

A Comparison Between On- and Off-Line Two-Dimensional LC for Impurity Profiling of Pharmaceutical Compounds

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INTRODUCTION

The pharmaceutical industry requires that impurities at or above 0.05 or 0.1% concentration of the active pharmaceutical ingredient are identified, depending on the daily dose.¹ The challenge in impurity profiling is to resolve all product related compounds. The use of 2-D LC techniques is beneficial for reducing the risk of coelution of impurities with the pharmaceutically active compound.

Here we describe a comparison between on- and off-line 2-D LC for impurity profiling of pharmaceutical compounds.

PEAK CAPACITY IN TWO-DIMENSIONAL LIQUID CHROMATOGRAPHY (2-D LC)

The goal of two-dimensional chromatography is to resolve complex samples beyond what is possible with one-dimensional separations. Where multiple one-dimensional separations may only partly resolve the analytes in a sample, 2-D LC enables the user to greatly enhance the peak capacity. Ideally, the maximum peak capacity in 2-D LC is the product of the peak capacity of the first dimension (1D) and the second dimension (2D) separation:²

$$(n_{c,tot} = n_{c,1D} \times n_{c,2D})$$

Figure 1 illustrates the concept of the 2-D LC workflow. The first separation (green) is fractionated and reanalyzed using the second separation method (red).

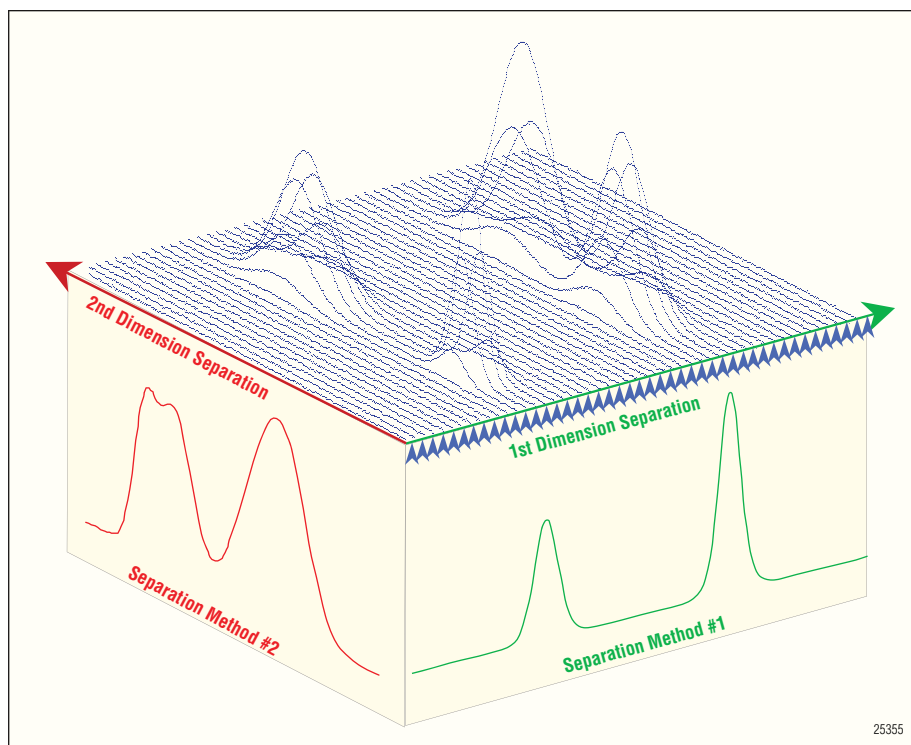


Figure 1. The surface plot (blue) illustrates the concept of 2-D chromatography: to enhance the peak capacity by coupling two separate methods with a different selectivity. The one dimensional chromatograms (red and green) are not able to resolve the sample independently.

THE EVALUATION OF CHROMATOGRAPHIC ORTHOGONALITY

It is useful to investigate the orthogonality of the retention mechanisms of the two dimensions prior to 2-dimensional analysis.

To achieve different selectivity and therefore accomplish a degree of orthogonality on the two C18 columns (with identical stationary phases) the separations were performed at two different pH levels (separated according to the pKa value of each analyte at pH 4.8 and pH 9.0).

