

EXPERT OPINION

The growing role of two-dimensional LC in the biopharmaceutical industry

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Introduction

Over the past two decades, three major trends have been observed in the bioanalysis world.

- First, in liquid chromatography (LC) two-dimensional liquid chromatography (2D-LC) has emerged as one of the most active areas of technology advancement [1–3]. In 2D-LC, a conventional separation is carried out in the first dimension and aliquots of the effluent are collected and injected to a second-dimension column that has very different separation selectivity compared to the first-dimension column. Therefore, much higher peak capacity, and thus resolving power, can be achieved in 2D-LC compared to 1D-LC. This increased resolving power can be used to increase the likelihood of separating a complex mixture, or decrease the time required to fully separate simpler mixtures. In addition, 2D-LC allows the coupling of two completely different separation modes (e.g. reversed-phase and ionic exchange) in one method. This makes it possible to measure multiple attributes of a target analyte in one method instead of two separate methods. Although 2D-LC research has been going on for more than three decades, the speed of innovation and commercialization has accelerated in the last ten years due to efforts at both universities and instrument companies.

- Second, Mass Spectrometry (MS) has become an indispensable tool in bioanalysis [4]. The ability of new MS instruments to more accurately characterize large molecules keeps improving. However, several challenges still remain. MS works best with volatile buffers of limited concentration. The ionization suppression effect still occurs when compounds with very different concentrations (e.g. several orders of magnitude) co-elute in chromatographic separations. These challenges to MS make LC separation a critical part of any bioanalysis workflow.
- Finally, in the application area, biopharmaceutical research has become the fastest growing area in the pharmaceutical industry. In particular, monoclonal antibodies (mAbs) are currently the most important class of biotherapeutic molecules. As of 2016, seven of the top-ten-selling drugs were biologics, and six of these were mAb related [5]. In particular, the number one drug Humira (adalimumab) had an annual sales of \$15.7 Billion dollars in 2016. Due to the large size of these antibodies at about 150 kDa molecular weight, analyzing them is very challenging. It often takes a suite of analytical tools to fully characterize the molecule and ensure good quality control of drug products involving these molecules.

These three trends are developing at the same time and at high speed. It is our opinion that the combination of 2D-LC with MS is emerging as an exciting and essential tool for efficient, high quality biopharmaceutical analysis. In this article, we will discuss examples that demonstrate the power of 2D-LC-MS in this application area.

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Instrument Development

2D-LC instrumentation

There are several ways to implement 2D-LC, in the order of decreasing complexity.

1. Online Comprehensive 2D-LC (LCxLC)
2. Online Heart-Cutting or Multiple Heart-Cutting 2D-LC
3. Offline 2D-LC

Comprehensive 2D-LC is predominantly used to analyze unknown, complex mixtures as in omic-type applications (e.g. proteomics and metabolomics), natural products, and the analysis of biomolecules. Every peak eluting from the first dimension (1D) column is sampled and transferred to the second dimension for further separation as illustrated in **Figure 1**. Dual loop interfaces are used most commonly for this purpose [6]. Sampling occurs frequently to avoid remixing of components that had been successfully separated by the 1D column. These conditions entail a 2D-LC operation with fast gradients, short columns, and high flow rates in the second dimension, and reduced flows in the first dimension, because the second dimension (2D) cycle time (analyzing red loop in **Figure 1**) is about equivalent to that of sampling (blue, 1D path).

Heart-Cutting or Multiple Heart-Cutting (MHC) 2D-LC is different from comprehensive chromatography in that it aims to determine only one, or a few target compounds, characteristically in samples of high complexity

[7]. **Figure 2** illustrates the setup of a MHC interface for 2D-LC, where only distinct fractions of the 1D peaks are sampled for further separation in the second dimension. The MHC interface has two valves attached to the modulator, each having a cluster of six sampling loops. These “parking decks” allow fractions to be stored while 2D cycles are running. This breaks the link between sampling time and 2D cycle time so that both dimensions can operate under optimal conditions. The use of longer 2D gradients and columns with higher separation efficiency often leads to improved chromatography compared to comprehensive mode, and it is not necessary to reduce the 1D flow rate to very low levels.

