MCSGP Process Development – Part 1: Gradient Development

MCSGP with AutoPeak® control (Multi-column Counter-current Solvent Gradient Purification) is a twin-column continuous chromatography process that has multiple advantages for oligonucleotide and peptide manufacturing compared to single-column batch chromatography. MCSGP is typically developed on the Contichrom® CUBE system using the in-built "MCSGP Wizard" software tool. The MCSGP Wizard provides an easy interface to quickly translate a standard "batch-design" chromatogram directly to a functional MCSGP operating point.

Before using the MCSGP Wizard, it is sensible to optimize the input batch-design run so that less time is spent optimizing MCSGP operating points directly, which can be more demanding in terms of time and feed consumption. In this application note we aim to provide a solution to the question "how do you create a batch-design gradient for programming the MCSGP Wizard that gives close to optimal results from the beginning?".

This application note provides a simple step-by-step flowchart template to guide the development of batch gradient conditions that result in optimal input data for the MCSGP wizard. Using simulation-assisted testing, we demonstrate that following this simple flowchart results in MCSGP setpoints that outperform those derived from a numerically optimized batch procedure.

Introduction

MCSGP is a cyclical chromatographic technique that enables the continuous counter-current separation of complex mixtures, such as peptides and oligonucleotides. MCSGP uses two identical columns to facilitate side-cut recycling during chromatographic purification. In general, a gradient elution is conducted on one fully loaded column and the pure "center-cut" product is collected. However, MCSGP's key feature is the direct automatic recycling of the impure side-cuts (product co-eluting with impurities) to a second

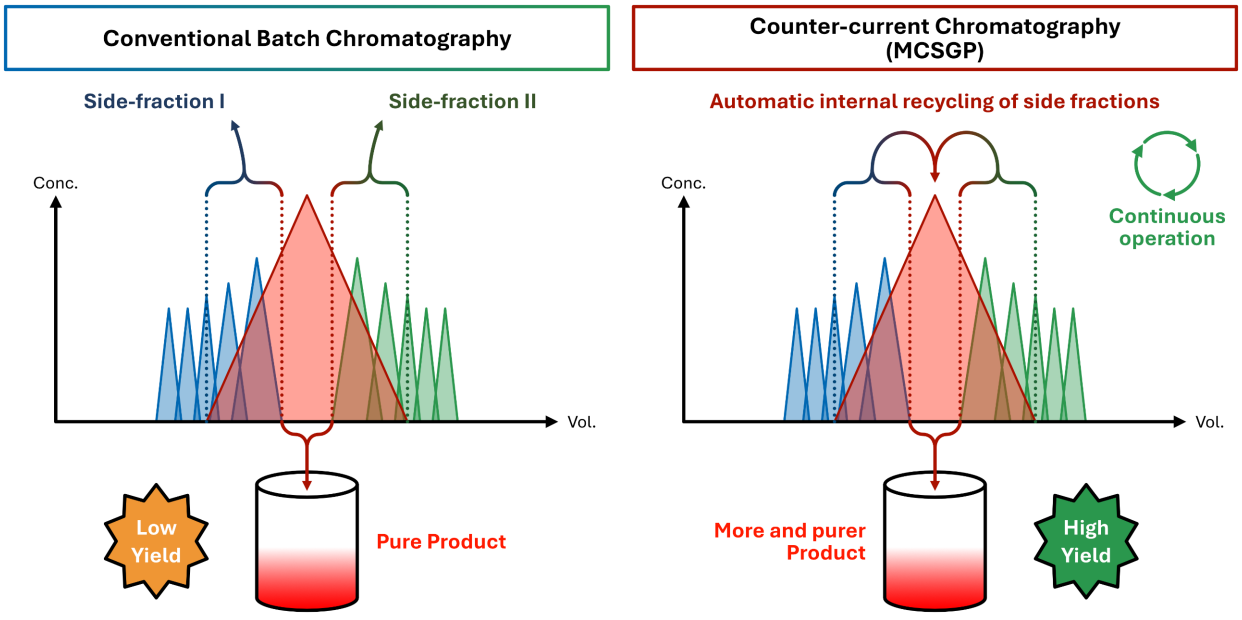

Figure 1 Traditional single column chromatography (left): side fractions are discarded or separately stored for re-chromatography. MCSGP (right): side fractions are internally recycled, continuously removing impurities and collecting pure product.

column for immediate re-purification (Figure 1). These recycled side-cuts are diluted inline and instantly captured on the second column, then supplemented with fresh feed material, and the next purification is started as soon as the previous one is concluded. This pattern repeats back and forth between the two columns, and with a good MCSGP design, a cyclic steady state is quickly reached. This results in an automated process that can be operated for 100's of consecutive elutions where high yield, high throughput, and high purity are maintained robustly throughout the run.

Overview of MCSGP Design Procedure

MCSGP generally uses the same columns, solvents/buffers and same washing and cleaning protocol as single column preparative processes. If you have a functional batch polishing process with a poor yield, then MCSGP is simple to try on the Contichrom CUBE and is likely to improve yield.

