Purification of a Therapeutic Oligonucleotide Using Twin-Column Chromatography (MCSGP)

This application note demonstrates a significant improvement in yield and productivity by converting an optimized batch chromatography method to Multi-column Counter-current Solvent Gradient Purification (MCSGP) for the manufacturing of a therapeutic oligonucleotide.

By automatically recycling low purity product side fractions on a second column of the same type, MCSGP eliminates the tradeoff between yield and purity intrinsic to single column batch chromatography. In this example, MCSGP vastly outperformed an optimized single column process with 94% yield at target purity, compared to only 60% yield for the batch process. Method development was streamlined using a MCSGP design wizard with Contichrom® HPLC hardware, allowing for rapid application of this technology.

Introduction

Chemically synthesized oligonucleotides have become a vital research tool, used for DNA sequencing, polymerase chain reaction (PCR), and molecular cloning. The untapped potential of oligonucleotides is their ability to modify gene expression in a targeted manner for novel and potent therapeutic benefits in humans. After decades of development to refine their pharmaceutical properties, oligonucleotides are now emerging as a major drug-development platform after small molecules, peptides, and protein biologics. It is anticipated that the oligonucleotide platform will soon be ready to treat a wide range of genetic diseases that were previously untreatable. Indeed, clinical trials using oligonucleotide therapies have steadily progressed resulting in substantial incentive to adopt and optimize better techniques for the long-term production at greater scale. Given the high cost of oligonucleotides synthesis, any increase in yield can have a major benefit upon the economics of production.

The production of oligonucleotides for therapeutic use requires that any undesired byproducts of synthesis be removed to obtain pharmaceutical grade purity. To this end, a chromatographic polishing step is routinely used, but this typically reduces the overall yield of production by 40-60%. Given the high cost of synthesis, poor chromatographic performance downstream has a major negative impact on the economics of the overall production. As countermeasure, lower purity "waste" products can undergo re-chromatography, however this greatly reduces process productivity and significantly increases the burden of analytical characterization of product fractions. Thus, manufacturers are looking for alternative, more advanced chromatographic methodology to boost yield.

MCSGP simultaneously achieves high purity and yield. It has been shown to increase yield at target purity by up to 90% compared to single-column batch chromatography in a scalable manner. Thus, MCSGP overcomes the yield-purity tradeoff typically seen in single-column batch chromatography and often operates with higher productivity and lower solvent consumption due to the increased yield. This application note shows the benefits of applying MCSGP versus single column batch chromatography for the purification of an oligonucleotide therapeutic, obtaining maximum yield at target purity.

Principle of MCSGP

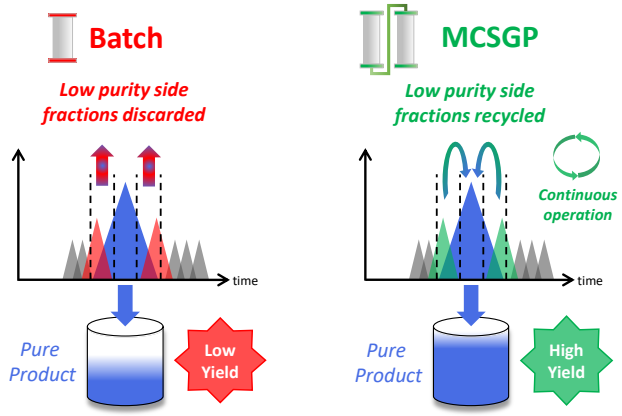


Fig. 1. Traditional single column chromatography (left): side fractions are discarded or kept for re-chromatography. MCSGP (right): side fractions are internally recycled, continuously removing impurities and collecting pure product.

MCSGP is a chromatographic method operated continuously using two columns, compared to the batch mode of single-column chromatography (Fig. 1). Much of the basic principle, however, remains the same. In a chromatographic process, the impure feed mixture is separated, giving fractions with pure product, fractions with a mixture of impurities and product, and

fractions containing only impurities. Pure product fractions are collected, and impure product-containing side fractions are discarded or re-processed. If product containing side fractions are discarded, valuable product is lost. Re-processing the side fractions accumulates impurities and applicability is thus limited. Moreover, reprocessing reduces overall productivity. MCSGP can isolate most of the product included in the starting material by internally recycling product-containing side fractions and repeatedly separating impurities and pure product. The recycling is done automatically, without the need for off-line analysis. The impure side fractions are transferred from one chromatographic column to another. Fresh feed material is added every cycle. This ensures continuous operation and leads to a product with high yield without compromising purity.

