the analytical scale, that is, without the analytes traversing a pump. As mentioned above, most people use either holding loops or arrangements that involve excessive pressure surges and even flow reversals.

Column switching in analytical liquid chromatography

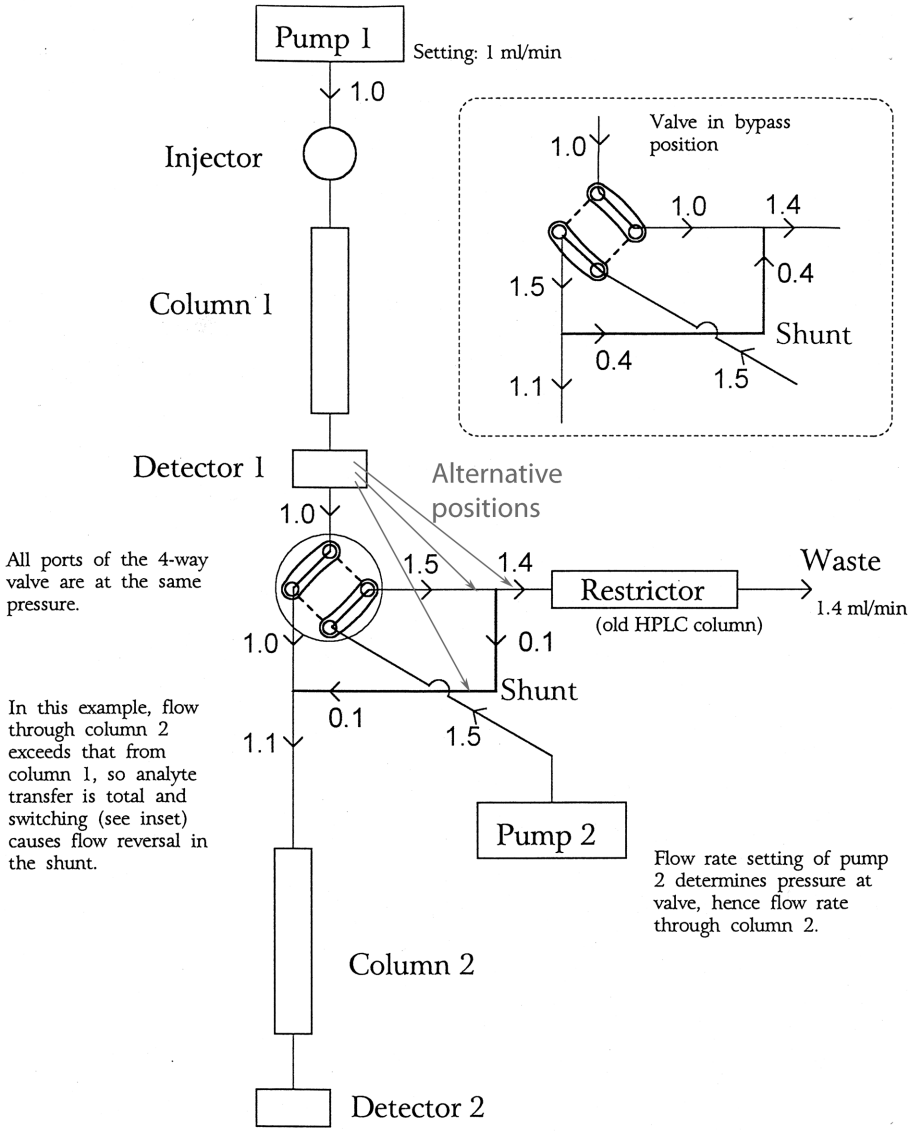
Pressure-balancing column switching

The first of our systems was also set up by Dominique Tessier. It involves pressure balancing as in GC, but it doesn't seem to have been published by others; the principle isn't mentioned in the wide-ranging 2008 review Guiochon *et al.* One difference with respect to GC practice is that you can have a valve in the flow stream, although a commercial realisation could easily be "valveless" (see below). The idea should be fairly obvious from the figure below. The two columns and mobile phases were identical.



Column switching in analytical liquid chromatography: pressure balancing.