The programming of MCSGP methods is supported by the "MCSGP Wizard", a module of the ChromIQ® software included in Contichrom systems. The wizard translates an input batch chromatogram (gradient or isocratic) directly into a fully functional MCSGP operating point. However, the performance of the input "design" method also determines the performance of the resulting MCSGP method. Moreover, a method previously optimized for single-column batch production does not automatically provide the best starting point for MCSGP design because MCSGP can frequently tolerate conditions, such as steeper gradients, without the performance tradeoffs experienced in batch chromatography. So how can we ensure the considerable benefits of MCSGP are fully realized?

Basic Recommendations for MCSGP Design

Our basic recommendation for generating a chromatographic gradient intended for transfer to the MCSGP wizard has the flowing features:

- Linearity – the gradient should be linear, avoiding varying slopes or steps during elution.
- Complete target elution – The main product should elute completely within the range of 10% to 70% of eluent B.
- Duration – the gradient should have a duration of 10 column volumes (CV).
- Target purity – The product pool should ideally contain at least 50% of the loaded product at target purity.*

When followed, these guidelines produce a batch gradient method compatible with the MCSGP Wizard. However, to find gradient conditions that are even better suited for transfer to MCSGP, we recommend following the "Gradient development flowchart" presented here.

In purification process development, obtaining product in specification is the primary objective and there are several product-specific Critical Quality Attributes (CQAs) associated with product purity. The secondary aim in process development is optimization of process performance, which can be evaluated and compared by a set of process performance parameters (Table 1). This application note will focus

* The ideal yield at target purity is strongly dependent on the purification task complexity. The range of 50-70% purity represents a standard compromise in most biopolymer purifications; however, lower or higher yield values would be considered as acceptable starting points. Accordingly, this requirement needs to be assessed on a case-by-case basis.

Table 1 **Overview of process performance parameters, optimization targets and effects.**

Parameter	Definition	Unit	Optimization Target	Effect in Manufacturing
Yield	$Y = \frac{m_{P,Prod}}{m_{P,Feed}}$	[%]	Maximize	Reduction in upstream synthesis batches
Productivity	$Prod. = \frac{m_{P,Prod}}{t_{Cycle} \cdot n_{Col} \cdot V_{Col}}$	[g/L/h]	Maximize	Reduction in production time and/or column size More production
Eluent Consumption	$EC = \frac{V_{Eluent}}{m_{P,Prod}}$	[L/g]	Minimize	Reduciton in costs, waste, and eluent tank size
Product Concentration	$c_{P,Prod} = \frac{m_{P,Prod}}{V_{Prod}}$	[g/L]	Maximize	Size reduction in subsequent unit operations

