WHITE PAPER

Use of 2D-Liquid Chromatography in GMP Regulated Laboratories

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Introduction

This white paper demonstrates how Two-Dimensional Liquid Chromatography (2D-LC) is used in a GMP regulated laboratory for either routine quantitative analysis or confirming results of a one-dimensional measurement. 2D-LC is applicable to both small molecule and large molecule analyses in the pharmaceutical and biopharmaceutical industries respectively. Used for pharmaceutical analysis since the 1970s, LC uses a single column to achieve analyte separation (1D-LC) with the option for gradient elution for more complex separation challenges.

However, there are situations where 1D-LC is inadequate due to complex separations such as co-eluting impurities, degradation products and/or trace cross contamination analysis. These are difficult to separate or require excessive run times; similar situations are the separations of chiral and other isomeric compounds. Where a satisfactory analysis cannot be achieved in ^{1}D, the combination of orthogonal separation principles can be applied such that results from 2D-LC separation is superior. Another application area where 2D-LC is superior is complex separations such as peptide mapping, as the technique provides a significant increase in peak capacity. This is where 2D-LC comes into its own, with the ability to provide additional resolution using a second, often orthogonal, separation column. The principle is that the effluent from the first dimension column (^{1}D) is transferred to a second dimension (^{2}D) using a column with a different chemistry and often different mobile phases to enhance separation and selectivity (i.e., a Size Exclusion Chromatography [SEC] for ^{1}D and a reverse phase separation for ^{2}D).

While 2D-LC has been described and the principles published since the 1990s, it is only since the availability of commercial 2D-LC chromatographic systems with good robustness that has allowed it to be considered for quantitative analysis in regulated laboratories. However, this also requires the availability of Chromatography Data System (CDS) software for method development and analysis with controls for ensuring compliance with applicable regulations and ensuring data integrity.

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There are two modes of 2D-LC:

1. **Comprehensive:** Where all the material eluting from the first column is subjected to further separation on the second column to gain a very detailed understanding of the components in the sample. Comprehensive 2D-LC is about peak capacity, Stoll et al have achieved a separation of 10,000 peaks in 4 hours [1].
2. **Heart-cutting:** Where one or more fractions of the effluent from the first column is transferred to a second column for further separation. Heart-cutting with long and shallow gradients allows a much more detailed analysis compared to comprehensive 2D-LC.

We will only consider heart-cutting here, as it is the more common technique in pharmaceutical analysis. An example is shown in **FIGURE 1** that is being used to determine peak purity and selectivity of a separation; we will look at examples of this with the Case Studies.

The principles of 2D-LC will not be discussed here; if you want more information, there is an Agilent Primer by Carr and Stoll [2] as well as published articles in Recommended Reading at the end of this white paper.

Chromatography Data System Control

It is important that any analytical procedure used for quality control analysis of raw materials and finished pharmaceutical products is robust and reliable. In part, this is due to the procedure and how well it has been developed, but also analytical instrumentation and application software that ensure highly precise and reproducible measurement for consistent operation. As the system will be installed in Analytical Development and Quality Control laboratories operating under GMP, the CDS needs to have the technical controls for regulatory compliance and ensuring data integrity. Agilent fully-automated 2D-LC instruments using 2D-LC Software for OpenLab CDS meet instrument control requirements. OpenLab CDS has technical controls for restricting access to authorized individuals, audit trails for ensuring integrity of data, and the ability for a reviewer to document that they have reviewed a run's audit trail entries and electronically sign reports.

The CDS provides the ability to seamlessly control all components (pumps, column heaters, injectors and detectors) of the chromatographic system for both dimensions plus the automated transfer of the peak cuts from ^{1}D to ^{2}D and capturing and managing the data from two detectors. For single heart-cutting and comprehensive, this is relatively straightforward. However, when handling multiple cuts from a single ^{1}D peak, sophisticated automation is required for controlling multiple valves, switching between the two dimensions, as well as the gradient that is formed by the second dimension pump, which requires the combination of data from several cuts for quantitative results. Both dimensions require sophisticated calculations to combine the ^{2}D data from the multiple cuts and connect this information to the ^{1}D peak, as we will see later.