Materials and methods

The therapeutic oligonucleotide was prepared at 45 g/L in 30% ammonium hydroxide and purity was 73.8%, as determined by analytical reverse phase HPLC (Fig. 2).

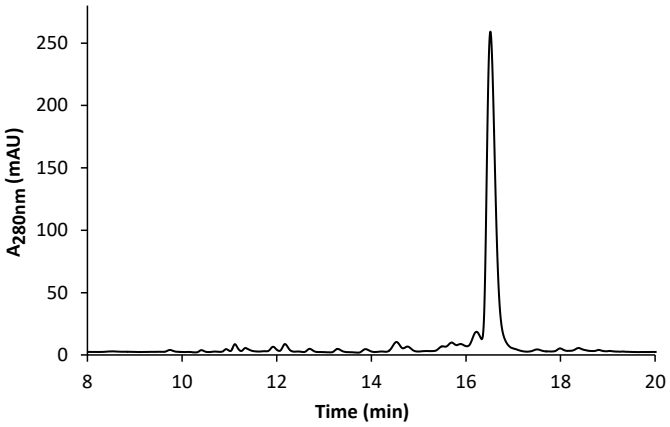


Fig. 2. Reverse phase analytical chromatogram of the feed material.

Preparative chromatography methods: The chromatographic method was scaled down from the single-column method used in large scale production. Oligonucleotide was purified in single-column batch and twin-column MCSGP modes. For both modes, a Contichrom HPLC 30 system was used (flow rate range 0.1-36 mL/min). UV absorbance was measured and recorded at 280 nm. Two HiScreen Q Sepharose FF columns of 0.77 cm inner diameter and a bed height of 10 cm were used (column volume of 2x 5 mL). The solvents for preparative chromatography were 25 mM NaOH (solvent A) and 25 mM NaOH + 2 M NaCl (solvent B). For single column runs, the load volume per injection was 1 mL feed per mg resin. Product elution was run under linear gradient conditions from 10% B to 90% B for 24 CV (column volumes). Three single column chromatography runs were carried out, one as a performance benchmark, and two as a basis for MCSGP process design.

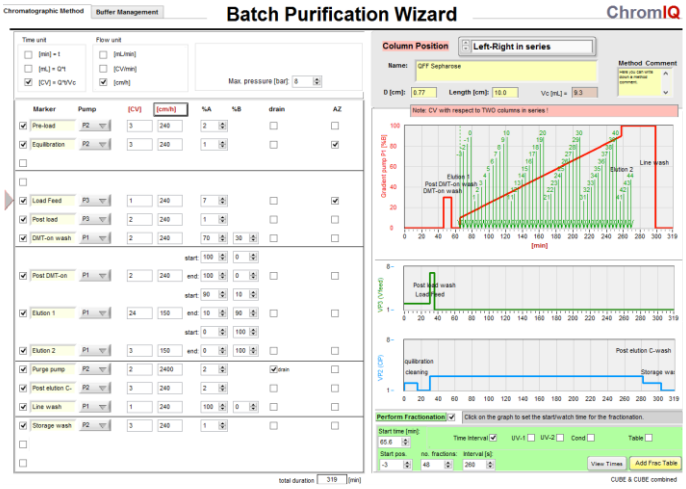


Fig. 3. User interface of the Batch Wizard of the ChromIQ software.

The batch methods were created using the Batch Wizard as shown in Fig. 3. Parameters that were varied are described in Table 1.

Table 1. Comparison of single column method parameters. The numbers in the method names (150 & 300) correspond to the elution flow rates.

		Benchmark	*Batch Run 150*	*Batch Run 300*
Bed height	[cm]	20	10	10
Loading Flow rate	[cm/h]	240	120	120
Elution flow rate	[cm/h]	150	150	300
Washing, cleaning flow rate	[cm/h]	240	480	480

The *Benchmark* batch run was carried out using 20 cm bed height and this provided reference process performance identical to that found at production scale. MCSGP was adapted from this reference batch experiment. For MCSGP two columns with half the bed height were used in order not to exceed a total height of 20 cm during recycling steps when columns are operated in-series. As a result of this, the steps that involve only a single 10 cm column (elution, wash, CIP, regeneration) can be run at 2x to 3x greater linear flow rates without exceeding pressure limitations of the columns. This translates into increased productivity. A single column run with elution flow rate of 300 cm/h (*Batch Run 300*) was carried out using a 10 cm column and used as basis for MCSGP design. The run was

fractionated, and the fractions were analyzed using offline HPLC to generate accurate product purity profiles from which MCSGP can be quickly adapted.

