# Preparative Chromatography: A Holistic Approach

**White Paper**

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## Abstract

Preparative Chromatography is only one of many vital techniques that can be applied as a pre-cursor to efficiently determine the structures of the unknown impurities or purify a sample to meet specification.

Dealing with these unknown impurities, for example in finished products, stability samples or discovery compounds, can be very challenging as failure to identify or remove them might interrupt product supply, delay clinical studies or interfere with toxicology studies. Very often there is a regulatory requirement to disclose structure of new impurities above a certain limit. The financial loss to the pharmaceutical, agrochemical, food and consumer goods and fine chemical industry can be enormous.

This White Paper discusses the key difference between isolation and purification, and then focuses on an efficient strategy for method development, scale-up and post-isolation treatment. If outsourcing, this highlights the benefits in establishing a supplier who have the full breadth of expertise spanning across Preparative Chromatography and all supporting techniques.

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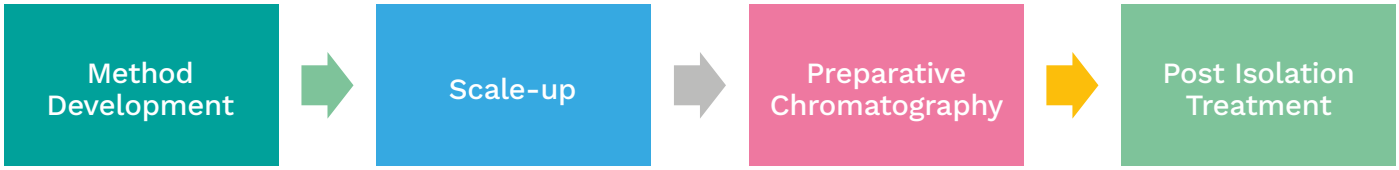



Figure 1: Flow diagram of strategic approach for Preparative Chromatography

## 1. Introduction

Before considering a strategic approach to isolation of an unknown impurity, the following questions should be answered:

### What is Preparative Chromatography?

Unlike Analytical Chromatography, the aim of Preparative Chromatography is its ability, with the right equipment, to collect any peak or area of interest in a chromatographic profile. To do this efficiently, it is key to work with the maximal sample concentration and injection volume that still affords reasonable resolution between the peak of interest and any other peaks/ artefacts within the chromatographic trace. In essence, the over-arching aim is to overload the column (which produces broad peaks) without sacrificing specificity of the peak of interest and maintain as short a runtime as possible.

### Is quality (e.g. purity) more important than quantity (e.g. amount)?

The answer to this question lies in the purpose of using Preparative Chromatography in the first place:

#### Structural Elucidation

If it is for Structural Elucidation, purity would likely be more crucial than quantity as it will make the elucidation step easier, but it is not essential to achieve the highest purity.

#### Purification

If the driver behind the Preparative Chromatography is to purify a sample then the purity of the material may be more important than the amount.

This highlights the value in spending some time on what is more important at the outset and defining the criteria before commencing with Preparative Chromatography. Depending on the outcome, the strategy may require some slight adjustments over the course of the process.

This White Paper will describe a typical flow path starting with an analytical method and progressing through to the isolation/purification. It will also consider any obstacles and pitfalls that may be encountered along the way. This, by no means, isn't the only way to tackle complex isolation/purification projects in chromatography but it is certainly the most common one.

It is also important to note that when talking about Preparative Chromatography, Semi-Preparative Chromatography is always included.

## 2. Analytical method development

A lot of the important work can be carried out without actually using a preparative High-Performance Liquid Chromatography (HPLC) instrument. If the analytical development is performed correctly, then the isolation step is straightforward and might only require minor adjustments.

Very often an analytical method already exists and the associated chromatogram shows the desired impurity. In other cases (e.g. where the impurity shows up in Nuclear Magnetic Resonance (NMR) analysis), a dedicated method might have to be developed.

A variety of information needs to be considered before starting with the development/optimisation of a method suitable for Preparative Chromatography:

- Are the mobile phase additives non-volatile?
- Is the column temperature controlled?
- Is the column not available in preparative mode?

If any of the questions (or indeed all of them) are being answered with "yes", then the strategy is to re-develop the method because it is essential to use non-volatile additives. It is very difficult to temperature control preparative columns and of course it is important to use analytical and preparative columns with the same stationary phase.