It's easiest to think of pump 2 operating at constant pressure, though one is more likely to use constant flow, which is technically more straightforward; turning the valve makes no difference to the pressure or the flow rates, apart from transients. Total transfer to the second column (with a little dilution) can also be arranged (figure below), though it may be advisable to include a low dead-volume mixer.



Pressure balancing with total transfer to the second column.

A pessimist would identify the following obstacles for routine use; they may partly explain the absence of a ready-made commercial offering:

1. Operating two columns in series is not a common or popular choice, partly because of the pressure/flow limitation. However, as explained above, chromatographic performance is sometimes better than expected and analysis time is not a serious issue here.

2. Recycling chromatography is not possible.

3. Unlike the case of GC, variable flow restrictors for LC are not readily available catalogue items. We had to choose a suitable restrictor by trial and error from the collection of columns that had been withdrawn from service and had not yet found their way to a university teaching lab. It's possible to use a short column in series

with a needle valve or back pressure regulator, but this still involves some "fiddling around". However, suitable equipment could be designed without requiring much innovation, because electronic pressure and flow monitors and regulators are available.

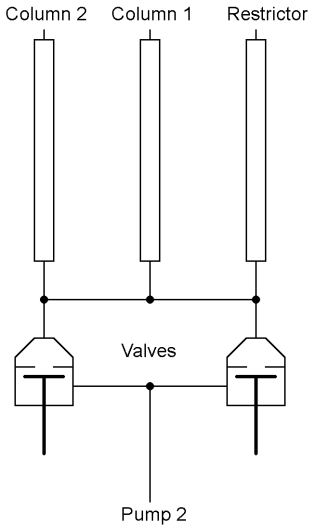
4. UV/vis detectors have a long capillary heat exchanger upstream of the cell, and somewhat indeterminate plumbing downstream. This leads to difficulties with timing and peak-broadening that were not serious on the preparative scale. Alternative positions for the first detector are indicated in the figures, but there is no ideal solution.

5. Detectors for the first column must be types that can operate with both the inlet and the outlet at high pressure.

6. Instrument control and data acquisition require specific software which takes acount automatically of factors such as detector lag (this is routine GC practice). Back in the 1990s we were still able to make ad hoc arrangements, generally using real-time computing integrators, and we assembled the valve interfaces ourselves. Nowadays, analysts are not expected to be able to configure their chromatographic data system to this extent, or even to connect a push-button as an event marker. Bytes of data may well visit a network server on the other side of the site or the world before they appear on the computer screen; apparantly that still counts as "real-time" processing.

7. Rotary valves are generally reliable, but they do have numerous failure modes that can be difficult to diagnose.

A commercial implementation might be of a "valveless" design as shown very schematically below. There is less plumbing in the solute flow-path and the volume of the valves is not critical; the valves could be designed to attenuate switching surges. The right hand valve is open during switching, and the left hand one the rest of the time.



"Valveless" column switching.

Alternating loop column switching

Sandra Firmin did some brief experiments with a different system that had already been described on several occasions, the first time, according to Guiochon *et al.* (2008) by Erni and Frei in 1978. Below, we show another realisation, that was not cited by Guiochon *et al.* (2008); as often happens, this design was presented simply as part of a practical application. The flow from the first column is directed to twin holding loops that are alternated between collection and injection into the second column. A standard 8-port valve is used.