Single column chromatography results

The chromatographic results of the preparative batch purification runs are shown below (Fig. 4 & Fig. 6). Additional analytical evaluation of the elution fractions was carried out using reversed phase HPLC to measure product purity and the product concentration was determined using a Nanodrop spectrophotometer (Fig. 5 & Fig. 7).

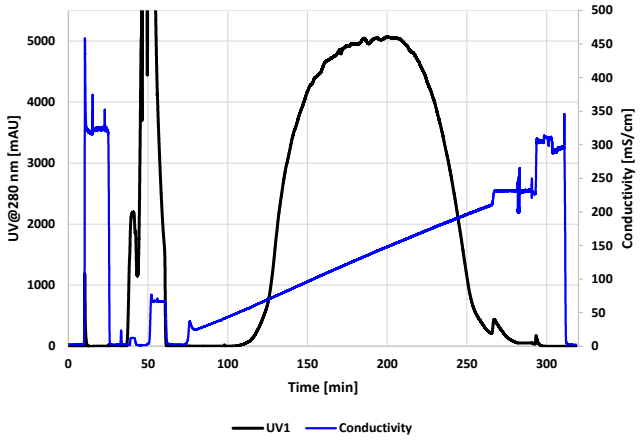


Fig. 4. *Benchmark*: Preparative batch chromatogram showing the Contichrom® online signals (black: A280, blue: conductivity).

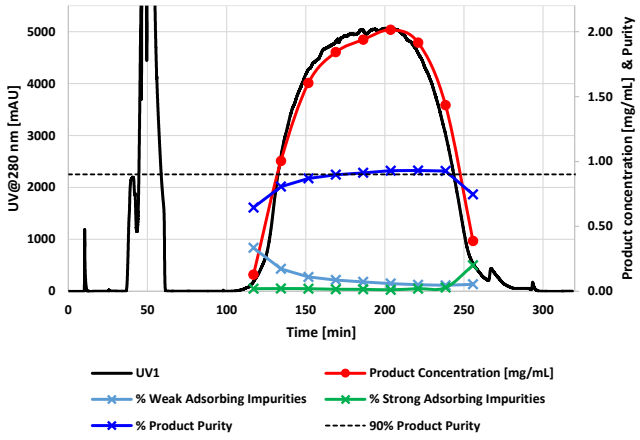


Fig. 5. *Benchmark*: Offline analysis of fractions taken from the elution phase of the preparative run.

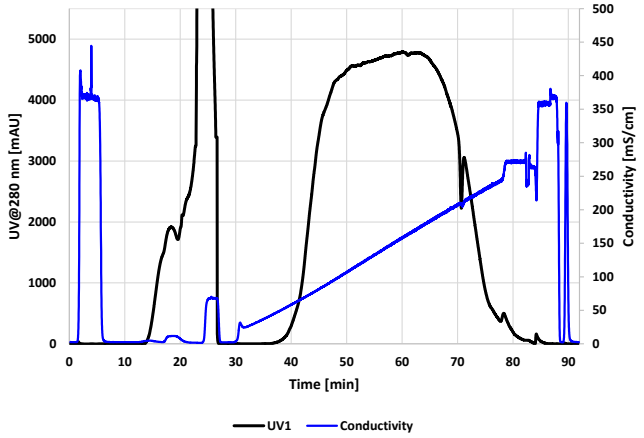


Fig. 6. *Batch Run 300* used for MCSGP design: Preparative batch chromatogram showing the Contichrom® online signals (blue: A280, black: conductivity).

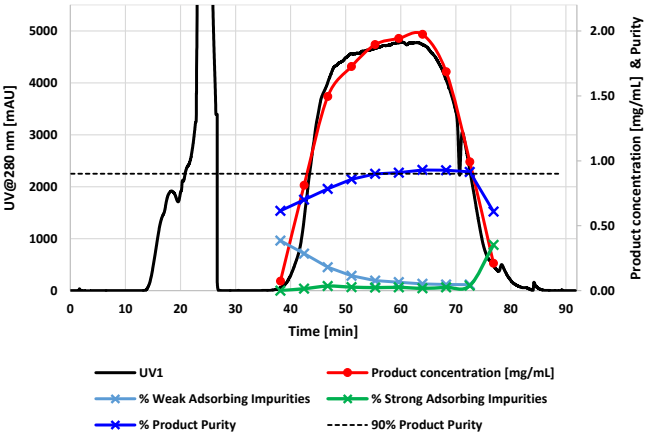


Fig. 7. *Batch Run 300*: Offline analysis of fractions taken from the elution phase of the preparative run.

