

In the Classroom

Two-Dimensional High-Performance Liquid Chromatography

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Classical two-dimensional chromatography is a powerful tool that helps to solve many of the current tasks.

The quest for a substantial increase in the peak capacity and, therefore, in the number of compounds that can be separated in a single chromatographic run, led to the development of multidimensional techniques in high-performance liquid chromatography (HPLC). The “classical” method of collecting fractions from the initial separation and separating them further in the secondary steps is very powerful but its fully automated “online” version requires quite complex instrumentation. In contrast, development of new chromatographic media with

In the past 20 years, the number of instrumental techniques available to the chemist has grown exponentially. In order to help our readers keep up with this rapidly growing field, tutorial articles on chemical instrumentation will be a regular feature of The Chemical Educator. The articles are designed to serve as a brief introduction to emerging instrumental techniques, with an outline of the underlying principles and major applications.

—Martin Schimpf, Series Editor

segregated bimodal chemistries allows the 2-D separations to be achieved in a single column with standard HPLC equipment.

Chromatography is a physical method of separations in which the components to be separated are distributed between two phases, one of which is stationary while the other, the mobile phase, moves in a definite direction. Tsvett first described this separation method at the beginning of this century. Martin's theoretical work in the early 1940s opened the route to practical use of many different modes of chromatography in which the mobile phase can be gas (gas chromatography) or liquid (liquid chromatography). The efficiency of liquid chromatography using columns packed with relatively large irregular particles of the stationary phase was initially quite low. This changed substantially after the introduction of microparticulate porous silica 30 years ago, which marked the advent of high-performance liquid chromatography (HPLC) and soon allowed a dramatic increase in both the separation power and the efficiency of chromatographic columns.

Despite the impressive increase in the resolving power of typical chromatographic columns, the number of compounds that can be separated into individual peaks in a single run (peak capacity) is limited by the peak width, which in turn depends on the efficiency of the column. Generally, peak capacity is proportional to the square root of the number of theoretical plates available for the separation. For example, a peak capacity of up to about 60 can be theoretically achieved on a 15-cm HPLC column with a plate count of 15,000. However, the theoretical calculation shows that less than 37% of the peak capacity can be used to generate peak resolution. This decreases the number of separated peaks to only 22, provided the selectivity of the column allows the separation of all of the components. When the selectivity is not sufficient for such a separation, some peaks can overlap and actually represent more than one compound. Overlap must also occur if the overall number of compounds in a sample exceeds the peak capacity of the column. Since the number of components in some complex biochemical or environmental samples may easily exceed the actual peak capacity of the available HPLC system, a method that allows their complete separation is desirable.

Multidimensional Separation Methods

Multidimensional separation techniques provide dramatic improvements in peak capacity. In a two-dimensional (2-D) separation, the total capacity is equal to the product of the peak capacities in both dimensions, resulting in much higher resolution. Two major fields of application for 2-D techniques are thin-layer chromatography and electrophoretic separations, in which planar media are typically used. Thus, the separation is effected in one direction first, the plate is rotated by 90°, and then the next elution is carried out. The electrophoretic 2-D format is one of the most powerful separation systems known, and the isolation of more than 1000 proteins from a complex mixture has been demonstrated. Unfortunately, this simple rotation approach cannot be used for chromatographic separations in columns.

Two-Dimensional Liquid Chromatography

Since HPLC techniques are essentially different from planar chromatography, alternative means must be used for 2-D liquid chromatography separations. Typically, the initial separation proceeds in a primary column and “cuts” of the eluent from this column are directed into one or more secondary columns. This can be done using an offline or online approach. In the former, the cuts from the first dimension are collected in vials and injected independently to achieve the second dimension of separation. The online approach is characterized by a direct coupling of the primary and secondary columns through switching valves. Although more complex, online techniques are preferred due to the ease of automation. Therefore, this report will focus only on this method.

In an ideal setup, all of the effluent from the primary column should be sampled into the secondary ones. In practice, however, only the eluent that contains peaks of interest monitored during the first dimension of separation is redirected to the second dimension of separation. Several important issues must be addressed to accomplish this operation.

Number of Columns in a Two-Dimensional System

One of the most important considerations when setting up a 2-D HPLC system is the number of columns required for a specific separation project. It is well known that a HPLC column is most efficient at a specific flow rate of the mobile phase. The

differences in optimal flow rates between various columns are not very large. Therefore, the primary column affords a much higher number of samples than the single secondary column can process. Let us assume for example, that the flow rate through the primary column is 1 mL/min and the separation is completed in 5 minutes. If the sampling volume into the secondary column is 50 μL , 100 samples are produced per run of the primary column. If both the flow rate and run time of the secondary column are similar to that in the primary column, 100 secondary columns would be required to accomplish this relatively simple task. The number of secondary columns will increase as the time of the primary run increases, the number of cuts increases, and the sample volume decreases. Obviously, this approach would be quite column intensive and its implementation very complex. Therefore, simplified methods serving the purpose of online 2-D HPLC with fewer columns have been developed. First, sampling of only the eluent that contains peaks may lead to a significant decrease in the number of samples directed to the separation in the second dimension. Since only a very narrow part of the first-dimension chromatogram, and often only one peak, is important for the separation in the second dimension, the number of samples may be reduced to one. This in turn leads to only a single column required in the second dimension. Thus, conventional 2-D requires a minimum of two columns.