The approach taken to re-develop methods depends very much on whether a) the analytical column is available in preparative mode and b) the resolution of the desired impurity compared to other peak(s) (Figure 2).

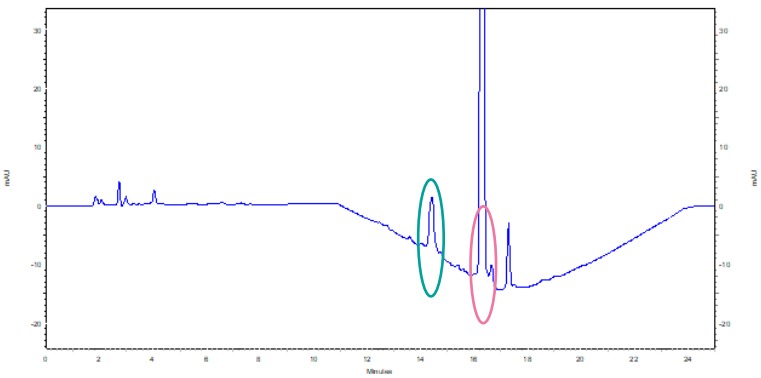


Figure 2: Typical chromatogram highlighting different resolutions

In regard to a), if a column is not available in preparative mode, then a new method would need to be developed and an equivalent chromatographic profile obtained. The use of Liquid Chromatography-Mass Spectrometry (LC-MS) to track the impurity would expedite the development of the new method.

In regard to b), the resolution can be much more of a challenge for example, the peak outlined in green (Figure 2) is considered to be relatively easy to isolate and scaling up is straight forward assuming that the analytical column is available in preparative mode. Isolating the shoulder peak in the pink circle (Figure 2) could be significantly more challenging. Potentially a full re-development would be required in order to overcome the likely issue of co-elution that may be seen when the method is scaled up and hence allow efficient isolation of the component.

One of the strategies that could be adopted to work through this problem would involve re-development of the method (with a chromatogram shown in Figure 2). A 4-6 column screen using orthogonal columns under different mobile phase conditions could be employed, with the appropriate instrument set up such as using a quaternary pump system and a 4-6-position column screen (see Figure 3). This would allow results to be obtained in a single overnight analysis, and then the column with the best 'hit' can then be further optimised e.g. in an isocratic method.