In addition, there are instrument qualification protocols available from Agilent to demonstrate that the 2D-LC chromatograph has been installed correctly and works according to laboratory user requirements as required by USP <1058> [3]. On the day of analysis, system suitability tests [SST] evaluate the performance of both the analytical instrument and the analytical method for two-dimensional measurements. A new feature of the system in the most recent release (2D-LC Software 1.1 for OpenLab CDS) will provide purity calculation for 2D peaks.

Pharmaceutical Analysis

To ensure product safety and quality, analysis is performed on raw materials and active pharmaceutical ingredients (APIs for small molecules), in-process and on the finished product. These tests fall into the following categories:

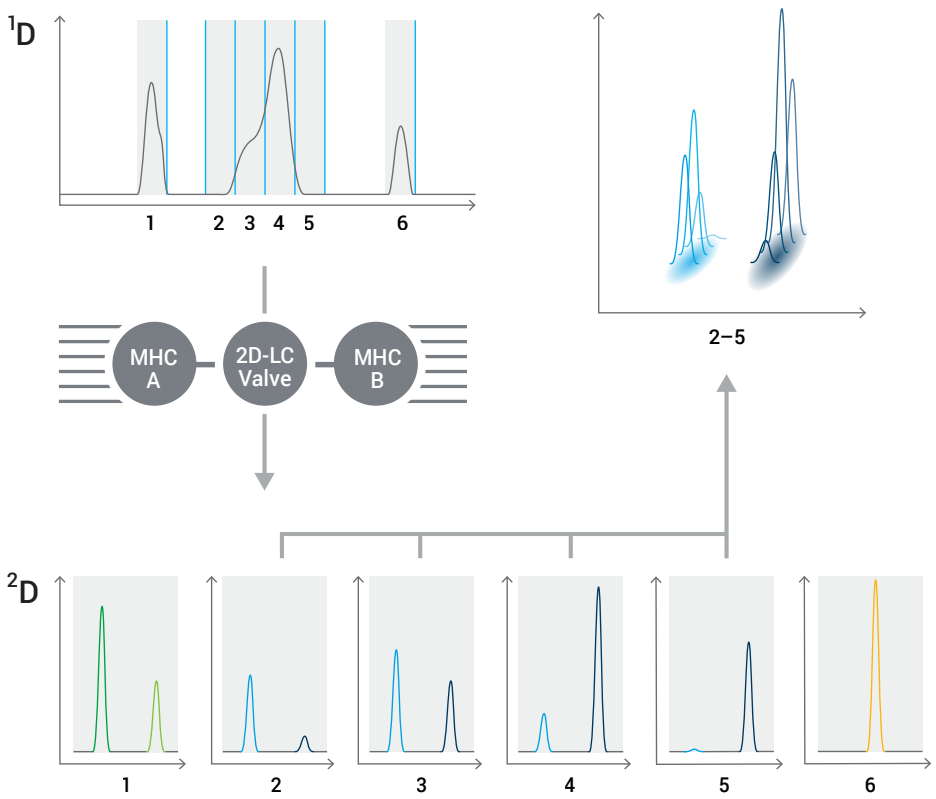
- Identity testing (typically on goods from trusted suppliers when they enter the warehouse)
- Purity of the API
- In process testing
- Label claim of the finished product
- Stability indicating assays assessing impurities over the shelf life of the product
- Degradation studies

Validation of analytical methods is guided by International Council on Harmonisation (ICH) guideline Q2(R1) [4]. This is currently under revision along with writing a new guidance ICH Q14 on Analytical Procedure Lifecycle. The first drafts were issued for public comment at the end of March 2022 [5, 6]. Currently the best integrated guidance for developing and validating an analytical procedure is USP <1220> on Analytical Procedure Lifecycle, effective from May 2022 [7] as method development is not mentioned in the current ICH Q2(R1) [4].