Timing in Online Separations

A typical system for online 2-D separation involves two complete chromatographs consisting of pumps, columns, detectors, and injection devices with their flow paths interconnected through a switching valve and controlled entirely by a computer. The eluent from the primary columns passes the first detector, and then through the loop of the switching valve. Once a peak is detected, the computer sends a signal to the valve that switches the flow through the loop from the primary to the secondary circle. This switch actually represents injection into the second-dimension column. Since some overlap of adjacent peaks may occur in real-life chromatograms, often only the central part of the peak is directed to the second dimension of separation system (“heart-cutting” scheme). Obviously, to obtain the desired peak cut, exact timing of the valve switch is essential to compensate for the delay between the detector and the loop.

Mobile-Phase Compatibility

Since selectivity is also an important factor in the separation of complex mixtures, different chromatographic modes can be used in each “dimension.” However, different mobile phases are characteristic of each of these modes and some of them may not be completely compatible. For example, size-exclusion chromatography (SEC) is a method of choice for the separation of molecules that vary widely in molecular weight. Therefore, SEC is very useful as the first dimension of separation in a multidimensional processes. A typical solvent for this separation is tetrahydrofuran, which is well-miscible with both water and hydrocarbons such as hexane. Therefore, SEC is compatible as the primary method with both reversed-phase (RPC) and normal-phase chromatographic (NPC) modes in the secondary separation. The combination of SEC and RPC has been used for the determination of pesticides in plant extracts. Other compatible modes are RPC and ion-exchange chromatography (IEC) and SEC–IEC. On the other hand, normal-phase separations are typically not compatible with those that use aqueous mobile phases such as RPC and IEC.

Single-Column “Pseudo 2-D” Separations

An approach involving single-column “pseudo-multidimensional” chromatography simplifies the concept of multidimensional HPLC to effect sequential separations in two different mobile phases. Typically, conventional reversed-phase C_{18} silica is used as the stationary phase for the sequential chromatography of charged and neutral small organic acids and peptides. First, a pH gradient in the aqueous mobile phase is used for the gradual ionization of the carboxyl groups of the individual charged species in the sample, resulting in both a lowering of their affinity for the nonpolar separation medium, and their elution. In the second dimension, a gradient of methanol in an aqueous buffer is used to elute the neutral hydrophobic compounds that were held at the top of the column during the first step. This type of elution is obviously controlled only by the composition of the mobile phase, because the stationary-phase chemistry remains the same for each of the separation steps.

In contrast to a “single-chemistry” approach, two or more functionalities can also be introduced intentionally in the separation system in order to achieve the equivalent of a two-dimensional separation. Stationary phases based on modified porous silica beads with both a hydrophilic outer surface and hydrophobic pores have been developed by Pinkerton to allow the direct injection of complex matrices such as blood plasma,

serum, saliva, and urine. The determination of drugs and metabolites can be achieved without any pretreatment, while preventing accumulation of the proteins that would eventually completely clog the column. In these so-called restricted-access media, the proteins are eluted at the void volume of the column, while the other analytes follow and are separated. In this case, the hydrophobic surface of the packings is protected from protein contamination by preventing their access to the inner part of the stationary phase through a sieving mechanism that only allows the proteins to interact with the hydrophilic nonadsorptive outer layer of the packing. This type of separation is not truly two dimensional because the high molecular weight portion of the sample is not separated.

Separation Media with Segregated Chemistries

Pore-size-specific functionalizations have been developed to prepare porous media in which pores within different size ranges may be endowed with different chemistries. The use of catalysts or reagents with defined molecular volumes that are able to perform a chemical modification only in those pores that are large enough to allow their access is the key concept of pore-size specific modification of porous materials shown in Figure 1. This new process relies on the same principle as size-exclusion chromatography.

For example, if we assume that the internal surface of porous beads that have a relatively broad pore-size distribution is lined with reactive functionalities **A**, a macromolecular reagent or catalyst added in solution to such beads will only penetrate the large pores of the beads. Any reactive group **A** that comes into contact with the polymeric reagent is modified into functionality **B**. In contrast, the hydrodynamic volume of the soluble polymer does not allow its permeation into small pores, which retain their functional groups **A** unchanged. In a subsequent reaction step, a second reagent, or catalyst, that has a smaller hydrodynamic volume than the previous one may be used to penetrate deeper into the smaller pores, transforming **A** into **C**. Thus, the different groups **B** and **C** of the final separation medium are all localized in different areas of the bead. The pore-size-specific modification process allows the preparation of separation media that can accommodate pairs of very different chemistries in small pores and large pores, for example hydrophilic and highly hydrophobic (SEC–RPC), hydrophilic and charged (SEC–IEC), and charged and hydrophobic (IEC–RPC).