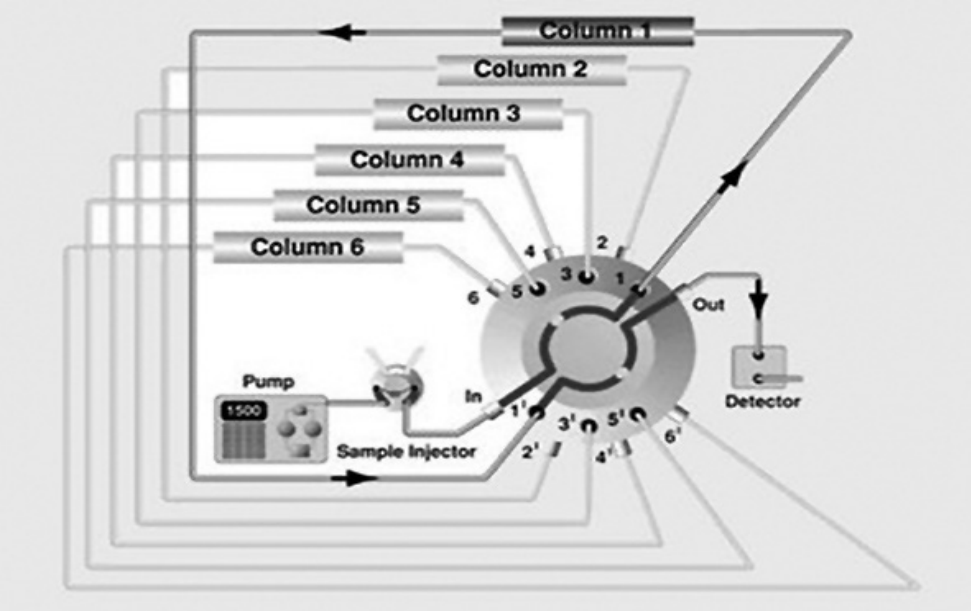
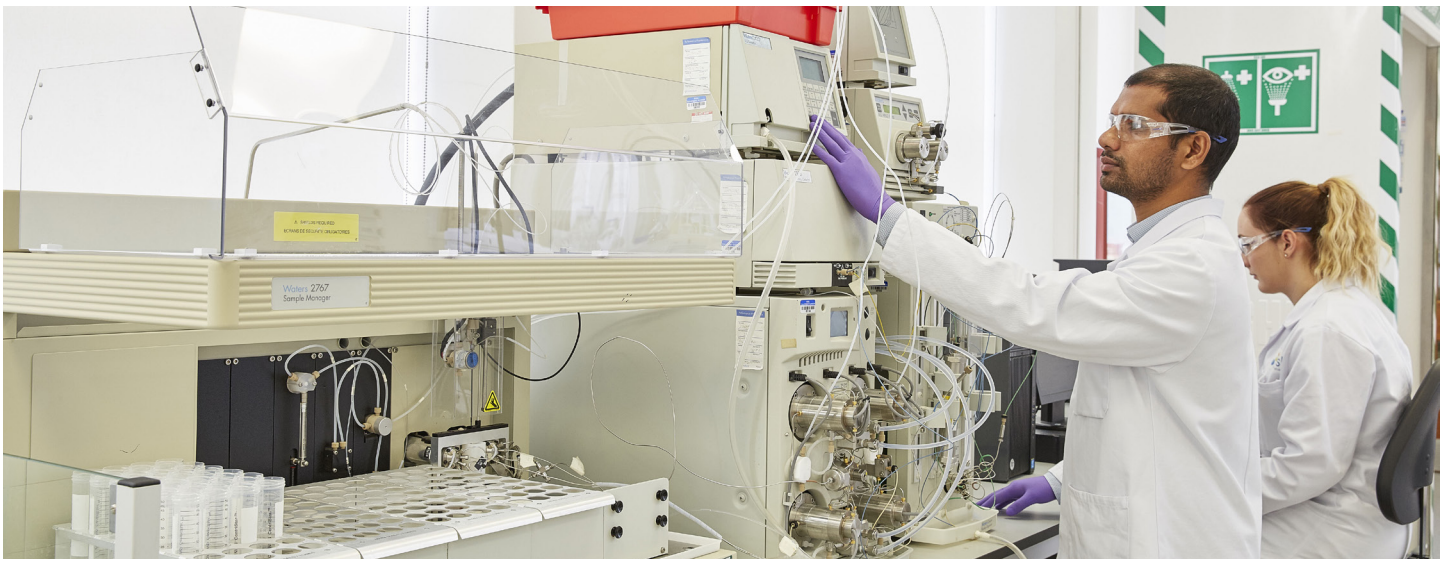



Figure 3: Example of a typical set-up of a column screen

Run time should also be adjusted to achieve as short a run time as possible to maximise efficiency, whilst also reducing solvent waste and its subsequent environmental impact.

One last, but equally important point, is the method run time. For efficiency reasons, it is essential to keep the run time as short as possible and depending on the resolution, shorter columns and higher flow rates can be the answer to this problem.

Shorter run times will result in using less solvent and generate less solvent waste and therefore, this results in a financial benefit to the running costs of a lab. It also reduces the amount of injections which is particularly important if larger amounts are to be purified. Very often this results in isocratic method because gradient methods require an additional equilibration time which is not necessary for isocratic runs.



## 3. Scale-up

In essence, scaling up involves imitating an analytical chromatogram on a preparative scale.

Once a method has been developed, there are two ways to scale-up to preparative mode:

If the separation is straightforward (e. g. see green circle in Figure 2) and assuming that sufficient then the method can be directly scaled-up on the prep instrument.

If the separation is more difficult (e. g. see pink circle in Figure 2) then a scale-up on the analytical instrument is a good strategy to determine the optimum sample concentration and injection volume. This would require a slight modification on the analytical instrument (by installing a larger injection loop) to facilitate larger volumes, which is important in understanding how the resolution behaves on the preparative scale.

Some very simple experiments can be set-up by preparing different sample concentration and injecting them at different injection volumes. This will generate a significant body of data that can be used to fine-tune the method moving forward.