To ensure accuracy and reproducibility (called Total Error or Target Measurement Uncertainty in USP <1220>), it is critical that quantitative methods for purity and stability have the required selectivity. It is also important to understand the influence of various factors, such as light or heat, on the stability of the API requiring degradation studies to be performed. As chromatography is a comparative and not absolute technique, 2D-LC can help immensely as with combination of two stationary (columns) and mobile phases (solvents) for different selectivities, which reduce risk to product and enhances process understanding by increasing the likelihood that there are no unseen or co-eluting components in the chromatogram.

The peak of the active component can be heart-cut into several sections as it elutes from ^{1}D (e.g., peak start, apex, and peak end) to see if there are any co-eluting peaks underneath the active moiety as shown in **Figure 1.**

Figure 1: Illustration of 2D-LC With Multiple Heart-cutting (See Case Study 1 for Context)



2D-LC can also be very useful in meeting ICH Q9 [8] risk management requirements for gathering product knowledge such as critical attributes including purity of raw materials and APIs during development, and helps to set specifications for and reduce variability of investigational medicinal products [IMP]. In QC analysis of product batches and stability, the technique can be used directly in testing as well as investigation of Out Of Specification [OOS] results.

Understanding the c in cGMP

Pharmaceutical analysis is regulated by the current Good Manufacturing Practice [GMP] guidance issued by authorities such as the FDA, European Medicines Agency [EMA], etc. One GMP requirement is the necessity to keep current with advances in science and technology [9], as noted on the FDA web site:

> *Accordingly, the "C" in CGMP stands for "current," requiring companies to use technologies and systems that are up-to-date in order to comply with the regulations.* ***Systems and equipment that may have been "top-of-the-line" to prevent contamination, mix-ups, and errors 10 or 20 years ago may be less than adequate by today's standards [10].***

The requirement to be current is also found in Article 23, §1 of European Directive 2001/83/EC that establishes GMP in the European Union:

> *The **marketing authorisation holder** shall, in respect of the methods of manufacture and control provided for in Article 8(3)(d) and (h), **take account of scientific and technical progress and introduce any changes that may be required to enable the medicinal product to be manufactured and checked by means of generally accepted scientific methods [11].***

Using 2D-LC is one way of keeping current and ensuring the quality of pharmaceutical products as we shall see in Case Study 3.

FDA Citations for Impurity Analysis Failures

FDA Warning Letters and 483 observations are good indicators of the FDA's views on key regulatory topics. Impurity and/or degradation analysis is one area of regulatory concern. Three FDA Warning Letters have been selected to illustrate problems that can occur with 1D-LC in terms of identity testing, poor peak integration, and inadequate OOS investigation and show how 2D-LC can prevent regulatory citations.

Citation 1: In August 2021, a company was given a Warning Letter for lack of HPLC method selectivity for impurity analysis:

> ***You have not provided an update whether the <redacted> assay/potency method used for testing Nalbuphine HCL is stability indicating and can detect impurities and that no degradants co-elute with active pharmaceutical ingredient or excipients.*** *Further, your <redacted> testing method is inadequate to be used for identification testing as the method does not have good discriminative ability.*
>
> ***Identification solely by a single chromatographic retention time,*** *for example, is not regarded as being specific. However, **the use of two chromatographic procedures, where the separation is based on different principles,** or combination of tests into a single procedure, such as high-performance liquid chromatography (HPLC)/UV diode array, HPLC/Mass Spectrometry (MS), or gas chromatography (GC)/MS, **is generally acceptable [12]**.*

This citation provides an excellent justification for 2D-LC. A single LC retention time is insufficient to identify an analyte and to assure that it is free from co-eluting impurities and degradants. Only the use of two chromatographic procedures based on different separation principles provides such selectivity.