A detailed process comparison of the batch runs is found in Table 5 on p. 7. Briefly, for a target purity of >91%, the 10 cm bed height showed increased productivity compared to the 20 cm bed height used in the *Benchmark* run due to the higher linear flow rates used. However, this came at the cost of significantly reduced yield (-5%) due to the narrower collection window in which the product meets the purity specification.

Designing an MCSGP run

The MCSGP Wizard facilitates the creation of twin-column MCSGP methods based on chromatograms generated from single-column preparative batch experiments (see Fig. 8). It is integrated into the ChromIQ operating software of Contichrom systems. The MCSGP process uses the same columns, solvents/buffers and same washing and cleaning protocol as the single column preparative process.

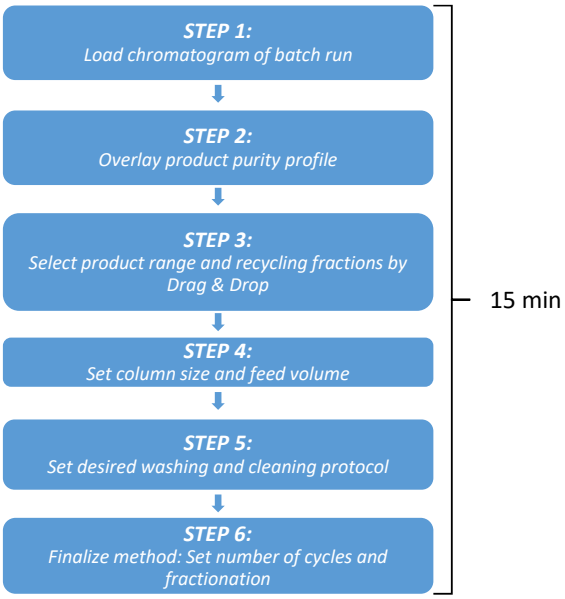


Fig. 8. Schematic of the guided MCSGP process design procedure provided by the MCSGP Wizard. Starting from a single column batch chromatogram, the overall design procedure was completed within 15 min.

In step 1 of the design procedure, the single column batch chromatogram was loaded into the MCSGP Wizard (Fig. 9). In step 2, the product purity and concentration data from offline analysis of the fractions of the single column run were overlaid with the chromatogram (Fig. 9).

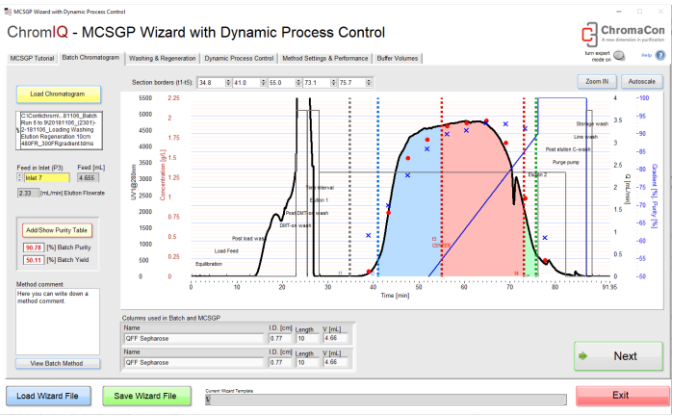


Fig. 9. Graphical user interface of the MCSGP Wizard of ChromIQ.

In step 3 of the design procedure, the chromatogram was divided into zones corresponding to fractions with pure product, impure product or impurities (Fig. 9 & Fig. 10), by drag & drop of the section borders. The expected pool purity is computed by the software and automatically updated based on the position of the section borders.