### 1. Concentration overload

In this scheme, the injection volume is being kept constant and the sample concentration increased.

| Sample concentration (mg/mL) | Injection volume (µL) |
|---|---|
| 0.1 | 5 |
| 1 | 5 |
| 10 | 5 |
| 0.1 | 50 |
| 1 | 50 |
| 10 | 50 |
| 0.1 | 100 |
| 1 | 100 |
| 10 | 100 |

Table 1: Example of typical injection sequence for concentration overload

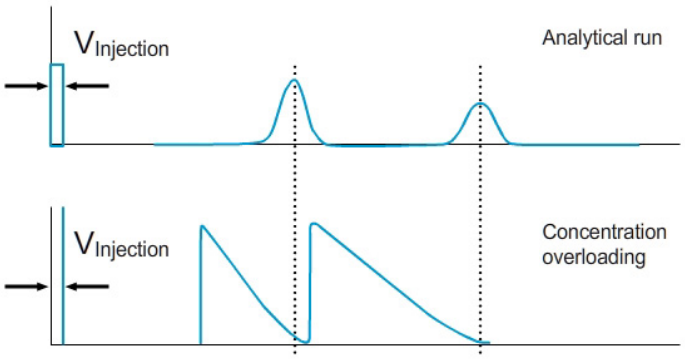



Figure 4: Chromatographic effects of concentration overload

### 2. Volume overload

In this scheme, the injection volume is being kept constant and the sample concentration increased.

| Sample concentration (mg/mL) | Injection volume (µL) |
|---|---|
| 0.1 | 5 |
| 0.1 | 50 |
| 0.1 | 100 |
| 1 | 5 |
| 1 | 50 |
| 1 | 100 |
| 10 | 5 |
| 10 | 50 |
| 10 | 100 |

Table 2: Example of typical injection sequence for volume overload

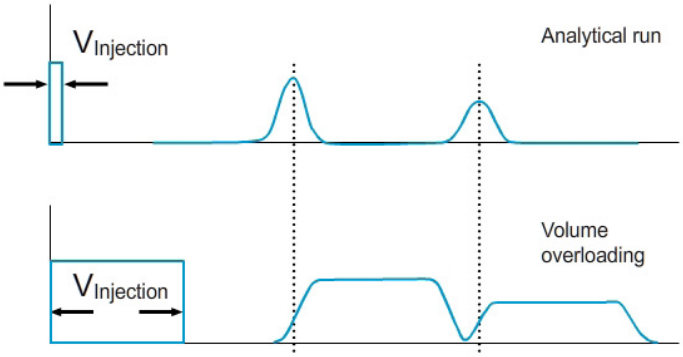



Figure 5: Chromatographic effects of volume overload

This results in peak broadening to a point where the peak height remains constant and peaks become rectangular.

Typically, concentration overload is favoured since it allows purification of more material. However, because solubility is often a limiting factor, a mixture of concentration and volume overload may be applied.

## 4. Preparative Chromatography

Before considering some of the key aspects of Preparative Chromatography, it is worth highlighting that it is key to any preparative task to perform the method development thoroughly and, if necessary, carry out an analytical scale-up procedure. Obtaining a robust analytical method will ensure that the preparative step is simpler and give confidence to the operator.

There are some important factors to be considered prior to starting the isolation process:

### Crude sample concentration and its diluent

In Preparative Chromatography, the aim is to work with the highest possible concentration and the largest injection volume. This will result in fewer injections and

therefore less mobile phase used (and less solvent waste generated). Of course, this is always dependent upon the complexity of the separation.

Consideration should be given to the solubility of the crude sample. Often this depends on its purity. Gross and trace impurities can dramatically affect the solubility. Ideally, preparing the sample in a solvent system to match the starting conditions of the chromatographic separation as closely as possible, is preferable. However this can result in lower than ideal sample concentrations. To achieve higher concentrations alternate dilution schemes could be employed, such as the use of different solvents.

Another important aspect is the use of additives (acid or base) in the diluent. This can dramatically enhance the solubility (depending on the chemical structure). Conversely, this can cause problems to a point where the sample (and impurity of interest) may decompose due to stability issues. The availability of any stability data prior to sample preparation (temperature, solvent compatibility, additivities, and light) is therefore key knowledge to have.

One potential issue that needs to be circumvented when working with the highest possible concentration/ largest possible injection volume is the precipitation of the sample in the system. This can sometimes be seen when using sample solvents that are different to the mobile phase composition If samples are poorly soluble in the mobile phase, when the sample meets the mobile phase, precipitation can occur, leading to blockages within the system. This can very often be seen on in or around the injector valve and is diagnosed by an increasing back pressure.