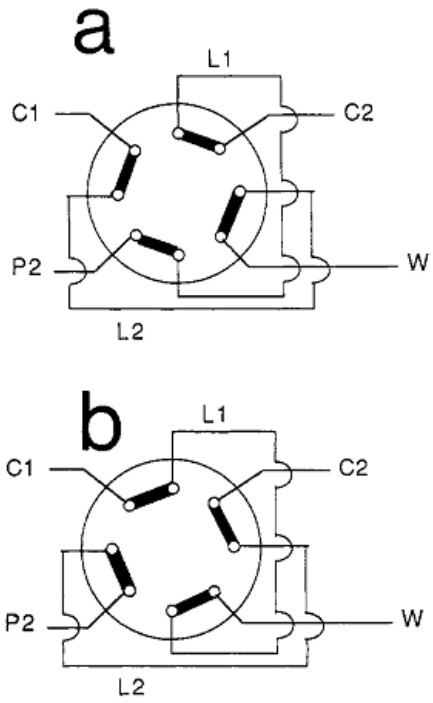


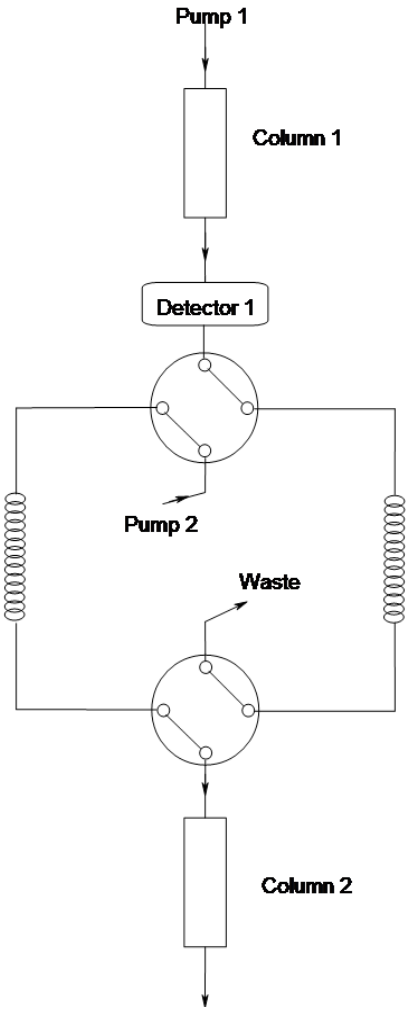
Figure 1. Two configurations of an eight-port, computer-controlled valve. C1 and C2 are columns 1 and 2, respectively, L1 and L2 are loops 1 and 2, respectively, P2 is pump 2, and W is waste.

M. M. Bushey & J. W. Jorgenson,
Analyt. Chem. 1990, **62**, 161-167.

An implementation of alternating loop column switching.

The arrangement may be easier to visualise with our set-up, shown below. We used pneumatically-driven 6-port valves recovered from old equipment, connected to operate as 4-port valves. Thomas D. M. Lee built a timing device for the pneumatic actuators, using a microcontroller.

Advantages of the alternating loop system are that the columns are not connected in series, and that the first column can be monitored (except during switching) using any kind of detector if it is mounted in the waste line.



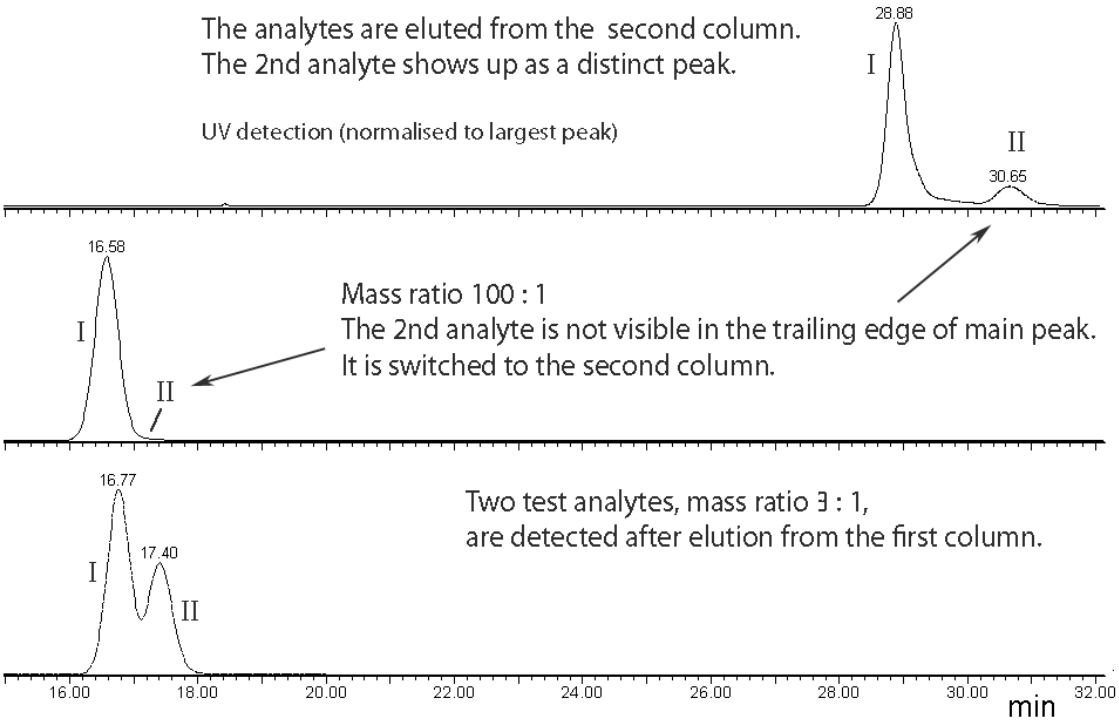
Column switching in analytical liquid chromatography: alternating loops.