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The chromatographic conditions are listed in Table 1, with the resulting chromatograms shown in Figure 2. Changes in retention time due to the application of different pH levels are apparent.

Table 1. Experimental Conditions for the Two Methods		
	1 st Dimension	2 nd Dimension
Column	C18, 120 Å, 150 × 1.0 mm I.D.	C18, 120 Å, 150 × 4.6 mm I.D.
Solvent A	10 mM NH ₄ OAc in H ₂ O + 0.05% CH ₃ COOH, pH 4.8	10 mM (NH ₄) ₂ CO ₃ in water, pH 9.0
Solvent B	CH ₃ CN + 0.05% CH ₃ COOH	CH ₃ CN
Gradient	5–100% B in 10 min	5–100% B in 20 min
Flow Rate	50 µL/min	1 mL/min
Oven	35 °C	35 °C
Injection Volume	4 µL of an 0.1 mg/mL test mixture	1 µL of the deposited fraction from the well plate

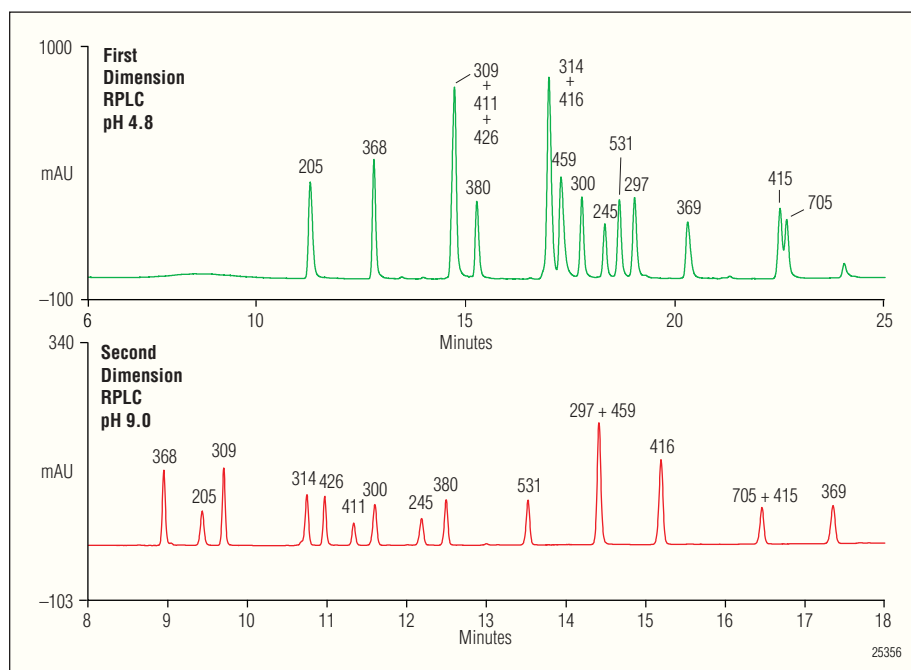


Figure 2. Two reversed-phase LC (RPLC) methods on identical C18 stationary phases yield different selectivity.

The visual and statistical evaluation of chromatographic orthogonality (or correlation) can be performed by means of a retention plot, which is created from the retention time tables of two one-dimensional chromatograms.

- A correlation coefficient close to a value of “1” means the two methods are correlated, or identical, and indicates 2-D LC with these methods is not useful.
- Full orthogonality is reached when the correlation coefficient of the two methods approaches a value of “0.” (This is rarely achieved in 2-D chromatography.)

The retention map for the selected methods is depicted in Figure 3. It gives an indication of the orthogonality of the two RPLC methods. The plot gives a visual impression as well of the retention behavior of the components in each method (e.g. Cinnarizine elutes third to last in method 1, and last in method 2).