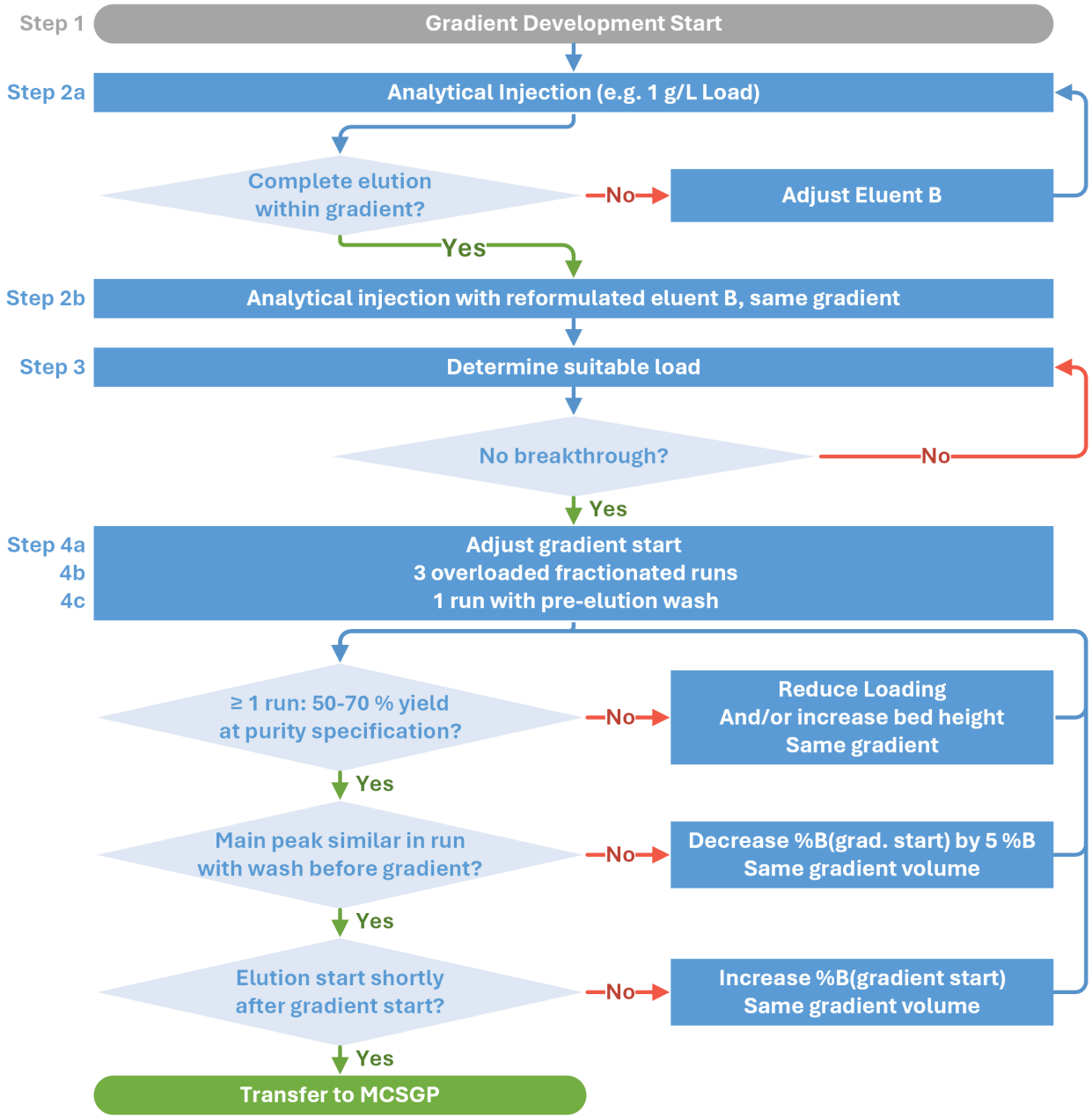

Figure 2 **Gradient development flowchart - abridged version.**

on the primary objective, while a procedure for optimization of process performance is presented in a second application note. In the context of meeting the target purity, the process parameter "load" is the most relevant.

Gradient Development Flowchart

The purpose of the gradient development flow chart is to guide development of a chromatographic gradient suitable for MCSGP design. Firstly, the initial starting parameters are chosen (e.g. bed height, flow rates, UV settings) and then optimal gradient operating conditions are established (e.g., loading and %B targets). An abridged version of the flowchart is presented in Figure 2. This approach is valid for a separation problem following a Langmuir-type isotherm.

Step 1: Selection of Starting Conditions

Suitable column lengths, flow rates, and UV detection settings are recommended before starting the gradient development: Specifically, we recommend 15 cm bed height columns to reduce feed demand and allow higher flow rates. The gradient flow rate should be selected below the maximum possible flow rate, e.g. 50 %, to give leeway for the inline adjustment in MCSGP without having to reduce the elution flow rate.

Furthermore, the elution flow rate needs to be selected to achieve sufficient separation. Lastly, all available UV channels should be recorded and set to different wavelengths to avoid duplicate data acquisition. Make sure that at least one of the selected wavelengths records the main peak maximum in the linear range, i.e. below 1000 mAU. This results in the most robust AutoPeak® control parameters to be used in MCSGP later. AutoPeak is a UV-based dynamic process control method, greatly improving robustness and scale-up of MCSGP.

Step 2: Gradient End Optimization

Once suitable starting conditions have been chosen, the gradient development begins using a standard screening method with a small injection of feed material onto the column (≈ 0.5-1 g/L Load), and a first test elution with 5-95 %B for 10 CV is performed (Step 2a in Figure 2). If complete elution is not obtained, an eluotropic strength increase of eluent B is required. However, if a complete elution is achieved it is advisable to reformulate eluent B, especially if the product peak is already eluted in the first 50 % of the gradient. Ideally, the tail of the product peak coincides with about 70 %B (Step 2b).

Step 3: Maximum Load Determination

After modifying gradient end conditions, one should determine upper limits for product loading. Here, two approaches are recommended: If enough feedstock is available, you may opt to intentionally overload the column and measure the dynamic binding capacity of the resin and determine the ≈1 % breakthrough value as a starting point. Alternatively, some molecule-specific standard Load values may be considered as a starting point. Typical standard Loads for a preparative single column run are approximately:

- Peptides: Between 5 and 20 g/L packed bed on RP stationary phases.
- Oligonucleotides: Loads up to 40 g/L are possible on AEX and up to 20 g/L on RP stationary phases.
- Proteins: Between 10 and 40 g/L on IEX resins.

Step 4: Fractionated Runs

Step 4a: Gradient Start Adjustment

The next step in the flowchart is to adjust the %B for gradient start. This parameter is consequential, as the %B gradient start serves as the target for calculating inline adjustment factors needed during recycling steps in MCSGP. The adjustment factor, when set too high, can adversely impact productivity and eluent consumption, as excessively large eluent volumes are delivered unnecessarily. This target is set as high as possible, i.e. where adsorptive conditions for the product are still robust. This means, if an isocratic pre-elution wash is done at the starting %B, the product remains bound to the column independent of wash duration (See step 4c for more information).

Step 4b: Test Runs with Fractionation (x3)

Having established the %B gradient boundaries and determined a maximum potential load, the next step involves performing 3 batch chromatography runs, fractionating the eluates, and analyzing the fractions for product and impurity concentrations. Three runs with different levels of product loading are carried out, the first one with at 95% Load (based on the 1% breakthrough value) or 100 % (based on standard loading values indicated in “Step 3”), respectively, the second one with 75% and the third one with 50% of the maximum Load determined in Step 3.