In the published applications, the twin loops appear to be regarded essentially as a means of collecting a fraction (usually a complete peak) while the previous one is being eluted through the second column. Guiochon *et al.* (2008) appear to dismiss the idea of switching the valve sufficiently rapidly (in relation to a typical peak width) that the twin-loop system becomes almost equivalent to the continuous-flow pressure-balancing system shown above.

For our very brief feasibility study, we found two test compounds that gave overlapping peaks with fairly long retention times of 16.8 and 17.4 minutes at a flow rate of 1 mL/min. Naturally, the equipment we were messing around with was fairly old and had no column thermostat, so there was a little retention time drift (see the figure below). Since we had "borrowed" the data system of a heavily-used LC/MS instrument, there wasn't time to measure the detector lag; switching times were determined by intelligent guesswork, and somehow the data system failed to record the timed events.

The loops had a volume of 200 µL and they were cut from narrow-bore tubing to limit peak dispersion. During heart-cutting, the valve was switched every 12 s, corresponding to the loop volume; in fact this time is not critical. The peak half-width on the first column was about 30 s and, as only a narrow slice of this was passed to the second column, the final peak widths were about the same.

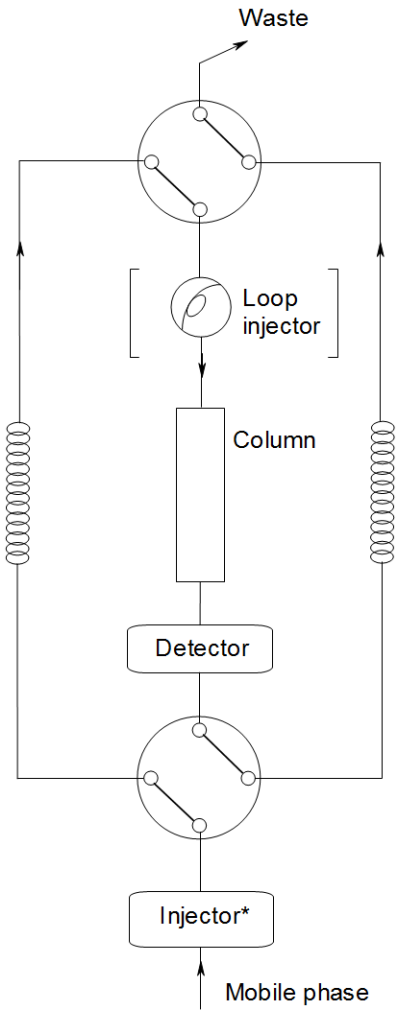
In the chromatograms below, the bottom tracing shows a 3:1 mixture of our test compounds (mass ratio). The ratio for the middle chromatogram was 100:1 and the peak of the simulated impurity with the larger retention time is not visible. In this run, Sandra switched the very end of the peak tail to the second column, on which the simulated impurity is well resolved from the remaining fraction of the main component (the chromatograms are normalised to the largest peaks).



Heart-cutting using the alternating loop system.

Recycling in analytical liquid chromatography

As shown below, the twin loop system is an obvious choice for recycling in analytical LC. Injectors commonly used in pharmaceutical analysis can not be placed in the recycling loop, and there would be less band-broadening during the first pass if a loop-type injector is used as shown. Unfortunately, we did not have an occasion to test this system.