Offline 2D-LC is the least complex form of 2D-LC from an instrumentation perspective. In this case, sample fractions from the first dimension are collected, stored in a physically separate device or container, and the 2D separations of all fractions are run later and sometimes much later. Higher peak capacity can be obtained than comprehensive 2D-LC because one can devote more time to each second dimension separation. However, offline 2D-LC is much slower [8] and may suffer from poor recovery and compound degradation because the first dimension fractions can be stored in the 1D mobile phase for an extended period of time, especially when buffers with extreme pH are used.

In this paper, the focus is on online comprehensive or multiple heart-cutting implementations of 2D-LC. These two implementations provide researchers with

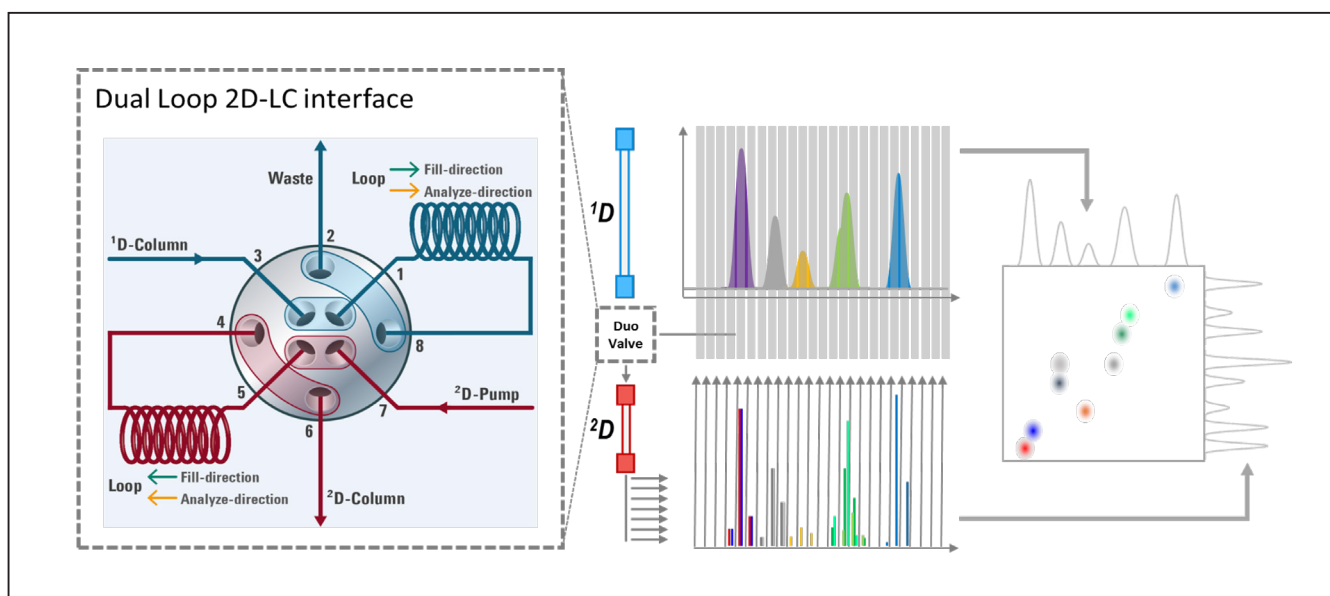


Figure 1. Comprehensive 2D-LC.