If none of the three evaluated runs deliver the target purity at an acceptable yield, additional runs further decreasing the load and/or increasing column bed height may be required. As mentioned earlier (see footnote above), a suitable batch yield should be chosen on a case-by-case basis. For example, for some very challenging purifications, > 50% yield at target purity may not be a realistic starting point.

Step 4c: Inline Adjustment Parameter Check

It is advisable to repeat a successful run from step 4b, but with an additional 2 CV isocratic “wash before elution” using the %B gradient start conditions as “washing %B concentration”. This precaution is taken to check that product remains bound to the column at the chosen %B target for in-line adjustment. If the peak profile is identical with and without the extra 2 CV wash, then no action is needed. If the product retention time significantly changes, a lower %B gradient starting point (i.e. a higher in-line adjustment factor) should be chosen and the test repeated.

Transfer to MCSGP

At the conclusion of this workflow, a suitable batch design gradient and chromatogram should now be available as crucial input enabling a robust Wizard-based MCSGP design. The chromatogram resulting from following this flowchart will have a region of interest where the main target compound is either not co-eluting or is only partially overlapping with other species (impurities). Additionally, its CQAs should meet the required specifications. Developing gradient conditions in accordance with these guidelines will effectively facilitate the subsequent MCSGP process setup.

Simulation-Based Flowchart Validation

To test the gradient development flowchart, a modeling-based approach was used. Batch simulation runs were done adhering to the flowchart instructions, and a batch design chromatogram was developed. The resulting chromatogram was imported into the MCSGP Wizard to generate an operating point. The MCSGP operating point was then simulated for comparison to batch chromatography. It is worth noting that the presented gradient development procedure does not require modeling and modeling was used only for verification and theoretical comparison in this application note.

Materials and Methods

Feed and Analytics

The material employed in this study was a 20-mer ssRNA oligonucleotide (5'-ATA CCG ATT AAG CGA AGT TT-3') solubilized in 20 mM ammonium hydroxide. Its starting purity was 78.9% and the target concentration was 25 g/L, as determined by analytical IP-RP HPLC with a YMC-Triart Bio C18, 1.9 µm, 30 nm, reverse phase column (150 x 2.1 mm ID) on an Agilent 1290 Infinity II LC with UV absorbance measurement at 260 nm. The equilibration buffer used in the analytical method was 100 mM hexafluoro-2-propanol (HFIP) and 4 mM triethylamine (TEA), while elution buffer was pure methanol. An analytical chromatogram of the feed mixture is presented in Figure 3.

Purification Conditions

Experiments were carried out on 5 × 100 mm columns packed with YMC BioPro IEX SmartSep Q30 resin on a Contichrom CUBE 30. UV absorbance was measured at 280 nm. The buffers for preparative chromatography were 20 mM NaOH (Mobile Phase A) and 20 mM NaOH + 1.2 M NaCl (Mobile Phase B).

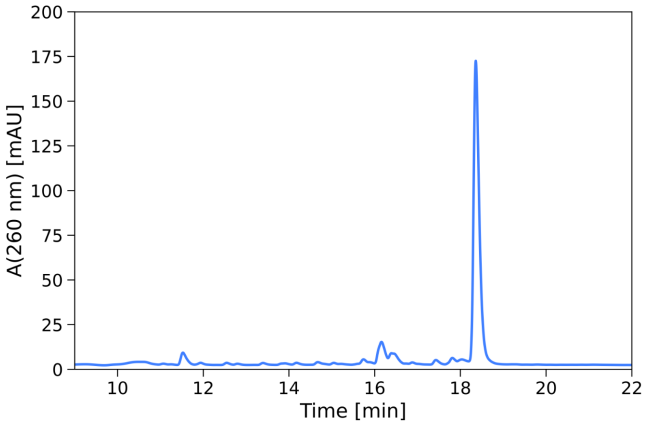

Figure 3 **IP-RP analytical chromatogram of the feed material.**

Model Calibration and Validation

Simulation runs relied on a lumped kinetic model, commonly used to describe processes for purification of large molecules such as oligonucleotides that display slow mass transfer kinetics. This approach facilitated a rapid progression through the different recommendations of the flowchart speeding up process development and saving feed material.

The model used in this study consists of a mass balance in the mobile phase accounting for axial dispersion, a transport equation in the stationary phase, and an adsorption equilibrium equation. A Bi-Langmuir isotherm was chosen as adsorption equilibrium model and the Inverse Method was applied to estimate the essential adsorption properties and parameters required for setting up the simulation.

Moreover, the procedure is based on the realistic assumption that the oligo components in the mixture have a similar adsorption behavior and mass transfer resistance. According to this, impurity isotherms are related to the main compound isotherm by means of their Henry value. Several minor impurities showing similar adsorption properties were grouped together and treated as a singular key-component (W1, W2, S1, S2).

To calibrate the model, five linear gradients were completed and fractionated under nonlinear preparative conditions. These were recorded varying product loading and gradient steepness, while keeping constant gradient start and end (10% to 90% of Mobile Phase B).