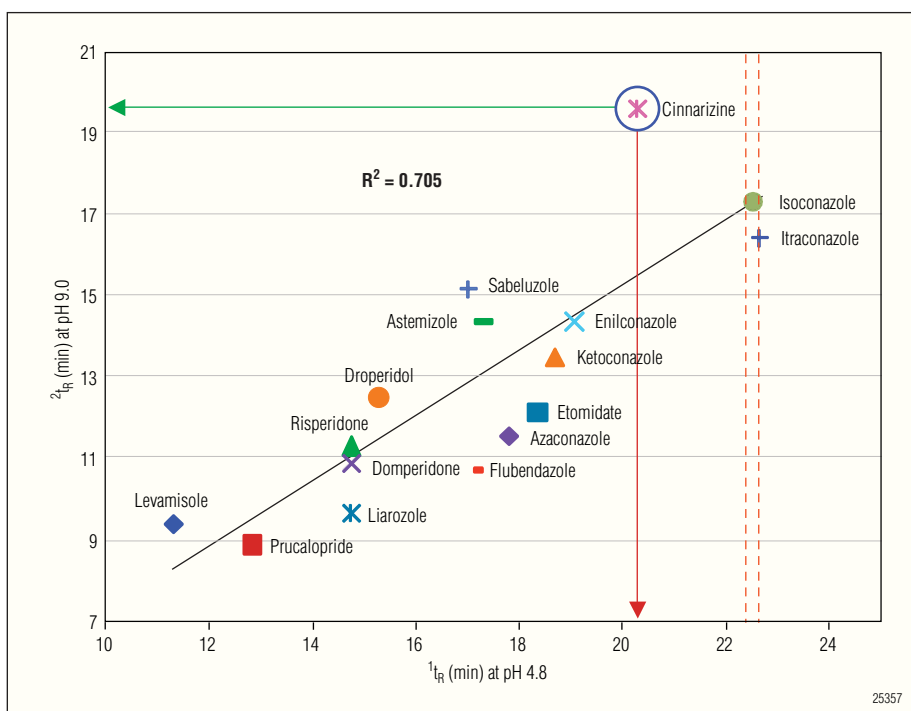


Figure 3. Retention plot for selected RPLC methods.

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AUTOMATED OFF-LINE 2-D LC SETUP

1. A sample is injected by the autosampler and separated on the 1st D column.
2. After detection the separated components are transferred back to the autosampler and fractionated into a well plate. The fractionation can be triggered on a time basis or by peak parameters.
3. After fractionation, Chromeleon software automatically generates the second-dimension injection table, the valves are switched, and the fractions are separated according to the 2nd D method.

The result of this workflow is a 2-D retention map (Figure 5).

The offline 2-D LC approach features an open system, allowing fraction manipulations (e.g. a derivatization/reaction step between the two dimensions, enrichment, multiple reanalysis of a fraction, etc.) at the cost of possible loss of volatile compounds and evaporation of the mobile phase, which can adversely effect quantitative analysis. Adsorption of hydrophobic analytes to the well plate walls should also be considered. When enrichment can be used, this approach is highly flexible with regards to column dimension selection and MS compatibility.

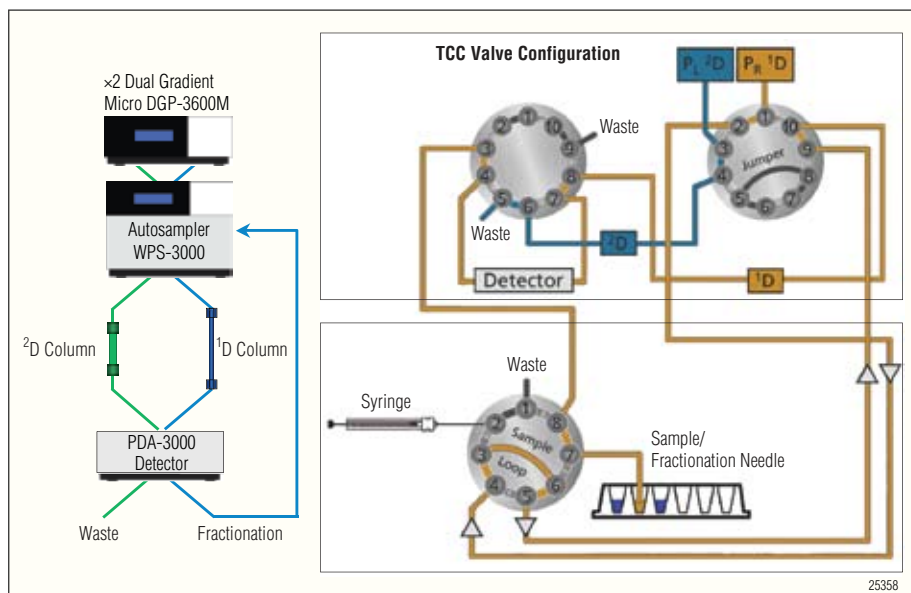


Figure 4. The workflow of automated off-line 2-D LC.