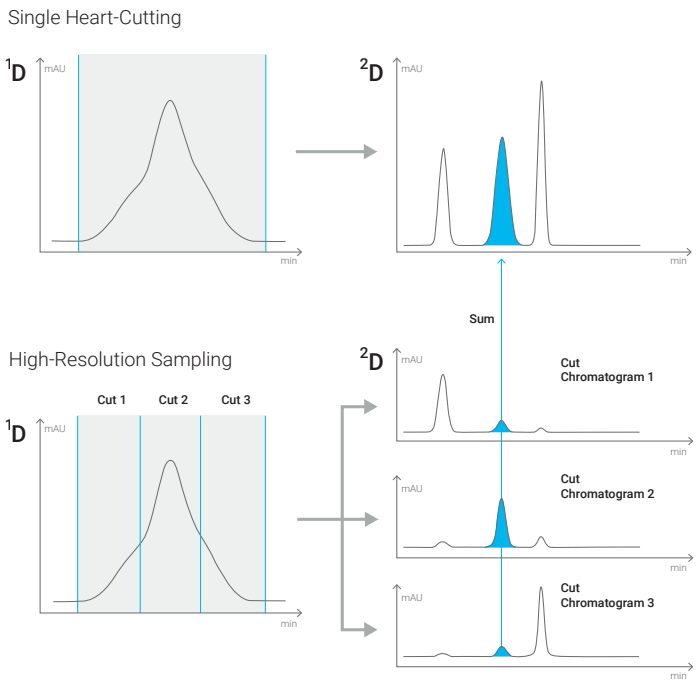
Citation 2: It is important to understand that inadequate peak resolution can generate OOS results that will trigger a laboratory investigation [13]. Another company was cited in an April 2020 FDA Warning Letter for an unscientific OOS investigation of co-eluting peaks.

> *Your firm's investigation of OOS results was closed without adequate scientific justification. For example, OOS results were obtained during testing of four <redacted>, United States Pharmacopeia (USP) samples starting on October 24, 2017.* ***Your investigation determined there was an unknown peak co-eluting with the <redacted> peak. However, this determination was not scientifically justified:*** *the sample solution determined to have a co-eluting peak was approximately 15 days old when it was tested. You lack data on solution stability to show that the co-eluting peak was not caused by the age of the sample solution [14].*

OOS investigations need to be conducted in a timely manner and waiting 15 days for reinjection is simply inadequate. One science-based approach for hypothesis testing to determine whether or not a coeluting peak caused the OOS result would be to re-analyze a peak in the second dimension (i.e., a different stationary and mobile phase) using heart-cutting 2D-LC. This approach could allow re-injection of samples with little or no delay of an investigation to help determine the root cause of the OOS result or determine that the OOS result is correct and there may be a production issue.

FIGURE 2 illustrates two options for 2D-LC to help investigate an OOS and establish hypotheses for testing. Option 1 is a single heart cut onto the 2D column to see if there is more than one compound. This is the most sensitive approach. The second option is to use high-resolution sampling and run each one individually and use the CDS to collate the data from the three runs shown in **FIGURE 2.**

Figure 2: 2D-LC Options for Resolving Poorly Resolved 1D-LC Peaks



Citation 3: Problems with co-eluting peaks should be identified and resolved rapidly to prevent regulatory problems. Our last example demonstrated how not to do this. A February 2020 warning letter cited a company for failing to integrate co-eluting peaks correctly, hiding OOS results going back to 2016.

> *Your firm* ***failed to properly integrate co-eluting peaks during impurity testing*** *of phentermine HCL capsules, which resulted in your analysis* ***failing to detect out-of-specification (OOS) results*** *for at least one lot of drug product. (21 CFR 211.165(e)).*
>
> ***You self-identified this problem in 2016.*** *In a deviation report, you wrote that* ***analysts were using "<redacted> methods" and the "reported impurity levels may not reflect the true concentrations found in the drug." Your firm conducted training on May 24, 2016,*** *reportedly to teach analysts to properly integrate and measure closely co-eluting peaks during impurity analysis of your drugs.* ***Despite this training, in December 2016 your analyst performed <redacted> integration to calculate the area of the peaks for a stability impurity test for Lot 12456A of phentermine HCL capsules. If the appropriate <redacted> integration had been performed, the test result for the drug product lot would have exceeded the impurity specification limits. This lot remained on the market until it failed <redacted> stability impurity testing on June 20, 2017.*** *Your secondary quality review of the December assay also failed to detect the error.* ***Multiple examples of your firm's failure to properly integrate closely co-eluting peaks were observed during our inspection [15].***