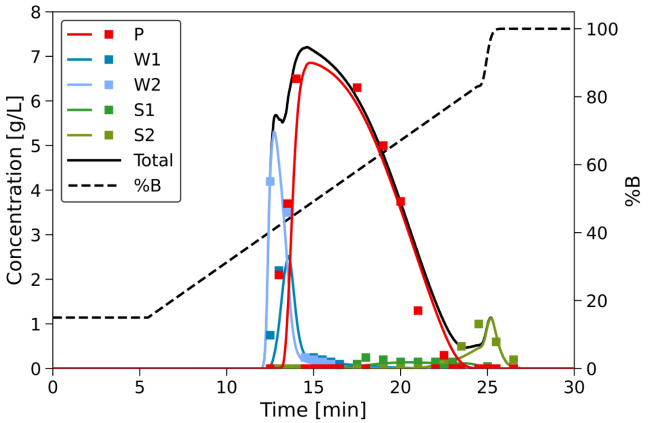

Figure 4 **Comparison between simulated and experimental gradient elution profile of the 20-mer ssRNA. Superimposition of total concentration (black trace), offline analytics (squares) and simulated elution (colored traces). "%B": gradient concentration, "P": Product concentration, "W1" and "W2": groups of weakly adsorbing impurities, "S1" and "S2": groups of strongly adsorbing impurities.**

Table 2 **Operating parameters of the flowchart-based and numerically optimized methods.**

Parameter		Flowchart Batch	Numerically Optimized Batch
Resin loading	[g/L]	25	21
Gradient volume	[CV]	10	8.1
Gradient slope	[%/CV]	7.5	9.6
Gradient	[%B]	15 – 90	13.6 – 91.5

Isotherm parameters were then iteratively regressed by fitting the recorded elution profiles until estimated and experimental profiles exhibited good agreement.

In conclusion, a new fractionated gradient experiment was performed, and Figure 4 presents a comparison between simulated predictions and the offline analytical data. This dataset highlighted accurate predictions of both elution times and peak slopes, confirming the quality of regressions on the adsorption isotherm and mass transport parameters for the oligonucleotide. The method was deemed validated.

The MCSGP process was modeled using the same equations and parameters as the single column process, only differing in initial and boundary conditions.

Results and Discussion

Flowchart-based gradient

After model validation, the flowchart introduced in Figure 2 was used to develop a suitable MCSGP batch design gradient using simulations. The workflow guided the optimization of gradient parameters but also supported the choice of other important process parameters, such as resin loading (g/L) and gradient flow rate (cm/h). The target purity for the gradient was 96 % at > 50 % Yield. The resulting batch method parameters are listed in Table 2.

Figure 5 shows the resulting simulated concentration profile overlayed with the profile of individual components (product + different impurities), and the linear gradient (%B). The precise description of the impurity distribution allowed analysis of the process performance and model-based design of MCSGP.

MCSGP Design and Operation

The MCSGP Wizard facilitates the creation of MCSGP methods based on chromatograms generated from batch processes. In this case, the flowchart-based batch chromatogram (Figure 5) served as design chromatogram. The chromatogram was divided into collection and recycling zones corresponding to regions with pure product or impure product and impurities (Figure 6).

To enhance process robustness, appropriate AutoPeak UV-based dynamic control parameters were chosen. Specifically, an absolute UV threshold value (4.5 g/L) was selected to trigger the start of the Weak Recycling phase, while two relative values (97% and 35% of peak maximum) were selected to initiate the start and end of the product collection phase, respectively.

The process parameters generated by the MCSGP wizard were transferred to the simulation program, which utilized a model structured with the same equations and conditions as the single-column one. This approach ensured a systematic and consistent modeling strategy across both single-column and MCSGP processes. The simulation was performed for ten cycles.

In Figure 7, the superimposition of multiple cycles is presented, and the consistent overlay of the chromatograms suggests a prompt attainment of cyclic steady state. The purity of the product pools was 97.3%.

Numerically Optimized Batch Process

A numerical optimization strategy was used to create a production-optimized batch process for direct comparison with MCSGP (using the flowchart development approach). For a fair comparison the target purity for the “production optimized” batch benchmark run was set to 97.0%, i.e., close to the purity reached in MCSGP simulations.

The numerical batch optimization procedure used a genetic algorithm to identify the optimal set of conditions within a given range of parameters. Optimization involves iteratively exploring and refining potential solutions to find the combination of variables that delivers the best overall process performance.

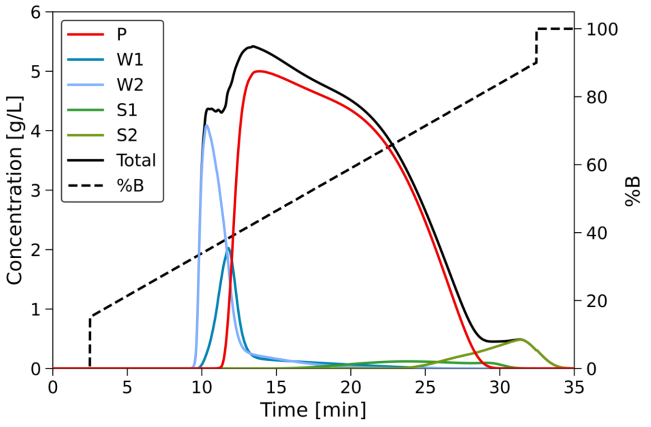

Figure 5 **Simulated single column gradient elution resulting from flowchart-based gradient development procedure.**

