On-Column Sample Focussing: a Personal Perspective

W John Lough, School of Pharmacy and Pharmaceutical Sciences, University of Sunderland, UK.

Academic researchers are often not entirely masters of their own destiny. A few are able to remain true to their primary interest, for others their interests evolve in a natural progression and many find themselves having to follow the interests of those willing to fund them. As someone who finds himself in the latter category, my 'research interests' within the broad area of separation science and its applications have been quite varied. One always tries to look forward but occasionally one permits oneself an occasional glance back over one's shoulder. In doing so I was quite intrigued to note that a publication from way back in the day [1] was somehow still accruing citations. I had never been a serious drug bioanalyst but funding for work in this area had allowed me to continue my interest in miniaturised LC, at the time, specifically microbore LC [2] and in particular the phenomenon known as peak compression aka on-column sample focussing *(Figure 1)*.

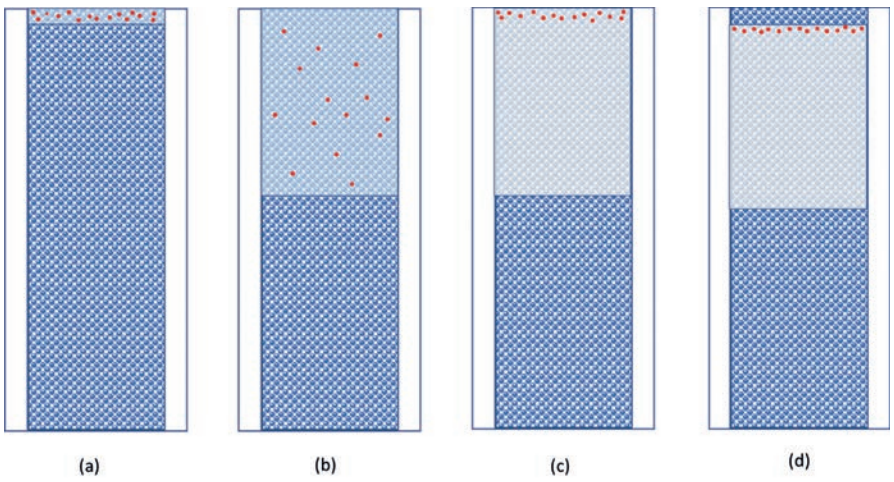

Figure 1. (a) Narrow band obtained by a low injection volume in mobile phase (b) Dispersed band obtained by large volume injection in mobile phase (c) On-column focussing obtained by injection in a non- or weakly eluting sample solution solvent (d) Further focussing as weakly eluting zone moves down the column followed by the mobile phase.

In short, low limits of quantitation and detection could be had by injecting large volumes of sample solution in which the solvent was less strongly eluting than the mobile phase. In this way there was no volume overload and accordingly none of the band broadening normally associated with the injection of large volumes. The paper, 'Assessment of Injection Volume Limits When Using On-Column Focusing with Microbore Liquid Chromatography' [1], which is still drawing citations, addressed, as the title suggests, an assessment of the actual extent to which large volumes of non- or weakly eluting sample solution solvent could be injected without introducing loss of efficiency through band broadening. As such, the publication involved a significant element of theory but in practice this was a transient diversion as our interest at the time was in the actual application of on-column focussing. One area in which there was an obvious need for concentration effects was in drug bioanalysis. Accordingly, on-column focussing was used in the injection of the eluent from solid-phase extraction cartridges directly onto a microbore LC column containing a more retentive alkyl-bonded silica in a fully automated method for the determination of a drug in human serum [3]. Less obvious was the role of the highly retentive LC stationary phase, Hypercarb, in facilitating on-column sample focussing in chiral drug bioanalysis [4]. Our interest in on-column sample focussing continued, but, more pertinent at this current time, having noted its value to others, is to consider how it has continued to be used over the years.

