Chromatography Solutions

Knowledge note #0038

The Scale-up Process: from Analytical to Preparative Scale LC Separations

INTRODUCTION

The scale-up process usually demands an initial investment in a relatively smaller-scale pilot study, to gain valuable information at a lower expense of cost, time, space, and labour. The process is not straightforward, and the key to preparative scale separations is understanding the transition from ideal to non-ideal overloaded separation conditions.[1] The process involves dedicated engineers and scientists to create a workflow that maximizes production efficiency and/or profit.

With respect to liquid chromatography (LC), the extraction of targeted compound(s) from a sample is often performed initially under ideal non-overloaded (analyte concentration and mass) conditions. After this methodology is established, the separation is run under non-ideal overloaded conditions and subsequently scaled up to a larger preparative column separation. The preparative scale separation can then be utilised to isolate the purified target component(s) and remove unwanted material/impurities.

This knowledge note, demonstrates how to achieve a high-yield preparative scale separation and confirm the purity of the resulting isolated fractions.[2] Large-scale preparative separations are important not only for small molecules, but also for large molecules with a growing interest in biopharmaceuticals.[3]

INITIAL ANALYTICAL SCALE METHOD DEVELOPMENT

Developing an ideal-non-overloaded analytical scale workflow (e.g. 4.6 mm ID), may be considered as a good starting point to compare the effects of the scaled-up overloaded experiments.[4] Moreover, this assay can be utilised later to confirm the purity of the final large-scale process. LC method development can be time and resource intensive, in particular for gradient separations, for both small and large molecules,[5,6] and is therefore best performed on the smaller column format to keep this critical step of the process as cost-effective as possible.

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The resolution between the compound of interest for the final purification and any adjacent peaks ideally will be optimised, as the scale-up to non-ideal overloaded conditions will ultimately lead to the loss of resolution. An example analytical scale separation for the purification of α-lactalbumin from whey protein is shown in Figure 1.[7] In this example, the separation has been developed to optimise the separation of α-lactalbumin (peak 1) and provide good resolution from subsequent eluting peaks. This approach then allows for scale-up to overloaded conditions.

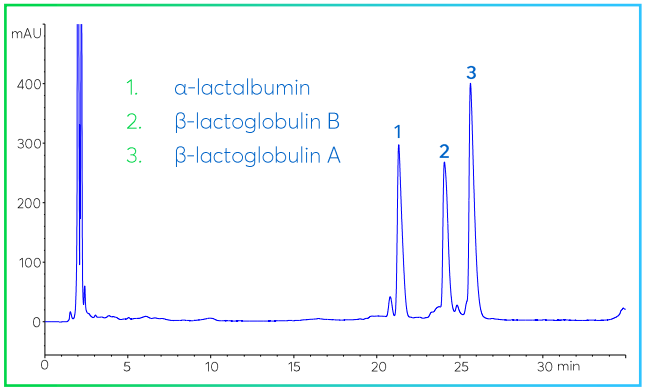

Figure 1: Separation of whey proteins from cow's milk purchased at a local market. Column: Avantor® ACE® 3 C4-300, 150 x 2.1 mm; mobile phase: A: 0.01% TFA (aq), B: 0.01% TFA in MeCN; gradient: 33% B from 0-5 min to 43% B at 32 min; flow rate: 0.2 mL/min; injection volume: 1 µL; detection: UV, 210 nm; Temperature: 45 °C; instrument: Agilent 1290 UHPLC.

SCALE-UP TO NON-IDEAL OVERLOADED CONDITIONS

Once the analytical scale method has been established, it can then be scaled up to non-ideal overloaded conditions using the same column format. A series of experiments to increase the sample loading (either by increasing injection volume or sample concentration) is carried out to understand the impact of sample loading on the separation profile (Figure 2). This highlights the chromatographic transition from an ideal non-overloaded assay to one where the column's capacity is overloaded (injected analyte concentration and mass) and a loss of resolution and peak shape occurs.[3] Whilst a loss of peak shape and resolution is observed, the priority for the final preparative scale process is to maximize the amount of the target analyte extracted from the injected sample with high purity.