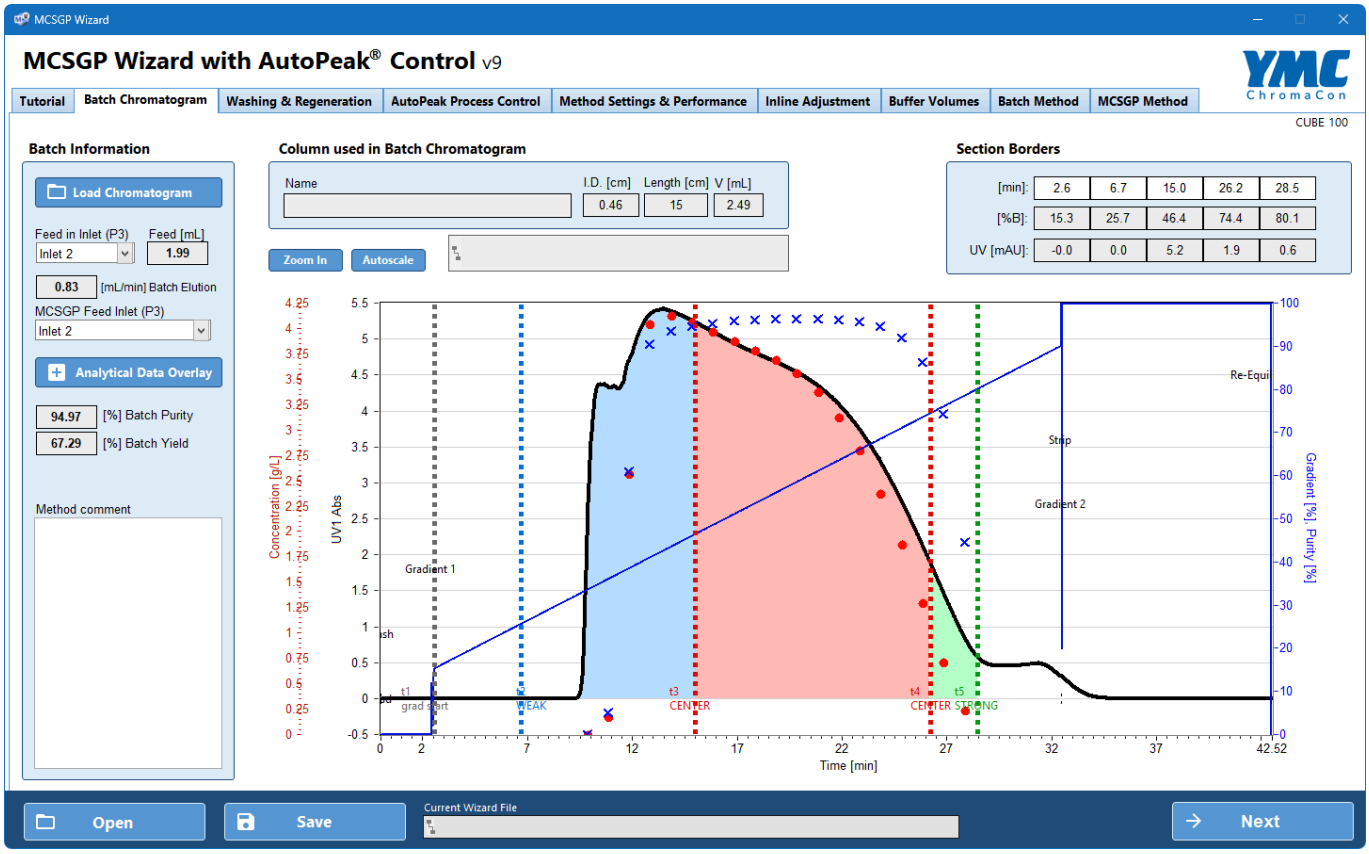

Figure 6 **Screenshot of the Batch Chromatogram tab with the flowchart-based batch chromatogram loaded and subdivided into recycling and product collection zones. Simulated fractions are overlayed. Note that the start of Weak Recycling (blue shaded area) is indicated earlier due to the active AutoPeak control strategy.**

This is guided by a defined objective function instructed to optimize both yield and productivity, assigning equal weights to the two parameters.

The varied parameters were resin loading, gradient duration, and gradient range (%B start and end). The population size of the genetic algorithm was set to 30 and the number of iterations to 30.

The optimized process parameters are listed in Table 2. The chromatogram and key-components distribution obtained with the simulation of the numerically optimized batch procedure are illustrated in Figure 8.

In summary, the numerical approach resulted in a process design with a steeper gradient along with reduced resin loading.