This continued interest in on-column sample focussing in LC is unsurprising. After all, in thin-layer chromatography (TLC), pre-concentration zones are commonplace and in gas chromatography (GC) on-column sample focussing takes place when splitless injection is used. The need for on-column sample focussing is also very much a feature of reduced volume separation techniques such as capillary electrophoresis (CE) and capillary electrochromatography (CEC).

To assess the degree of continuing interest in on-column sample focussing in LC by monitoring the citations of the assessment of injection volume paper [1] is a bit of a stretch, but, nonetheless, it is apparent that while the record is uneven it is continuous and shows little sign of abating *(Figure 2)*, the most recent year, 2022, notwithstanding. The nature of the citing papers *(Figure 3a)* is perhaps more relevant and more revealing. Given that the cited paper involved theory, there is a significant proportion of theory-based papers. However, mirroring our own interests, papers on applications predominate with bioanalysis being clearly an area where on-column sample focussing is needed. With respect to the distribution by mode of liquid separation technique involved (Figure 3b), general LC papers predominate but also noticeable is the need for on-column sample focussing in nano-LC and capillary LC.

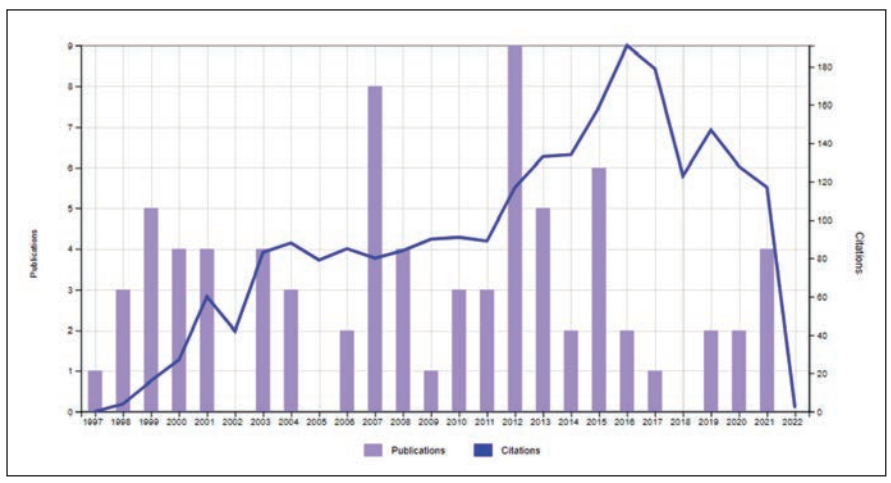

Figure 2. Citation report publications table for reference (1): times cited and publications over time.

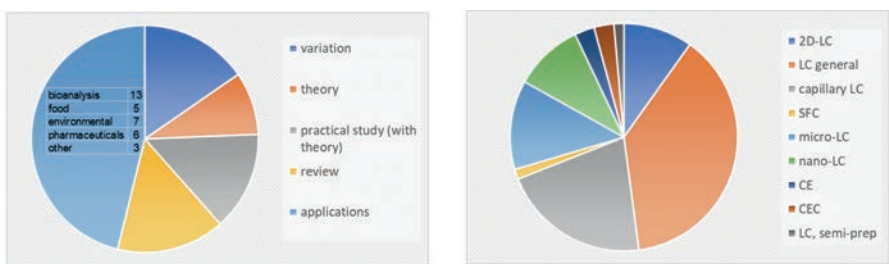

Figure 3. (a) Distribution of citing articles by article type. Figure 3 (b) Distribution of citing articles by mode of liquid separation technique.