There are two ways to avoid sample precipitation:

**1. Sandwich injection:** the sample is sandwiched between two plugs of sample solvent. The mixing with the mobile phase only occurs at the beginning and end of the plugs where the sample concentration is zero:

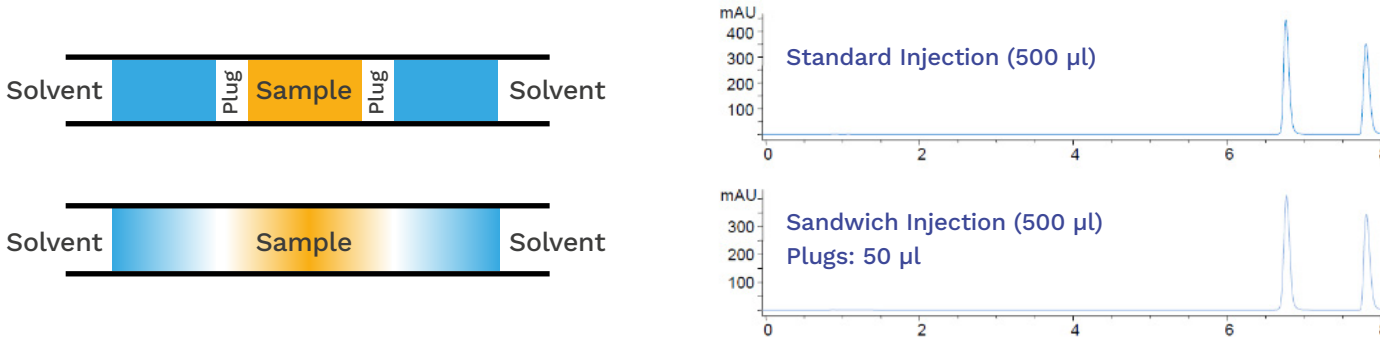



Figure 6: Illustration of a sandwich injection and how it affects the chromatographic output

**2. Organic phase injection:** in this case, the system is reconfigured so the sample is delivered by a stream of strong organic solvent into the top of the column. At this point the sample stream is continuously diluted with the aqueous stream. The rate of transfer is so high that no precipitation can occur.

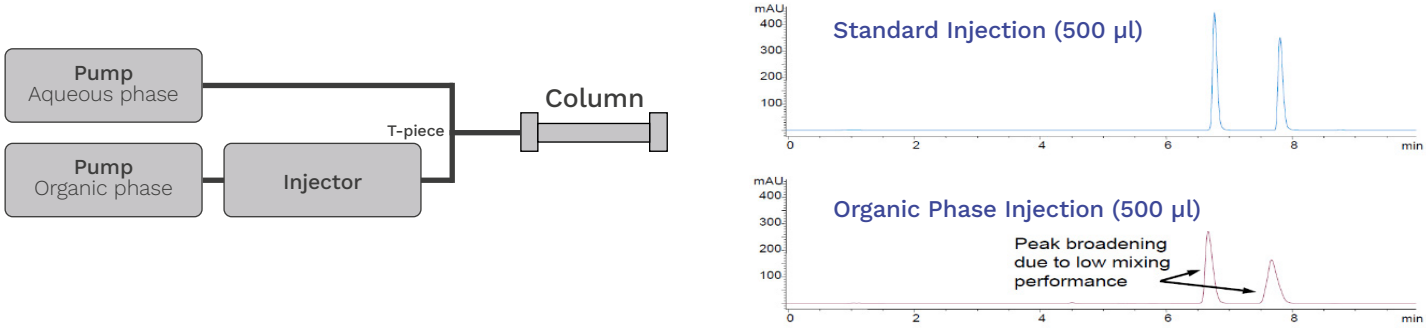



Figure 7: Illustration of an at-column dilution injection and how it affects the chromatographic output

Both injection techniques have advantages and disadvantages. Overall, sandwich injections are preferred for highly concentrated samples where the chromatography is not important.

Organic phase injection would tend to be utilised for medium concentrated samples with a requirement of good chromatography.

**Injection volume –** Not only is it important to work with the highest possible concentration of the sample in order to maximise the yield, using the largest possible injection volume produces a highly efficient isolation process. It is therefore recommended to install a larger injection loop on the instrument to accommodate larger injection volumes.

**Flow rate –** The flow rate is an essential part of the scale-up procedure and can easily be adjusted based on the conditions which were developed in the analytical optimisation.