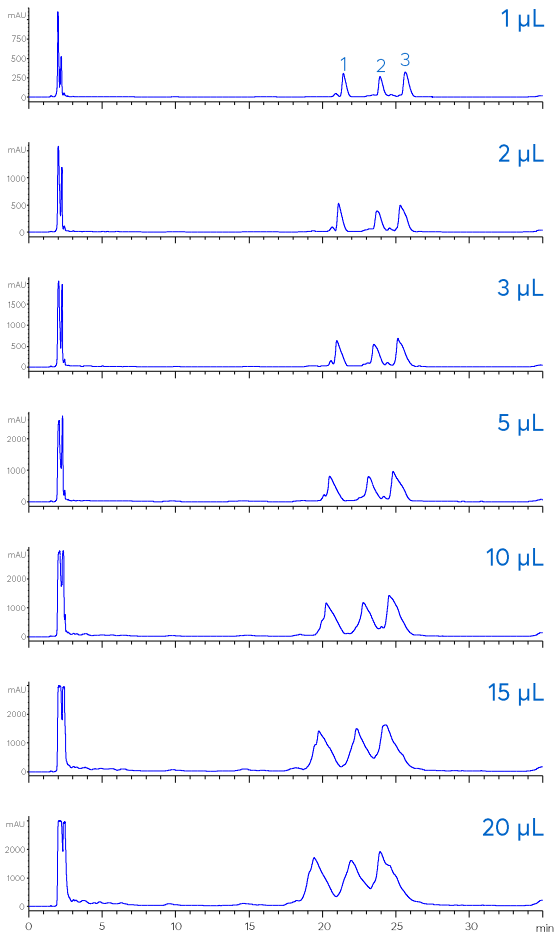

Figure 2: Figure 2: Loading study for the separation in Figure 1, showing sequential increase of the injection volume from 1 to 20 µL.

Once the desired loading is established at the analytical scale, the separation can then be scaled up to preparative scale. It is highly advantageous to scale up the method to a prep-scale column packed with similar material (same stationary phase, surface area, coverage and bonding) from the same manufacturer to ensure that the selectivity obtained in the analytical scale separation is preserved. Often a larger particle size is utilised for the prep-scale column as the absolute column efficiency is not as critical as at analytical scale and additionally, for cost and back pressure considerations. To scale the analytical separation to preparative scale, the flow rate and injection volume may be scaled to the preparative column dimensions using equations 1 and 2 respectively.

For gradient separations, if the same length column is used for the preparative scale separation, no change to the gradient profile is required. If the column length changes, then the gradient profile time points should be scaled according to equation 3.

$$F_{i2} = F_1 \times \frac{d_{c2}^2}{dc_1^2} \quad (1)$$

F_1 = flow rate of the analytical scale method (mL/min)
F_2 = prep-scale flow rate (mL/min)
dc_1 = internal diameter of the analytical column (mm)
dc_2 = internal diameter of the prep column (mm)

$$V_{inj2} = V_{inj1} \times \frac{(L_2 d_{c2}^2)}{(L_1 dc_1^2)} \quad (2)$$

V_{inj1} = injection volume of analytical scale method (µL)
V_{inj2} = prep-scale injection volume (µL)
L_1 = length of the analytical scale column (mm)
L_2 = length of the prep column (mm)

$$t_{G2} = t_{G1} \times \frac{F_1}{F_2} \times \frac{(L_2 d_{c2}^2)}{(L_1 dc_1^2)} \quad (3)$$

t_{G1} = gradient time point for the analytical scale method (min)
t_{G2} = gradient time point for the prep scale method (min)

Figure 3 shows the analytical scale separation from Figures 1 and 2 scaled to a 21.2 mm ID preparative scale column packed with the same stationary phase. The column length was kept the same, therefore the gradient profile did not require scaling. In this case, it was found that for the final prep scale separation, the injection volume could be further increased by 50% from the scaled injection volume of 2 mL to 3 mL without compromising the purity of the isolated target fraction.