Reliable and automated peak integration requires adequate separation power of the chromatographic method [16]. Therefore, the failure of the method to resolve the co-elution of the impurity adequately is the main cause of poorly performed peak integration. Separation of co-eluting components is dependent on the amount of work performed during method development and here 2D-LC can provide the extra separation by a heart cut of the co-eluting compounds to a second column to adequately resolve and quantify the two compounds.

Pharmaceutical Case Studies

Let's look at 2D-LC in practice in GMP regulated laboratories. We will present three case studies using 2D-LC to show the advantages of the technique in pharmaceutical analysis. The applicable FDA regulation to remember is 21 CFR 211.194(a)(2):

> *The suitability of all testing methods used shall be verified under actual conditions of use [17].*

This applies equally to in-house developed as well as pharmacopoeial methods. We will look at the use of 2D-LC in method development as well as QC testing.

1: Optimizing 2D-LC Method Development for QC Methods

All analytical procedures must be devised, developed and optimized so that any validation experiments are merely confirmation of good method development. The better the method development, the easier it is to validate and transfer to QC laboratories supporting production.

The current ICH Q2(R1) does not have any mention of method development and is only focused on validation of the procedure [4]. However, the recently effective USP <1220> on Analytical Procedure Lifecyle [7] places much importance on the design of the procedure and its development from the definition of the Analytical Target Profile (ATP) to identifying and controlling critical method parameters. The use of software for the Design of Experiments [DoE] is crucial to define a design space for validation, and typically this is performed using an additional application.

Although many laboratories consider method development outside of GMP, the current moves with USP <1220> and ICH Q14 show that the regulatory landscape is changing [6, 7]. If a design space is not defined, then all changes post release must be validated. In contrast, if a design space has been defined adequately in development and validated, any changes within the design space made during routine use are simply documented and applied as the design space is validated.

One laboratory's use of 2D-LC during method development and troubleshooting workflow has been described [18]. 2D-LC provides the means to ensure selectivity of an LC method especially for degradation studies to ensure confidence in the results generated. This is achieved by using a heart cut to determine if a peak representing a particular compound is pure and there are no co-eluting peaks. A selected peak is cut from ^{1}D and run under different conditions with ^{2}D. To provide further confidence that only the peak of interest is present, several successive heart cuts can be made over the time that the peak of interest elutes from the first column. If only one peak is seen in each of the cuts, the peak is pure as shown in **FIGURE 1**. An MS detector to monitor the effluent from the second system could provide additional assurance of purity.

If the 2D-LC method is quantitative, splitting impurity peaks into several cuts could cause problems close to the limit of quantification [LOQ] as sensitivity may be lost. However, a recent enhancement in OpenLab CDS includes a feature that is compliant with the regulations to collect and store the 1D eluent from several heart cuts and aggregate them into a single injection to improve sensitivity.

2: Validation of a 2D-LC method for QC testing

Yang et al. published the validation of a 2D-LC method for the separation and quantification of a candidate product from its regioisomer impurity for QC testing [19]. A single heart cut was used to transfer the peak eluent representing several analytes from the first to the second column. The best column for the separation of the product and its co-eluting isomer impurity from other impurities was a reverse phase C18

column. This resulted in a single peak of product and impurity. This was then heart-cut onto a 2D column for a baseline separation of the product and the isomer impurity.

Although the criteria for method validation (e.g., intermediate precision, accuracy, linearity, etc.) are documented in ICH Q2(R1) [4], the use of 2D-LC required additional assessment. Using separate Design of Experiments [DoE] software, Critical Method Attributes [CMA] were identified and tested to define the operating ranges to ensure a robust analytical method suitable for reliable QC testing. Results from these experiments defined the design space with operating ranges for each CMA so that the method is sufficiently robust for routine QC analysis.