It is worth noting that the particle size of the analytical column and the preparative column can play an

important part in the calculation of the flow rate. Assuming that the particle size of the analytical column is 3 µm and the prep column 5 µm (which is typically commercially available), this would almost halve the required flow rate.

Once all of these factors have been addressed, the Preparative Chromatography process can commence.

One of the most important instrument parameters to consider is use of the optimum delay time/volume. If this is incorrect, significant amounts of the target impurities may be lost, or too low a purity may be obtained.

The type of collection is also imperative. There are two main approaches for collecting peaks: time-based and based on threshold.

1. The most common approach is time-based collection. For this approach, a time window is set around the peak to be collected. Depending on the resolution, this can be a very tight or wide window. The latter can help to compensate for retention time shifts but the adverse effects are other small impurities being collected together with the desired peak, and higher collection volumes.
2. The threshold approach is based on the absorbance of the peak. This automates the collection of fractions based on their detector response, with criteria set in the method for desired threshold before starting and ending a collection window. This approach can automatically account for any retention shifts, however appropriate threshold triggers and consistent chromatographic profile must be achieved to ensure it collects the desired peak.

In general, it is a good idea to have flexibility to fine tune the method during the process. The first injection is usually carried out at a lower volume and, if the amount is crucial, the complete chromatogram should be collected in appropriate fractions. After the first injection has been assessed (and – if necessary – the method further optimised), the injection volume can be increased with subsequent injections to a point where the optimum injection volume is reached. From then onwards, a sequence can be set-up to process as much of the sample as necessary until a required amount of collections and material is obtained.

## 5. Post isolation treatment

Once the fractions are collected, they need to be combined. The final step in the Preparative Chromatography process is the solvent removal, which can be demanding. This step is of particular importance because the process so far may have taken a significant time, and failing to remove solvent(s) and additive(s) efficiently can turn out to be costly error.

Removing solvent(s) and additive(s) can be very challenging and time consuming, especially if water is present. One of the most common techniques involves rotary evaporation. Using a good vacuum pump will allow the removal of any aqueous solution. Even then, this can be a slow process, and one which is dependent upon the water bath temperature (which should be set >40°C). The disadvantage of rotary evaporation is not only its time-consuming nature, but also if the stability of the collected fraction is unknown, there is a risk of decomposition when operating at higher temperatures. Unless a very large rotary evaporator is used, the amounts to be evaporated are rather small.

There are more efficient instruments available that operate on the basis of centrifugal evaporation, meaning that they are more effective in removing larger amounts of organic solvent/aqueous mixtures and volatile additives. They can also freeze dry after trapping organic solvents in a fully automated process.

Freeze drying is an important technique to remove aqueous mixtures, especially for compounds that are suspected to be unstable at elevated temperatures. This approach is therefore deemed to be a very gentle way of solvent removal.

Once a purified sample has been obtained, further analytical testing such as NMR or LC-MS/MS can be carried out to elucidate the structure or check for purity.

## 6. Conclusion

Preparative Chromatography is a very effective and efficient technique for the isolation of impurities from complex mixtures. However, some time should be invested prior to starting any preparative project to carefully consider the importance of quality vs. quantity and how this relates to the specific project being undertaken.

This White Paper describes a strategic, step-wise approach detailing how to carry out successful Preparative Chromatography and to isolate an impurity or purify a sample. Successful Preparative Chromatography depends on a thorough and robust method development and scale-up procedure, ensuring that any problems that arise during the early steps are addressed and eliminated, thus allowing the preparative step to be carried out smoothly.

## References

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Our expertise ranges from all stages of the isolation process: from initial method screening and optimisation to the isolation itself and all the way to the Structural Elucidation using a minimal amount of sample by applying our established expertise in LC-MS/MS and – if necessary – NMR.

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To find out more please contact us on: **+44 (0)118 918 4076**, email **enquiries@rssl.com**, or visit **www.rssl.com**.

## About the author

**Ravi Muppa**
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Ravi Muppa has over fifteen years' experience in pharmaceutical analytical chemistry covering early phase development to commercial release. Ravi has supported clients through cleaning validations, stability studies and ensuring that requirements as per regulatory guidelines are achieved. Ravi's knowledge also extends to impurity isolation using preparative HPLC to aid elucidation of impurities by secondary techniques such as LC-MS and NMR.

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