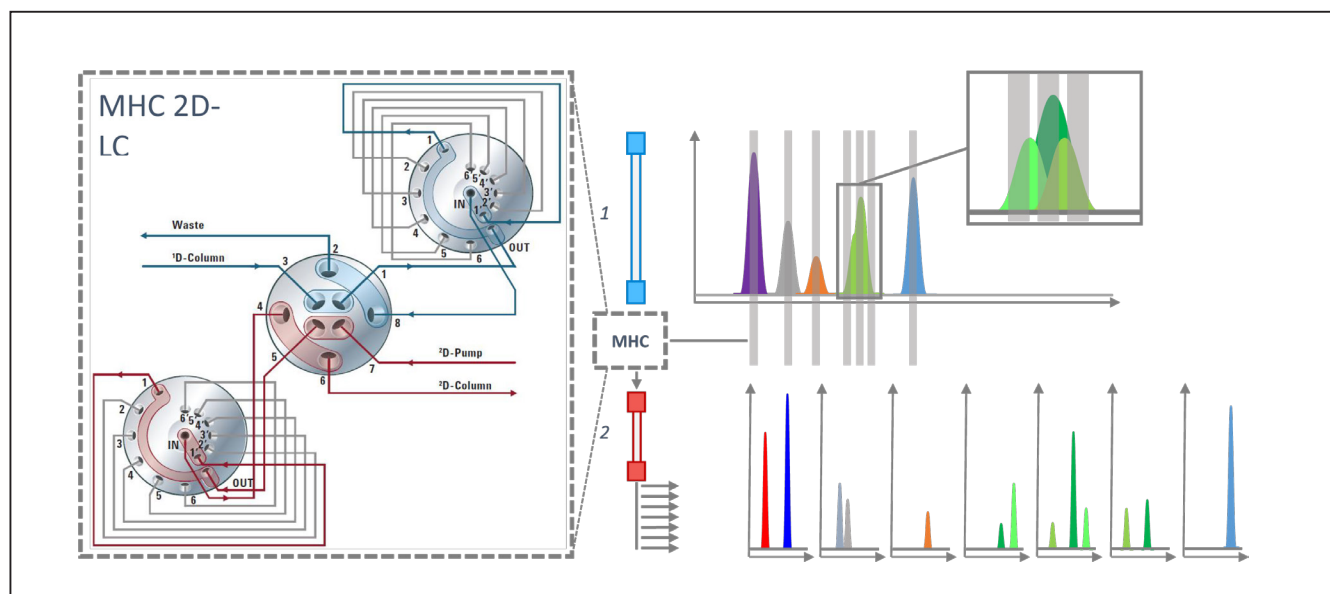


Figure 2. Multiple Heart Cutting (MHC) 2D-LC.

powerful, yet flexible analytical tools that can be highly automated, and are available as highly robust commercial instruments.

2D-LC-MS

Mass spectrometry with high resolution and accurate mass capabilities (such as Q-TOF) has become an essential analytical tool for identification and characterization of biological entities. However a low concentration of volatile buffers must be used with all MS. Many separation modes for proteins including mAbs are carried out with highly concentrated buffers in order to preserve the native state of the biomolecules and obtain therapeutically relevant information, or to improve peak shape in reversed-phase chromatography. This prevents direct coupling of these separation modes to MS.

Table 1 summarizes the most commonly used LC modes, the corresponding mobile phase buffers used and compatibility with MS detection [9]. Reversed-phase (RPLC) and hydrophilic interaction (HILIC) liquid chromatography are most suitable for coupling to MS detection due to their compatibility with low concentration, volatile buffers. Other separation modes typically use non-volatile salts, often at high concentration, and cannot be directly coupled with MS detection. These include Size Exclusion Chromatography (SEC), Ionic Exchange Chromatography (IEX), Affinity Chromatography and Hydrophobic Interaction Chromatography (HIC). Recently there have been a number of developments in column technology for biopharmaceutical separations, however a detailed discussion is beyond the scope of this short piece. Interested readers are referred to Ref. [10] that addresses some

Table 1. Different LC separation modes commonly used in protein analysis.