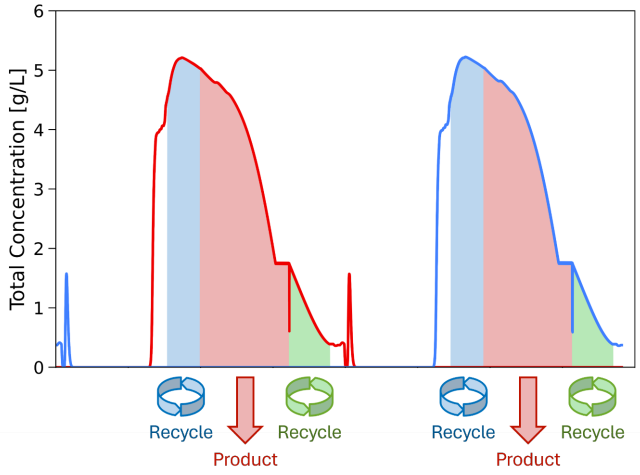

Figure 7 **Superimposition of 5 cycles in the simulated MCSGP process. The first set of product elutions corresponds to column 1 (in red), followed by the second set from column 2 (in blue).**

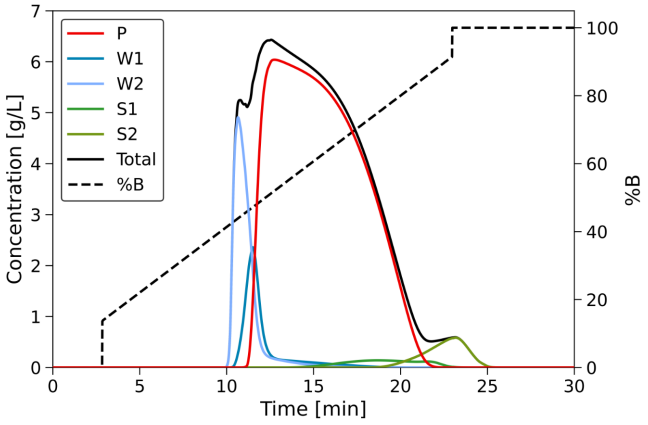

Figure 8 **Simulated single column gradient elution applying numerically optimized batch procedure.**

Process Comparison of Single-Column and MCSGP Processes

The process performances of the flowchart-based batch, the numerically optimized batch, and the twin-column MCSGP procedure were computed and compared based on parameters such as Purity, Yield, Productivity, Product concentration, and Eluent consumption. The pool purity reached in the MCSGP simulation (97.3 %) significantly exceeds the initial purity requirement of > 96.0 %. As a result, this value was chosen as the purity benchmark for comparing the two processes and as a constraint in the development of the numerically optimized single column run.

Table 3 provides a numerical comparison between the MCSGP setpoint and the performance of the numerically optimized batch process. The flowchart-based batch process could not deliver product at a comparable purity level and was thus excluded from the numerical comparison.

The superior purity achieved by MCSGP can be attributed to the combined effects of the countercurrent nature of the MCSGP process, which enhances separation efficiency, and the cycle-to-cycle depletion of undesired compounds. This depletion results in a growing displacement effect of the main product towards more weakly and strongly adsorbed impurities. Together, these factors can contribute to a consistently higher purity of the eluate in MCSGP processes. This can prove particularly advantageous, providing the opportunity to eliminate a second-dimension purification step and thereby contributing to a considerable reduction of the

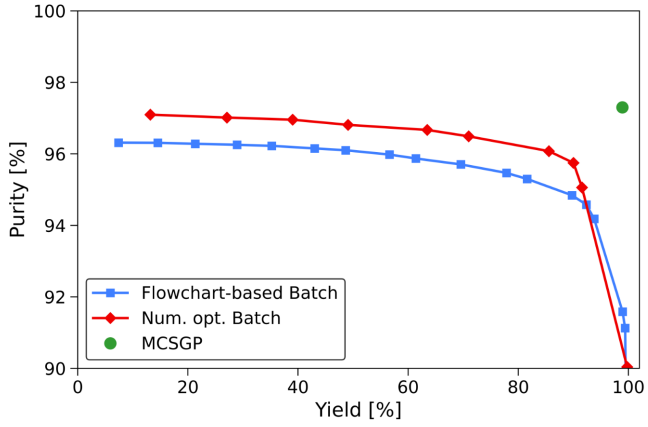

Figure 9 **Pareto curve of the MCSGP and the single-column batch reference runs. The individual data points represent different options for pooling product-containing fractions. MCSGP performance showed that the Yield/Purity tradeoff of the batch runs is overcome.**

Table 3 **Process comparison of numerically optimized batch vs. MCSGP simulation runs, under a purity constraint of 97.0%.**

Parameters		Numerically Optimized Batch	MCSGP
Load	[g/L]	21	23
Pool Purity	[%]	97.0	97.3
Pool Yield	[%]	27.1	98.9
Pool Conc.	[g/L]	5.8	4.0
Productivity	[g/L/h]	5.4	10.7
Eluent Cons.	[L/g]	2.7	1.5

Process Mass Intensity (PMI) index of the entire purification procedure.

As described by the Pareto curve shown in Figure 9, the numerically optimized batch showed superior performance compared to the flowchart-guided batch design. Specifically, it led to a pool with higher purity and higher product recovery. These improvements are mainly attributable to the reduced resin Load, whilst the observed increase in Productivity and reduction in Eluent Consumption is an effect of using steeper gradient and shorter elution time.