As there is a lack of systematic 2D-LC method validation in the literature, the focus was on the experimental design using an external DoE application for optimization of the second-dimension separation and measurement to ensure that it is robust enough for GMP analysis. Parameters that were investigated were:

- Heart-cut window: This was defined by retention time of ^{1}D separation and was set from method development and validation studies. This approach leads to better peak yield and reproducible results. Confirmation of the retention time window is part of the system suitability requirements before the start of the analytical run.
- Three parameters that were considered critical for further extensive experimentation were:
  1. Percentage of composition of the mobile phase at the start of the gradient in the 1D column.
  2. Volume of the switching loop holding the heart cut.
  3. Mobile phase composition, specifically the percentage of formic acid, of the heart-cut sample plug. This CMA was deemed critical in order to avoid chemical incompatibility between the mobile phases used in each dimension.

As the discussion of the CMA criteria and their selection is very important, readers are strongly encouraged to read the article and understand the logic for ensuring a robust method [19]. The approach described is consistent with the new USP general chapter <1220> on Analytical Procedure Lifecycle, effective from May 2022 [7].

3: Protein Aggregation Characterization

Size Exclusion Chromatography is widely used in biopharma to measure monoclonal antibody [mAb] aggregation. If the sample comes from a bioreactor, then isolation of the product from cell impurities and media is required first. Williams et al. published the use of 2D-LC for the characterization of mAb protein aggregation from bioreactor samples [20]; the authors are from the Emerging Technologies Team in the FDA's Office of Product Quality.

This work is part of the FDA's evaluation of new technologies as part of the current criteria in cGMP, discussed above. The aim is to assess how 2D-LC can be used as a component of Process Analytical Technology [PAT] in mAb production. Instead of traditional QC testing at the end of a process, PAT is used to change the manufacturing process to control product quality. Parametric release is described in EU GMP Annex 17 as real-time release testing consisting of in-process monitoring and controls that may provide, when authorized, substitute for end-product testing as part of the batch release decision [21].

Rapid and robust testing techniques are required to provide information to make real-time decisions to control cell culture within predefined limits of Critical Quality Attributes [CQA]. One such CQA is protein aggregation that occurs early after starting a cell culture process. Several factors can affect aggregation, therefore detecting and inhibiting this early in the culture is key to ensure reliable product quality. This publication reports an automated 2D-LC method that can quantify protein aggregation. It consists of a ^{1}D protein A affinity column responsible for separating the bioreactor media and impurities and then a heart cut of the mAb to a ^{2}D SEC column to quantify soluble protein aggregates [20].

Registered Analytical Procedures

The registration process for novel medicines requires that non-pharmacopoeial analytical procedures are described along with the validation data. Typically, the description of the procedures are not particularly detailed in order to avoid problems. For example, a specific make and packing of HPLC column is not given in case it is not available in the future; only details of column dimensions, type of packing, and particle size are given.

A 2D-LC procedure can be registered if it has been developed and validated for routine QC testing as outlined by Yang et al [19]. Documented modifications to the procedure within the validated design space can be made [7] without the need to revalidate and are traceable in the CDS to comply with 21 CFR 211.194(b) [17].

However, modification of an existing registered 1D-LC procedure to 2D-LC requires validation as well as cross-validation against the registered method. The 2D-LC performance should be as good, if not better, than the original. Dependent on the extent of the changes made to the method, it is highly likely that a Type 2 variation needs to be filed with regulatory authorities, although a Type 1B variation might be possible.

The General Notices section 6.30 of the USP discusses Alternative and Harmonized Methods and Procedures which include registered methods:

> *An alternative method or procedure is defined as any method or procedure other than the compendial method or procedure for the article in question. The alternative method or procedure must be fully validated (see Validation of Compendial Procedures {1225}) and must produce comparable results to the compendial method or procedure within allowable limits established on a case-by-case basis.*
>
> *Alternative methods or procedures can be developed for any one of a number of reasons not limited to simplification of sample preparation, enhanced precision and accuracy, improved (shortened) run time, or being better suited to automation than the compendial method or procedure.* ***Only those results obtained by the methods and procedures given in the compendia are conclusive [22].***

If a 1D-LC is the registered procedure, then any 2D-LC procedure developed should be demonstrated to be equivalent or better than the original as part of the validation effort. See USP <1010> and <1210> for statistical methodologies [23, 24].