Mode of separation	Suitable analytes	Common buffer	MS compatibility
Reversed Phase Liquid Chromatography (RPLC)	Proteins, peptides, amino acids	Low concentration volatile buffer	Excellent
Hydrophilic Interaction Liquid Chromatography (HILIC)	Peptides, glycans	Low concentration volatile buffer	Excellent
Size Exclusion Chromatography (SEC)	Proteins, peptides	Concentrated buffer	Poor
Ion Exchange (IEX)	Proteins, peptides	Concentrated buffer	Poor
Affinity Chromatography	Proteins	Concentrated buffer	Poor
Hydrophobic Interaction Chromatography (HIC)	Proteins	Very concentrated buffer	Incompatible

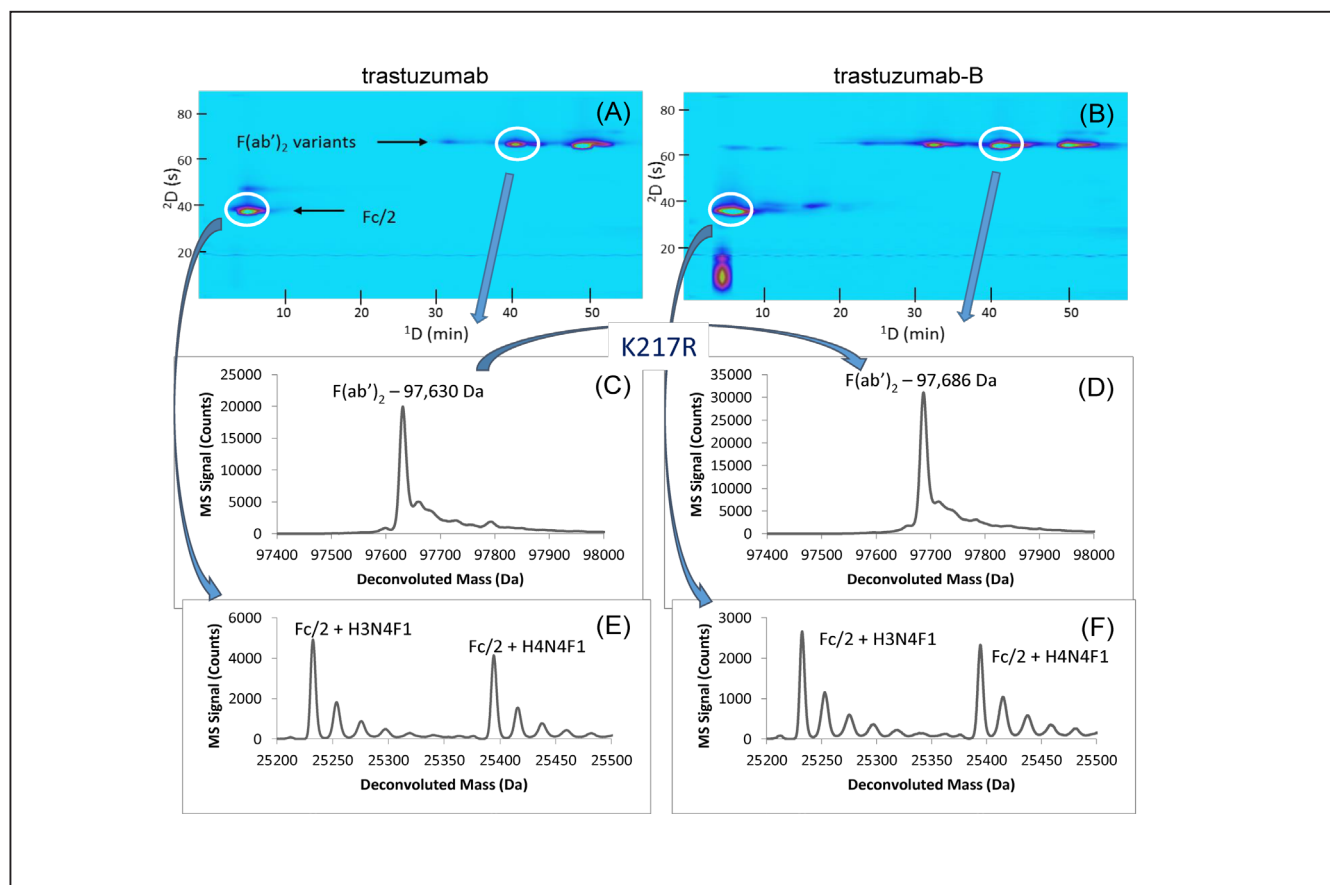


Figure 3. LC×LC-MS separations of IdeS digested trastuzumab (A) and biosimilar candidate trastuzumab-B (B), with IEX and RPLC separations in the first and second dimensions, respectively. Adapted with permission from the work of Ref. [10].

of these advances.

As a result, it is very powerful to use MS incompatible modes (e.g., SEC, IEX, Affinity, HIC) in the first dimension of a 2D-LC separation and use MS compatible modes (i.e., RPLC or HILIC) in the second dimension. The buffer from the 1D dimension fraction elutes in the dead volume of the 2D separation and can be diverted to waste before the 2D dimension effluent enters the MS. In such combinations, intact or reduced proteins can be analyzed by 2D-LC-MS, which would be otherwise impossible in traditional one-dimensional LC. In addition, the resolving power of 2D-LC provides more information on the analytes. In our view, these are the most convincing reasons for implementing 2D-LC-MS for biopharmaceutical analysis.

Application Examples

Protein level analysis

Traditionally the suite of chromatography-based methods for analyzing mAbs has included: 1) SEC methods

to characterize aggregation and fragmentation of the protein; 2) IEX methods to characterize charge heterogeneity; 3) Affinity methods to selectively determine protein concentration (i.e., titer); 4) HIC methods to determine the extent of protein oxidation, or to determine drug-to-antibody ratios (i.e., DAR) in the specific case of Antibody-Drug-Conjugates (ADCs); and 5) RPLC methods for mAb subunit separation. Two-dimensional separations enable coupling of