As mentioned in the Introduction section, even though superior in batch performance, an optimized batch procedure does not necessarily translate to an optimal starting point for designing an MCSGP process. The higher performances of the described optimized run are mainly attributed to a reduction of the column loading. In MCSGP, this approach is not advantageous as the Yield loss resulting from coelution of product and impurities is mitigated through the automatic recycling and repurification of the impure side fractions. Hence, employing such an approach would ultimately hinder process output and Productivity.

The MCSGP setpoint, represented with the green point in the upper right corner in the Pareto chart in Figure 9 strongly outperformed both the design batch process and the benchmark optimized batch simulations with respect to Yield, achieving almost complete recovery of the input material.

As expected, MCSGP alleviates the typical tradeoff between Yield and Purity of single column chromatography. This MCSGP setpoint exhibited superior performance not only in Yield but also in terms of productivity and buffer consumption. In comparison to the benchmark optimized batch, it proved to be about twice as productive and required less eluent per mass of purified product.

Conclusion

The selection of appropriate gradient conditions to design MCSGP is a pivotal step in the development of a successful twin-column continuous process.

A flowchart has been developed to guide Contichrom CUBE users towards a faster and more efficient development of appropriate starting conditions and gradient. Adhering to this flowchart while working with an oligonucleotide system, a design gradient chromatogram was implemented and then used as initial step to establish an MCSGP Wizard procedure.

The results of the MCSGP run were compared to those of a numerically optimized single-column process, revealing several advantages for the MCSGP process:

- It achieved a pool purity higher than initially required (97.3 % vs. 96 %), primarily due to the countercurrent nature of the process.
- In comparison with the optimized batch run, it delivered 3-fold higher recovery values.
- Productivity was almost doubled compared to the numerically optimized single-column benchmark.

In conclusion, adhering to the flowchart (Figure 2) enables a guided, straightforward and safe transition from single-column chromatography to MCSGP. Overall, the results confirm how the primary advantage of MCSGP lies in significantly improving product yields, especially at the elevated target purity, when compared to single-column chromatography.

In application note 2 of this series we introduce a second flowchart with strategies to optimize MCSGP directly.

YMC ChromaCon Modeling Services

YMC ChromaCon offers a standardized modelling service package for initial MCSGP development based on a predefined customer-supplied experimental dataset as well as services for subsequent MCSGP optimization. Additionally, customized modeling services are available upon request.

Table 4 YMC ChromaCon Modeling Service Package

Product	Order #
Model-based MCSGP Feasibility Study Service	700034

Table 5 Contichrom CUBE 30/100 System Specifications

Parameter	Value
Flow rate range	0.1 – 36 / 0.5 – 100 mL/min
Pressure rating	100 bar
Number of columns	1-2
Number of buffers	Up to 18
Fractionation	Fraction collector with multiple rack options
UV	4-channel external detector (200-600 nm) behind each column
Conductivity	1 conductivity behind each column
pH	1 pH probe at product outlet

Table 6 Contichrom CUBE 30/100 Ordering Information

Product	Order #
Contichrom CUBE 30	220060
Contichrom CUBE 100	220062

Contichrom CUBE for Lab-Scale Development

The MCSGP process with AutoPeak control can be operated by all Contichrom CUBE systems. The Contichrom CUBE is a versatile preparative laboratory-scale chromatography system for single- and twin-column processes with 100 bar (1450 psi) pressure rating. ChromIQ, the operating software of Contichrom systems, contains a wizard for designing and operating the MCSGP process.

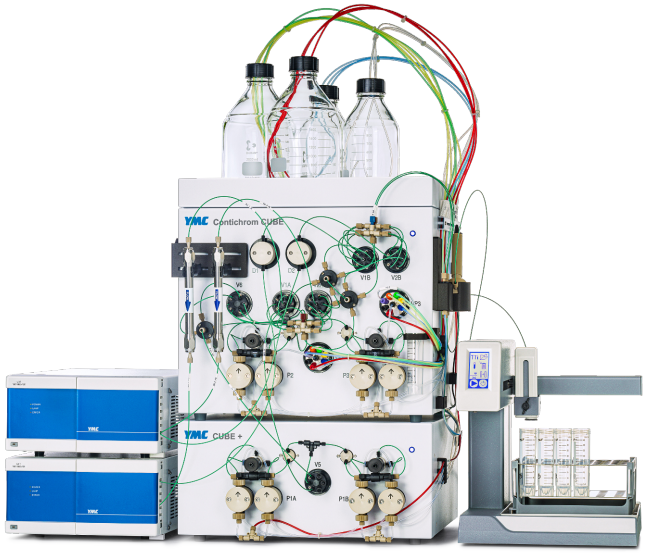

Figure 10 Contichrom CUBE benchtop chromatography system

Contichrom TWIN HPLC Scale-Up Systems

With the Contichrom TWIN HPLC series from YMC America, MCSGP with AutoPeak control is available for manufacturing under GMP conditions. The twin-column scale-up systems have been co-developed by YMC America and ChromaCon AG to ensure easy process transfer and scale-up.

For inquiries regarding the Modeling Services and Contichrom systems, please visit www.chromacon.com or contact sales@chromacon.com.

Figure 11 Contichrom TWIN process scale chromatography system

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