The theory papers took the understanding of on-column focussing to a much higher level of sophistication. Indeed, as would be expected following several studies of the situation by theoretical 'heavyweights', the original [1] assessment of injection volume limits has been superseded, or even 'disproved'. Groskreutz and Weber [5], as a principal example, developed a more refined treatment which matched experimental data. Also, they noted that the element of peak compression arising from incoming more strongly eluting solvent moving the back edge of the peak before the front edge of the peak was greater than expected. One of the other theory papers [6] was by Desmet, a doyen of separation science theoreticians. His theory was based on experimental data and then verified experimentally. The focussing in this case was refocussing, with a trap column receiving peaks from a preceding analytical column. The trapped peaks were then 'remobilised' using a solvent mixture of equal viscosity and a nano-LC pump to elute them with a ballistic gradient with a sufficiently steep gradient. While this set-up was not used for a real application, an impressive peak enhancement of 17.3 was achieved under ideal conditons and the results fitted the theory. In the most recent theory paper [7], by Rutan et al., the impact of solvent volume overload in gradient LC (which inherently involves on-column sample focussing) was studied. By taking into account effects of sample volume overload and a mismatch between the sample solvent and the initial mobile phase composition for the gradient, it was possible to obtain much more accurate predictions of retention times and peak widths than hitherto had been possible.

As with Desmet's post-column refocussing work [6], some of the application work involved an experimental variation different from simple on-column sample focussing at the head of an LC column. The case of the work of Pan et al., [8] could be considered as an advance on the SPE work of Mills et al. [3] in that the SPE was integrated, online and involved subsequent uHPLC. Other interesting "variations" included focussing in SFC [9] and the use of temperature to bring about focussing in capillary LC [10].

As already indicated, bioanalysis was an application area where there was a clear interest in on-column sample focussing. As is often the case, as found by Marta et al. [11] in their analysis of steroid hormones from human plasma after protein precipitation, it is necessary not only to use high injection volumes on micro- columns but also to use MS-MS detection. Just like the analysis of pharmaceuticals in biological fluids, the analysis of impurities in active pharmaceutical ingredients (API's) can also present a detection challenge. 2D-LC can be part of the solution to separating large numbers of structurally-related impurity peaks from one another and from the much larger main peak. However, as pointed out by Stoll et al. [12], 2D-LC had been perceived as being inferior to 1D-LC from the point of view of detection sensitivity. This was easily remedied by diluting the first column effluent with weak solvent (water in this case) prior to injection into the second-dimension column. This approach was sufficient to allow the quantification of 0.05% w/w impurities when using UV detection. The use of a more retentive phase in the second dimension could also have been used to bring about the desired focussing effect. Aflatoxins etc. in food, wine constituents and traces of environmental pollutants are other obvious cases where on-column sample focussing might be used to improve the detection and quantification of low level analytes. The latter area has been reviewed [13]. To give but just one example in the former areas, a very complex method involving isotope dilution, sample preparation on ion-exchange resins, nano-LC and tandem MS was required for the determination of glutathionylated and cysteinylated precursors of 3-mercaptohexan-1-ol and 4-mercapto-4-methylpentan-2-one in white grape juices [14]. Buried in the detail but integral to the success of the method was the use of focussing on a C18 pre-column prior to separation on the C18 nano-LC column.

Conclusions

Clearly the use of on-column focussing in LC will continue to be of interest to practitioners. This is evident not only from the steady stream of citations of the 1997 article [1] on the practical limits of the phenomenon but also quite simply because it is difficult to avoid. When injecting solutions into an LC system the sample will be dissolved in the mobile phase or a solvent more or less strongly eluting than the mobile phase. Use of the mobile phase as a solvent is not always possible, use of a stronger solvent is to be avoided unless solubility is a significant issue and, so, a more weakly eluting solvent may frequently be used, even if not specifically seeking out the benefits on on-column focussing. Further, there is no shortage of application areas for which limits of quantitation is not an issue and there is little sign that the trends towards miniaturisation and multi-dimensional LC will abate. Indeed, with a growing interest [15] in sustainable separation science, there is every possibility that the trend towards acceleration will continue. Accordingly, the exploitation of on-column focussing will continue, irrespective of whether 'Assessment of Injection Volume Limits When Using On-Column Focusing with Microbore Liquid Chromatography' ever again is cited, being replaced as it might well be by the deliberations of Groskreutz and Weber (5).

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