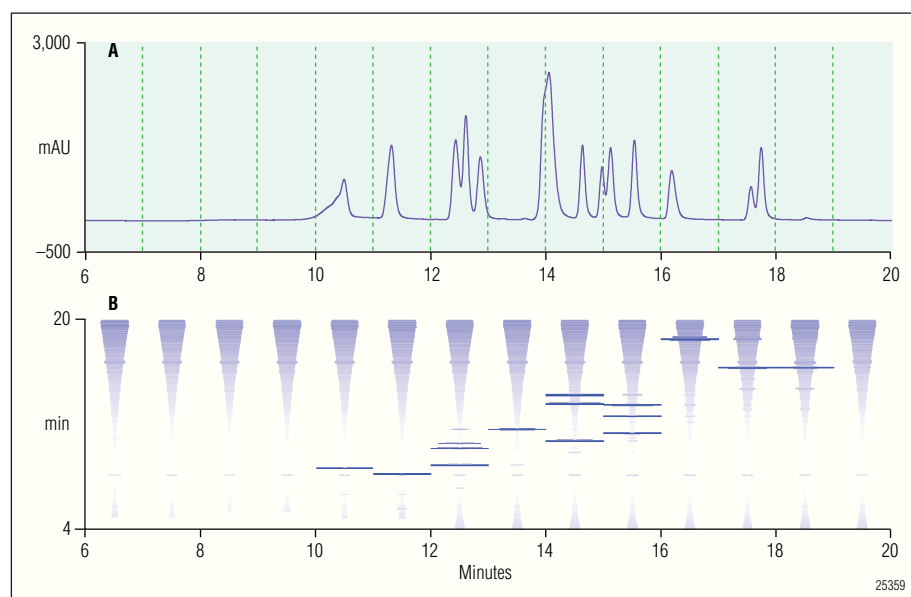


Figure 5. The upper chromatogram (A) is fractionated in the well plate sampler and reanalyzed. The retention plot (B) is a representation of each re-injected fraction.

ON-LINE 2-D LC SETUP FOR HEART-CUTTING

1. The sample is injected by the Autosampler and separated on the ¹D column.
2. After detection, the separated components of interest are transferred back to an open loop.
3. The valve is switched and the selected fraction is reanalyzed using the second-dimension method. The entire chromatogram was fractionated on a time basis and reanalyzed.

Several options exist to fractionate and reanalyze the ¹D separation.

- The user can decide which fractions to reanalyze, and can choose to take the fractions based on a time frame, or by applying peak triggers.
- A stopped-flow (¹D) separation can be an alternative to repeating the ¹D analysis again for each fraction.²
- It is also possible to perform fully comprehensive 2-D analysis, as required when all peaks require reanalysis.

1. The sample is injected by the Autosampler and separated on the ¹D column.
2. After detection, the separated components of interest are transferred back to an open loop.
3. The valve is switched and the selected fraction is reanalyzed using the second-dimension method. The entire chromatogram was fractionated on a time basis and reanalyzed.

The on-line heart cutting approach offers a simple closed system, avoiding sample loss or sample degradation, and allowing quantitative analysis. The components of interest can be selected by the operator and easily reanalyzed, and automation is simple using conventional hardware. In the on-line mode however, there is less flexibility in terms of column dimensions and LC conditions. In particular, fraction loop volume must be chosen carefully.