When considering the warning letter citations earlier in this white paper, a 2D-LC method could be used as part of an OOS investigation towards supporting the isolation of an OOS result from a 1D-LC procedure in the same way that a statistical outlier test might be used. However, neither can be used to invalidate the OOS. If the root cause cannot be established then a replicated retest protocol, approved by QA, must be executed using the registered method on the same laboratory sample with predetermined acceptance criteria in accordance with FDA OOS Final Guidance 2006 [13].

An update to USP <621> on all types of chromatographic analysis (e.g. paper, thin layer, column, LC, GC etc.) becomes effective in December 2022 [25]. This defines the permitted changes to pharmacopoeial LC methods for columns (including translation based on column length and particle size to either fully porous or superficially porous particles), mobile phases, flow rate and gradients.

Summary/Conclusions

2D-LC is a reliable and robust technique to be used for routine Quality Control analysis, especially for the reproducible separation of close running peaks that are incompletely separated by a 1D-LC method. Control by a chromatography data system designed for operation in regulated laboratories ensures compliance with GMP as well as data integrity.

Recommended Reading

For further information on 2D-LC and applications, the following publications are recommended:

Overview and State of the Art

D. Stoll, P. Carr, Two-Dimensional Liquid Chromatography: A State of the Art Tutorial, Analytical Chemistry. 89 (2017) 519–531.

D. Stoll, Introduction to Two-Dimensional Liquid Chromatography – Theory and Practice, in: M. Holcapek, W.C. Byrdwell (Eds.), Handbook of Advanced Chromatography /Mass Spectrometry Techniques, Elsevier, London, 2017: pp. 227–286.

Review of 2D-LC for Analysis of Monoclonal Antibodies

D.R. Stoll, K. Zhang, G.O. Staples, A. Beck, Recent Advances in Two-Dimensional Liquid Chromatography for the Characterization of Monoclonal Antibodies and Other Therapeutic Proteins, in: Advances in Chromatography, 2018: pp. 29-63.

Review of Recent Developments

B. Pirok, D. Stoll, P. Schoenmakers, Recent Developments in Two-Dimensional Liquid Chromatography – Fundamental Improvements for Practical Applications. Analytical Chemistry 2019, 91 (1), 240–263.

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12. *FDA Warning Letter: Maitland Labs of Central Florida.* 2021, Food and Drug Administration: Silver Spring, MD.
13. *FDA Guidance for Industry Out of Specification Results*. 2006, Food and Drug Administration: Rockville, MD.
14. *FDA Warning Letter: International Trading Pharm Lab Inc*. 2020, Food and Drug Administration: Silver Spring, MD.
15. *FDA Warning Letter: KVK-Tech, Inc* 2020, Food and Drug Administration: Silver Spring, MD.
16. R.D.McDowall, *Where Can I Draw The Line?* LCGC Europe, 2015. 28(6): p. 336-342.
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18. R.Shadbolt. *Editorial Article: Optimizing 2D-LC method development for QC test methods.* 2021; Available from: https://www.selectscience.net/editorial-articles/optimizing-2d-lc-method-development-for-qc-test-methods/?artID=55889
19. S.H.Yang, J.W., K.Zhang, *Validation of a two-dimensional liquid chromatography method for quality control testing of pharmaceutical materials.* Journal of Chromatography A 2017. 1492: p. 89-97.
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23. *USP General Chapter <1010> Outlier Testing.* United States Pharmacopoeia Convention Inc.: Rockville, MD.
24. *USP General Chapter <1210> Statistical Tools for Procedure Validation.* United States Pharmacopoeia Convention Inc.: Rockville, MD.
25. *USP General Chapter <621> Chromatography.* United States Pharmacopoeia Commission Inc: Rockville, MD.

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