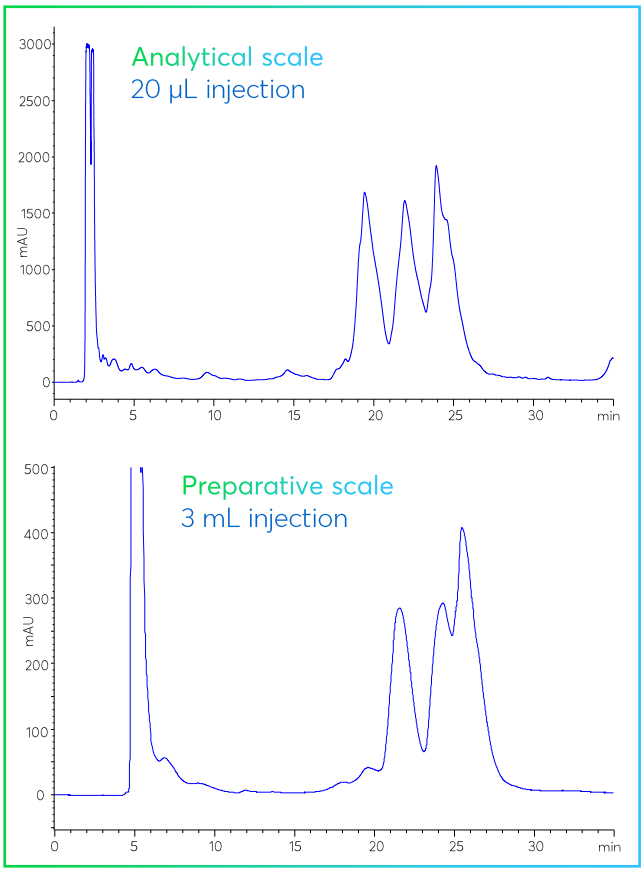

Figure 3: Analytical and preparative scale chromatograms highlighting the overloaded separation conditions of whey from com's milk (see Figure 1 for conditions). Preparative scale separation conditions: Avantor® ACE® 5 C4-300, 150 x 21.2 mm; mobile phase: A: 0.01% TFA (aq), B: 0.01% TFA in MeCN; gradient: 33% B from 0-8.1 min to 43% B at 35.1 min (sample loading 0-3.1 min; injection at 3.1 min); flow rate: 21.2 mL/min; injection volume: 3 mL; detection: UV, 210 nm; temperature: ambient; instrument: Knauer Azura PrepLC Premier system.

HIGH PURITY CONFIRMATION

The final overloaded conditions and separation process under preparative scale conditions may sacrifice resolution and peak shape but must not compromise the high level of purity. Hence, empirical fractionation must be carried out, collecting specific segments of the targeted peak. To confirm purity, simultaneous mass spectrometry detection during the fractionation process may be used. Furthermore, it may involve programming the fractionation of different segments under the targeted peak's area for automated collection and purity confirmation (Figure 4). Small aliquots from each fraction are separated under analytical scale column separation conditions to confirm the fraction purity (Figure 1). Depending on the purity specifications of the targeted final product – the large-scale process may segment more or less of the targeted peak. The amount of material required of a desired purity is also critical to the number of fractions that need to be collected and this impacts the number of repeat runs that will be required.

While this knowledge note summarizes the basic concepts / fundamental aspects of a scale-up process