Use of alternating loops for recycling in analytical liquid chromatography

There seems to be no obstacle to the use of much smaller loops, with more rapid alternation. Wear of the valve is not a serious issue, because it is operated for short periods. We used pneumatic actuators to ensure fast (though noisy) switching. Since that time, electric actuators have improved. A commercial implementation might perhaps rely on a precise stepping motor for aligning the rotor instead of mechanical end-stops; stepping motors allow controlled acceleration and deceleration. Perhaps a valve that rotates continuously and smoothly could be designed, but that is a specialised subject. Finally, a membrane valve manufactured by Vici could be considered. The membrane is of polyimide, which resists bullets and many solvents, but probably not nucleophilic attack by certain mobile phase additives.

"Orthogonality"

We have emphasised heartcutting between identical chromatographic systems, partly because this can be implemented with no additional chromatographic development. If we need better selectivity, two different reversed phase columns could be the most difficult option unless, perhaps, the second column uses a stronger mobile phase than the first. Another possibility, with identical reversed phase columns, could be to add an ion pairing agent to the second mobile phase.

Ion exchange chromatography, used alone, has limited possibilities for optimising separations, even if all the analytes are ionised. However, since the reversed phase separation is relatively insensitive to the ionic compostion of the mobile phase, the two modes can be optimised almost independently. Mixed-mode columns used to be available. The purely ion exchange columns we have used had inconveniently large capacities, and mobile phases must be of high ionic strength.

One separation mode worth reconsidering for the second column is size exclusion. When used alone, the separating power is insufficient, but this is of secondary importance here.

CONCLUSION

We present a number of reasons why current practice in chemical and pharmaceutical analysis may lead to unexpected impurities being missed. The situation could possibly be less secure than in the days of thin layer chromatography.

Not discussed here are extraneous factors such as a tendency to outsource or set rigid deadlines for analytical development. Once every two or three company reorganisations (that is, quite often), someone with no understanding of competing priorities may merge analytical development with formulation or chemical development. This is not a serious cause for concern, as such decisions are soon reversed.

Concerning laboratory practice, there seems to be little discussion about the current reluctance to use the wide range of mobile phase additives that were common some years ago. Reasons for this include compatibility with mass spectrometry and the risk of wear and damage to the equipment, particularly with phosphate salts. One consequence for the selectivity of separations is that ion exchange mechanisms are not so easily "covered" by ion-pairing reversed phase chromatography.

Recent improvements in the chromatographic efficiency of liquid chromatography columns are welcome but, as is well known in gas chromatography, columns of varied selectivities remain essential. Paradoxically, as column manufacture becomes more sophisticated and difficult, the range of choice could become more limited. Also, as the instruments become more sophisticated (higher pressures, lower flow rates), the analyst is further distanced from the practical and theoretical aspects of instrumentation, and it is out of the question to modify even the fluid connections.

As a partial response to these concerns, column switching techniques should be available for routine use during method development. Since this is already the case in numerous other fields, the present situation in pharmaceutical analysis should be considered anomalous. Two likely obstacles are the lack of equipment and software adapted to a regulated environment, and pervasive quality systems that inhibit innovation, encourage over-simplification, and dissuade innovative scientists from working in the field.

We have described two basic configurations for column switching in analytical liquid chromatography; each has specific advantages and disadvantages. Experimental column switching systems are not difficult to set up. While they may be a little disconcerting at first, analysts soon get used to them. On the other hand, some further instrumentation and software development is needed, and the software should be compatible with existing systems.

Analysts have little opportunity to participate in the development of instruments and new kinds of methods. Such projects are of great value to student interns, but in a busy laboratory it is difficult to take take developments to a point where they can be introduced into routine practice.

References

Our incomplete studies were presented in the following article in LC-GC Europe magazine, in the forlorn hope of stimulating development.

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Teoha, H. K; Sorensena, E; Titchener-Hookerb, N. Optimal operating policies for closed-loop recycling HPLC processes. *Chemical Engineering Science* 2003, 58, 4145-4158.