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Online and Offline Methodologies

The absolute majority of HPLC and LC/MS applications are based on single column platforms. If the analyzed sample is too complex and/or separation power of the column used is insufficient, the usual choice is optimization (enhancement or re-design) of off-line sample preparation (such as replacement of precipitation or LL with SPE) or less often, implementation of an additional, separate off-line chromatographic step. During off-line purification, samples are collected, followed by the next purification step. Often, collected fraction(s) require additional technical steps such as fraction analysis, pooling, drying, desalting, filtration, derivatization, etc. Completing these steps can be even more time consuming and labor intensive than chromatographic separation itself. As an alternative to manual/semi-automated routines for off-line sample handling, on-line strategies allow direct automated transfer of selected fraction(s) to the second column.

Off-line techniques, however, are usually more rugged, provide less carryover, do not require advanced operator experience and have no special LC hardware or software requirements. From the viewpoint of capital investment, off-line purification such as commonly used solid phase extraction (SPE), liquid-liquid extraction or supported liquid extraction followed by liquid chromatography can be the least expensive and simplest approach. A properly designed, optimized (and especially partially automated) off-line separation could be an optimal choice. However, for certain applications, an on-line (column switching-based) methodology might represent a more efficient solution. Within the past decade, software supporting 2 (or more) gradients and switching valves has become a standard option from most manufacturers of LC equipment.

2D OR NOT 2D

The most commonly used applications of column switching are comprehensive two dimensional chromatography, peak heart cut, on-line trapping/back-flush and alternating column regeneration. There is no consensus on how to define column switching and multidimensional chromatography (two dimensional (2D) as the simplest example). The broad definition of 2D (or multidimensional) chromatography is a "selective transfer of analyte of interest from a first column to the second column (by means of switching valve)" [1]. According to my understanding of that definition, any sequence of off-line separation techniques should not be considered multidimensional chromatography, because there is no selective on-line transfer from column to column involved. It is just multi-step chromatography (as is typical for protein purification process, which is consists of sequence of various chromatographic steps), regardless of possible differences in separation mechanisms involved in these separate steps. This moment is critical for understanding. 2D is defined primarily as selective on-line transfer and NOT as a combination of different selectivities. Selective transfer means that the process is controlled and optimizable. As we can see, the 2D definition proposed above emphasizes the technical realization of the 2D platform and the automatic aspects of the analyte transfer process. However, this definition neglects the column's selectivity aspects and the orthogonality of the retention mechanisms. There is a second school of thought, which considers multidimensional chromatography a "combination of two methods of chromatographic separations characterized by widely different retention mechanisms" [2]. According to this solely separation mechanism-oriented definition, multidimensional separations could be performed either on-line or off-line.

The well-known alternating column regeneration approach does not fit into either definition. This column switching-based platform helps to reduce method time and allows the regeneration and equilibration of one column, while a second, identical column carries on injection and separation steps. As we can see, within such a column switching platform, analyte transfer from column to column does not occur at all, and that platform can only be considered as a column switching application, but not 2D. Parallel chromatography also does not fit either 2D definition-this approach is based on simultaneous or sequential sample injection into identical columns (no selectivity changes), and typically shares one autosampler and one detector (usually a mass spectrometer). Implementation of parallel chromatography can vastly improve sample throughput; however, in such column switching platforms, analyte transfer from column to column does not occur.

The majority of reported reversed phase-reversed phase (RP-RP) applications are usually based on a short RP enrichment column, pre-column, guard cartridge or short trap columns for analyte pre-concentration, followed by switching to an analytical RP column, often in the reverse direction (also termed back-flush). This pre-concentration design is often implemented for the direct analysis of biological fluids and complex samples, and utilizes on-line SPE, restricted access materials (RAM) or turbulent flow chromatography approaches in the first dimension. The first dimension column serves to remove bulk impurities and for analyte enrichment. The analyte is retained at the top of the first column and is not separated from other hydrophobic components; therefore transfer from the first to the second column is not expected to be very selective. An high-resolution chromatographic separation [by gradient elution] of analyte is conducted only on the second dimension's analytical column. In general, since both columns are reversed phase, there is not much difference in their...
separation mechanisms. Therefore, such trap/backflush-based on-line SPE applications could be considered column switching-based applications, but not as 2D. To some extent, applications based on the negative adsorption of analyte in the first column are related to this approach. The analyte of interest is not retained in the first column, and passes through (along with the void volume) and is retained in the second dimension column, while some unwanted sample components become trapped in the first dimension column. In such applications (similar to on-line SPE), high-resolution separation of the analyte of interest is also conducted in the second column only. Unlike on-line SPE, both columns have different separation mechanisms. Typically, first dimension utilizes ion exchange, second dimension–reversed phase. Negative adsorption is a simple, technical approach, which can be implemented on-line. Such applications are uncommon in the HPLC field, being more abundant in protein purification (low/middle pressure chromatography). Negative adsorption fits into both of the 2D definitions cited above, due to the on-line transfer and orthogonality among both dimensions. However, transfer of the unretracted fraction, eluted with the void, is not very selective. It is a binary [YES or NO], low-resolution type of separation in the first dimension. For true 2D chromatography, I would expect not equal, but similar degree of separation power in both dimensions. Unfortunately, this fundamental point is overlooked by the definitions cited above. Perhaps, approaches with heavy disbalance in separation efficiency between dimensions should be considered as multidimensional separations (either on-line or off-line), but not as 2D chromatography. The combination of chromatography and any previous [off-line or on-line] low resolution sample preparation step, even if the both dimensions are completely orthogonal, cannot be termed multidimensional chromatography. Instead, I suggest it should be considered only as multidimensional separation.

In conclusion, only comprehensive 2D and peak heart cut approaches fit both definitions of multidimensional chromatography described above. I propose that the balance of separation efficiency in both dimensions be considered the key factor distinguishing multidimensional chromatography and multidimensional separation.

References