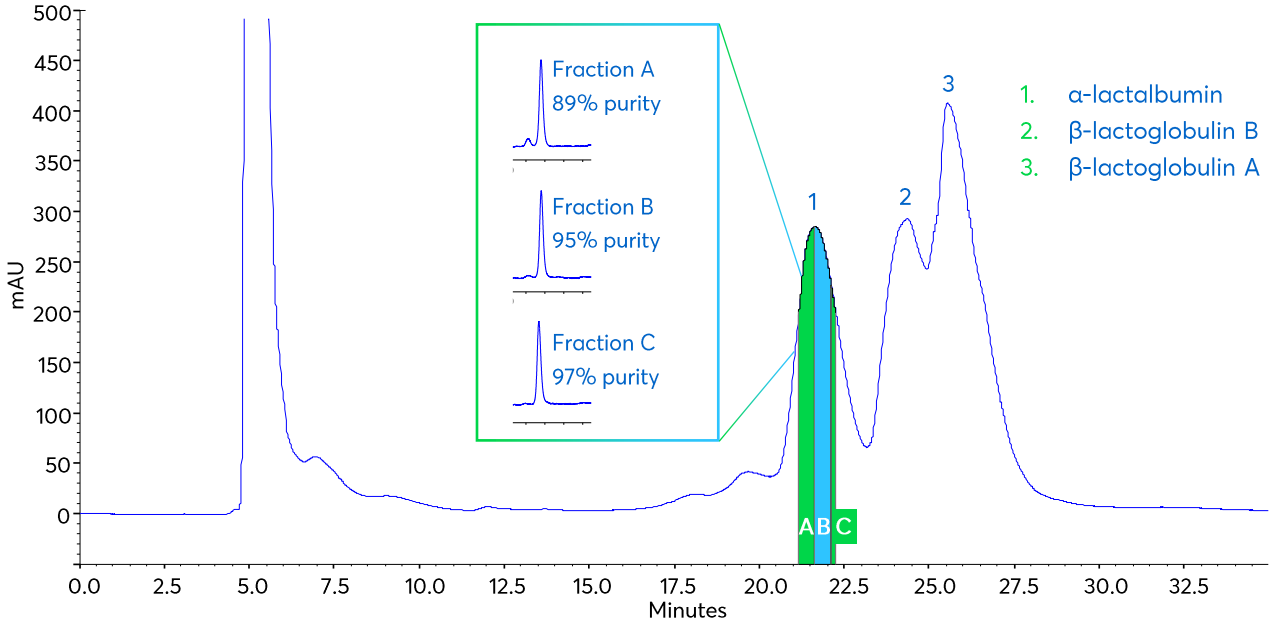

Figure 4: Preparative scale fractionation (conditions: see Figure 3) of α-lactalbumin with high purity analytical scale confirmation (conditions: see Figure 1). The fractions collected from the α-lactalbumin peak are highlighted in green and blue. Preparative instrument: Knauer Azura PrepLC Premier system with Foxy fraction collector.

from analytical to preparative scale chromatography, scale up is, in reality, often not as simple. Additional analytical scale methods may need to be employed to confirm the purity of fractions before the validation of the final preparative scale workflow.[7]

CONCLUSION

In this knowledge note, the main aspects involved in the chromatographic scale-up from an analytical to a preparative scale column have been demonstrated. The final preparative scale process must maximize the yield and quantity of the target analyte. This involves understanding the compromises between peak shape and resolution when separations move from ideal non-overloaded conditions to non-ideal overloaded conditions. The final fractionation process must then be established with peak fractionation experiments and high-purity confirmation before the final large-scale workflow can be validated. It is important to note that most industries have dedicated engineers and scientists working together during the process development, highlighting the importance of creating cost and time-efficient workflows.

REFERENCES

1. 'Preparative liquid chromatography' – A Review, G. Guiochon, *J. Chromatogr. A* (2002) **965** 129–161.

2. Avantor® ACE® Technical Note #034 'High purity and yield purification of α-Lactalbumin from cow's milk using preparative liquid chromatography' (https://av.cmd2.vwr.com/pub/apl/chrom/main?key=C-13243).

3. 'Separation science is the key to successful biopharmaceuticals', G. Guiochon, L.A. Beaver, *J. Chromatogr. A* (2011) **1218** 8836-8858.

4. Avantor® ACE® Knowledge Note #0018 "Step-by-step Protocol for Streamlined Reversed-Phase Method Development using Avantor® ACE® MDKs" (https://uk.vwr.com/cms/ace_knowledge_notes)

5. 'Peak capacity optimization of peptide separations in reversed-phase gradient elution chromatography: Fixed column format', X. Wang, D.R. Stoll, A.P. Schellinger, P.W. Carr, *Anal. Chem.* (2006) **78** 3406.

6. 'Optimization of gradient reversed phase chromatographic peak capacity for low molecular weight solutes', A. Soliven, I.A. Haidar Ahmad, M.R. Filgueira, P.W. Carr, *J. Chromatogr. A* (2013) **1273** 57.

7. 'Purification of N-acetylgalactosamine-modified-oligonucleotides using orthogonal anion-exchange and mixed-mode chromatography approaches', A.A. Kazarian, W. Barnhart, J. Long, K. Sham, B. Wu, J.K. Murray, *J. Chromatogr. A* (2022) **1661** 462679.