different combinations of these separations, typically with one or more of three goals in mind. First, as was discussed above, use of a RPLC separation in the second dimension enables characterization of peaks eluting from separation modes that would otherwise be considered incompatible with MS detection. Second, two of these methods may be combined in a single 2D-LC analysis to save time and improve overall data quality. Third, a well-developed 2D-LC separation may yield more analytically useful information than two different separation modes operated independently. Several different combinations of separation modes have been applied to separations of therapeutic proteins and

related materials in creative ways; these have been discussed in detail elsewhere [3]. Here, we briefly discuss a few examples that are representative of recent work in this area.

Exemplary results from an application that achieves all three of these goals [11] are shown in **Figure 3**. In this case, trastuzumab and a candidate biosimilar mAb (i.e., trastuzumab-B) were compared using 2D-LC-MS analyses involving 1D and 2D separations based on IEX and RPLC, respectively. In this work the mAb samples were treated with the IdeS enzyme yielding Fc/2 and F(ab')₂ subunits of the protein. In this case the cation-exchange separation used in the first dimension both separates the subunit types, and separates any charge variants of a particular subunit. Fractions eluting from this 1D separation are then desalted and again separated by subunit. Although the IEX does a pretty good job of separating the subunits first, there are regions in these 1D separations where late eluting Fc/2 subunits overlap with early eluting F(ab')₂ subunits, thus the additional RPLC separation is useful for resolving them. The MS detection at the end of the RPLC separation quickly reveals a one amino acid difference between these mAbs that is located in the F(ab')₂ region, as shown by the mass spectra in panels C and D. On the other hand, the mass spectra in panels E and F show that the Fc/2 regions of these molecules are very similar. This example shows how 2D-LC-MS can be used to efficiently discern both differences and similarities between closely related mAbs.