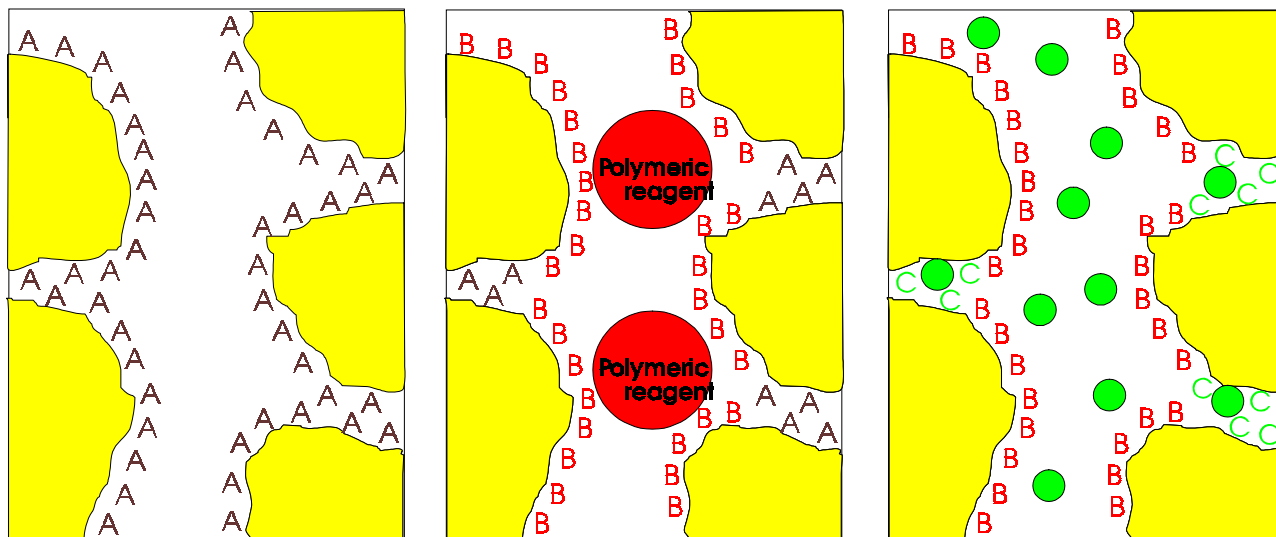


FIGURE 1. SCHEMATIC VIEW OF PORE-SIZE SPECIFIC FUNCTIONALIZATION.

Genuine Single-Column Two-Dimensional Chromatography

Separation media with bimodal chemistries are generally designed for the complete separation of complex samples that typically contain molecules differing in properties such as size, charge, and polarity. For example, the composition of the high molecular weight portion of a sample of blood plasma has traditionally been thought to be less significant. This is partly because current columns for direct injection do not allow the separation of proteins. However, knowledge of the protein composition may be important. Using classical techniques, the total separation of such samples with dissimilar components would rely on the use of multiple columns and the complex system of valves, pumps, and detectors mentioned above. In contrast, a single column packed with a multimodal separation medium with multiple segregated chemistries can be used for this separation without requiring any special chromatographic equipment.

Let us consider the separation of a complex mixture consisting of proteins and drugs using a medium with small and large pores differing in their hydrophobicity. After injection into the mobile-phase stream that does not elute any of the compounds, all of the components of the sample are quickly adsorbed at the top of the column. However, the adsorption is size selective, the sample is separated during adsorption into two portions according to both the hydrodynamic size of its components and their affinity toward the pore surface. Thus, proteins remain in the large pores, which contain just

enough hydrophobic phenyl ether groups in a hydrophilic environment to provide for subsequent elution in hydrophobic-interaction mode. In contrast, the small drug molecules penetrate the small pores, where they interact strongly with the highly hydrophobic surface. This process—the separation of two classes of components in different pores where they are held by different interactions—is actually the first dimension of the separation. The proteins are then eluted by a decreasing ammonium sulfate gradient in the hydrophobic-interaction mode, whereas the segregated small molecules are retained within the hydrophobic small pores. These are not eluted until a mobile phase containing acetonitrile is applied, and isocratic reversed-phase mode elution is used. The consecutive elution of the two parts of the original sample represent the second dimension of the separation.

Conclusion

The increasing complexity of samples requires development of novel means for their complete separation. Classical two-dimensional chromatography is a powerful tool that helps to solve many of the current tasks. However, the instrumentation that is essential for the multiple-column approach is rather complicated. The use of the novel single-column 2-D separation media significantly decreases the instrumental requirements and in some applications can provide better separations than the significantly more complicated multicolumn systems.

FURTHER READING

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