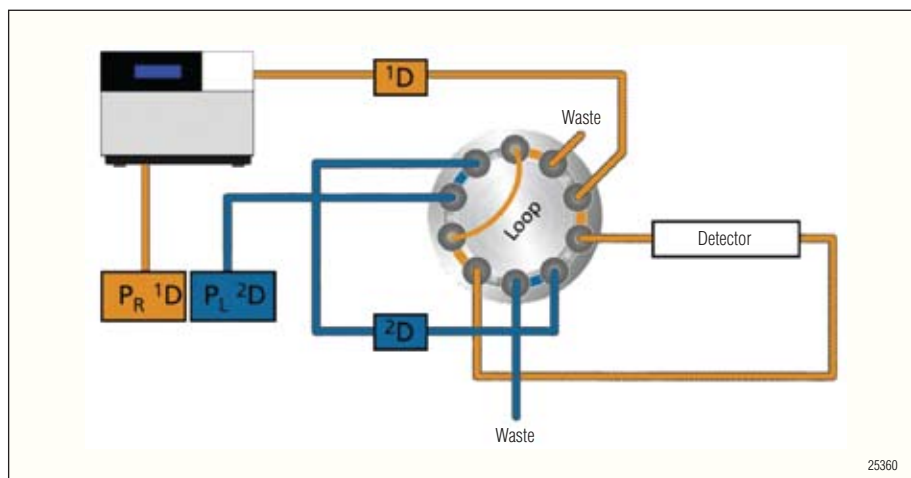


Figure 6. This configuration illustrates a straightforward approach to online 2-D LC.

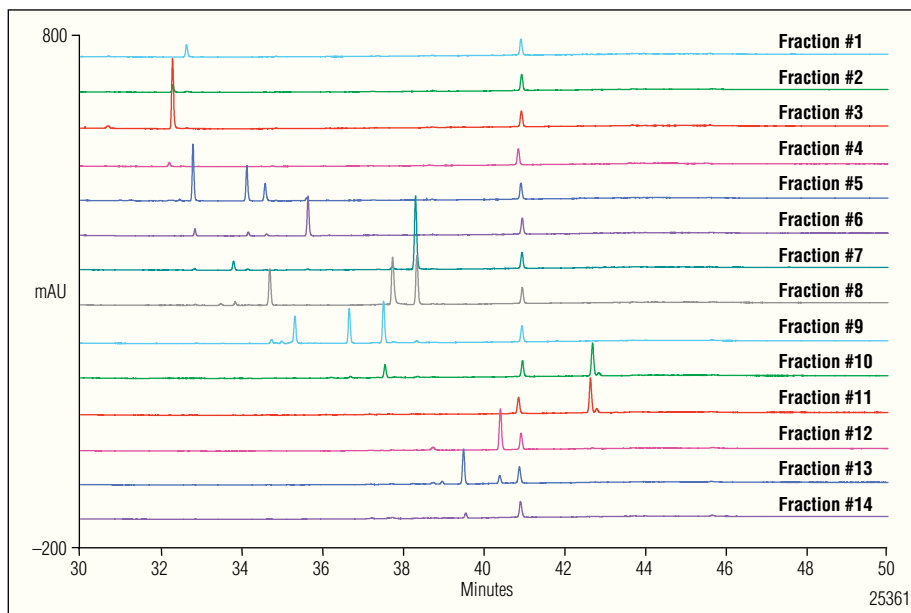


Figure 7. The overlaid reanalyzed fractions resulting from on-line heart cutting 2-D LC. The entire chromatogram was fractionated on a timebase and reanalyzed.

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CONCLUSIONS

Automated offline 2-D chromatography is an easy and relatively fast way to enhance resolution for complex mixtures. This method is most useful when enrichment is performed between ¹D and ²D (e.g., when combining an IEX separation with a RP separation). This method is less suited for quantitative analysis. If thermal stability of the sample is good, raising the temperature of the autosampler can overcome this. This allows the mobile

phase to evaporate, which can be followed by redilution of the fraction in a mobile phase of choice. These autosampler procedures can be readily programmed using Chromeleon®.

On-line heart cutting LC is easy to perform on a basic system, and is the choice of interest when a single product related compound needs to be further resolved from its parent peak with a second separation. It provides a fast, easy platform for routine impurity profiling.

REFERENCES

1. ICH impurities in new drug substances, *Q3A(R2)*, October **2006**.
2. P.J. Schoenmakers, G. Vivó-Truyols, W.M.C. Decrop, *J. Chromatogr. A* 1120 (**2006**), 282–290.
3. Bedani F., Kok WTh., Janssen H-G, *J. Chromatogr A* 1133 (**2006**), 126–134.

The nomenclature was adapted from Schoenmakers et al. *LC-GC Europe* (**2003**), 16(6), 335–336, 338–339.

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