The results of a recent study in 2017 by Williams, Bronson, et al. demonstrate how affinity and SEC modes of LC separation combined in a single 2D-LC analysis were used to efficiently obtain information about mAb concentration and aggregation level in crude in-process samples pulled from a mAb bioreactor [12]. In this case the 1D separation utilized a Protein A-based media. A multiple heart-cutting approach was then used to capture and re-inject up to twelve fractions from the Protein A separation into the 2D SEC column. UV absorbance detection was used following separation by the SEC column, and the authors demonstrated the ability to construct a calibration curve for antibody quantitation based on the 2D-LC data. In this way the single 2D-LC method yielded information about both the total antibody concentration in the bioreactor samples, and the extent of antibody aggregation. Finally, the authors suggest that such a method could be very useful as a Process Analytical Technology (PAT) tool to provide near real-time feedback on protein quantity and quality for in-process control of bioreactor conditions.

Finally, several recent studies have highlighted the utility

of 2D-LC separations for separating drug formulations and related materials containing different combinations of surfactants, intact protein, and small molecule drugs. For example, Li, Zhang, et al. have used IEX and mixed-mode IEX/RP separations to first separate polysorbate surfactant from mAb protein, followed by 2D RPLC separation coupled with MS detection for deep characterization of the surfactant material [13]. In another study, researchers have used a first separation to resolve protein and unconjugated small molecule drug in an ADC material, followed by quantitation of the free drug in a 2D RPLC separation [14]. These are examples of the utility of 2D-LC for addressing very complex separation challenges in the biopharmaceutical research area.

Peptide level analysis

Even before the dramatic growth of biopharmaceutical research, 2D-LC was used extensively for separations of peptides in proteomics research [15]. Now, this prior knowledge in the literature is being applied and extended as researchers use 2D-LC to detect and understand small differences between therapeutic proteins. For example, Vanhoenacker, Sandra, et al. have used 2D-LC to rapidly determine which amino acid residues of trastuzumab were deamidated as a result of pH stress of the protein molecule [16]. As part of this work, 2D-LC separations involving RPLC separations in both dimensions were shown to be superior to those involving 1D IEX separations. More recently, the same group demonstrated the use of 2D-LC to understand conjugation patterns in ADCs where small molecule drugs are linked to lysine residues in the antibody protein [17]. As part of an effort to help users understand the conditions under which 2D-LC provides more resolving power than 1D-LC separations for peptide analyses, the Heinisch group recently demonstrated that the peak capacity of 2D-LC separations is superior to that of 1D-LC separations for analysis times longer than about five minutes [18]. In the area of Host Cell Protein (HCP) analysis, Donenau and coworkers have demonstrated the use of heartcutting 2D-LC [19,20] and offline comprehensive 2D-LC [21] to detect trace-level peptide markers of HCPs. This is very important to quality assurance for biotherapeutic mAbs. We believe that 2D-LC will be important for the future of therapeutic protein analysis at the peptide level.

Summary

Analysis of biopharmaceuticals is very complex. It requires a suite of analytical methods and state-of-the-art instruments with maximal resolving power. 2D-LC provides high chromatographic selectivity and resolving

power by combining complementary separation modes and enabling direct coupling to mass spectrometers. As shown in the application examples, many different combinations are possible in 2D-LC, and it is possible to combine two traditional 1D-LC separations into a single 2D-LC method that yields more than twice the analytical information gained from a single 1D-LC analysis. This is very attractive for biopharmaceutical analysis.

2D-LC-MS has already been evaluated and implemented in many R&D groups of biopharmaceutical companies [4,12–14,16,22]. Future development of 2D-LC-MS will further simplify the instrumentation, method development and data analysis. This will enable implementation of the technique in the QA/QC environment. In fact, there have already been such reports of this transition, which is expected to continue and accelerate [22]. Finally, it is likely that 2D-LC-UV will be used in both R&D and QA/QC groups when analytes have been thoroughly characterized and UV detection is sufficient for quality control purposes. We look forward to continued implementation of 2D-LC in these areas.

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