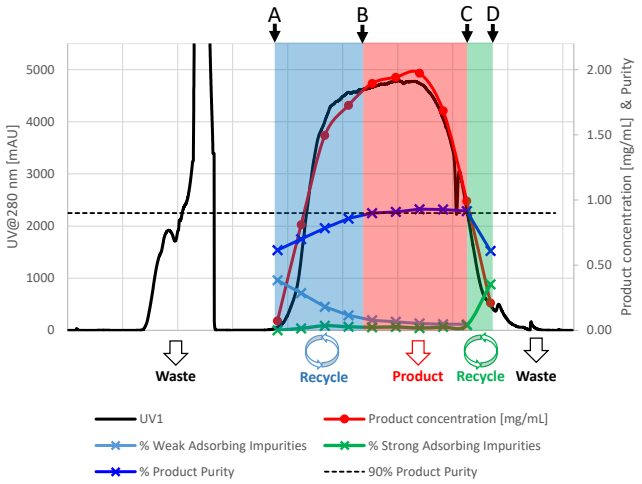


Fig. 10. Schematic of step 3 of the design procedure of the MCSGP Wizard, which encompasses the definition of the zones of the chromatogram selected for internal recycling (A-B in blue and C-D in green), and the product elution window (B-C in red).

Three zones are highlighted (Fig. 9 & Fig. 10), corresponding to pure product (red) and impure, product-containing side-fractions (blue and green). For MCSGP operation, these zones correspond to tasks of recycling of impure product fractions in front of the peak (from A to B in Fig. 10), to product elution (from B to C in Fig. 10), and to recycling of impure product fractions in the peak tail (from C to D in Fig. 10). In summary, the zone borders A-D were defined. The MCSGP wizard assigns the following tasks to these points:

- A: start recycling at the time when product starts eluting from the column
- B: start product collection when product reaches 90% purity
- C: stop product collection and start product recycling when product falls below 90% purity
- D: stop recycling when product elution is complete

In step 4 of the design procedure, the column dimensions and feed volume were defined. Two columns of the same type and dimensions (per column) as in the single-column batch run were used for MCSGP. The Wizard sets the feed volume relative to the batch design run, whereby a % of the original feed volume is fed based upon the size of the product collection window (Fig. 9 red zone) relative to the recycling phases.

In step 5 of the design procedure, the washing and cleaning protocols for MCSGP were entered. The same protocols as in batch chromatography were used. The MCSGP Wizard then automatically calculates suitable in-line dilution factors to

ensure that impure product is re-adsorbed on the downstream column during the recycling phases.

In step 6 of the design procedure, the MCSGP Wizard displays the expected consumption of starting material, allowing adjustment of the number of cycles. Furthermore, the Wizard automatically generates start-up and shutdown methods.

Finally, the predicted process performance parameters are displayed, including product purity, productivity, feed and buffer requirements, and product pool concentration. The complete design procedure for each run was completed within 15 min.

Three MCSGP runs were carried out with conditions shown in Table 2 with elution flow rates of 150, 300 and 450 cm/h, respectively, to test performance within the pressure limits of the columns.

Table 2. Comparison of MCSGP method parameters. The numbers in the method names (150, 300 & 450) correspond to the elution flow rates.

		MCSGP Run 150	*MCSGP Run 300*	*MCSGP Run 450*
Bed height	[cm]	2x 10	2x 10	2x 10
Loading Flow rate	[cm/h]	150	150	150
Elution flow rate	[cm/h]	150	300	450
Washing & cleaning flow rate	[cm/h]	480	480	480

MCSGP operation

As a representative example, the chromatogram for *MCSGP Run 150* is shown in Fig. 11. The chromatogram shows a repetitive pattern of product elutions from the two columns. There is one product elution from each column per cycle.

The ChromIQ operating software also allows the superimposition of multiple consecutive cycles of a continuous process such as MCSGP (Fig. 12). The good overlay fit of the chromatograms indicates that cyclic steady state is reached by cycle 2.

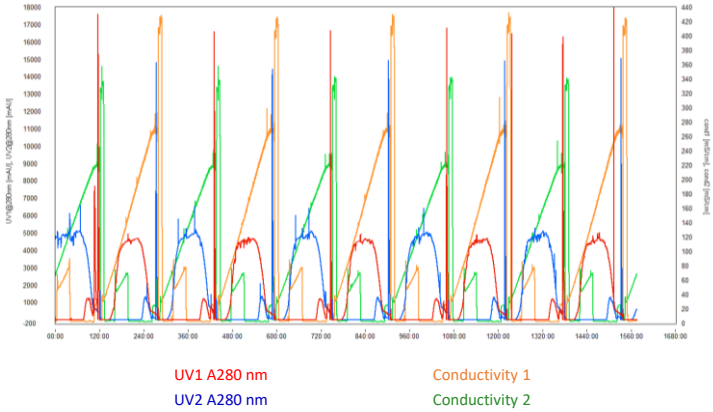


Fig. 11. Example chromatogram of *MCSGP Run 150* showing 5 consecutive cycles.

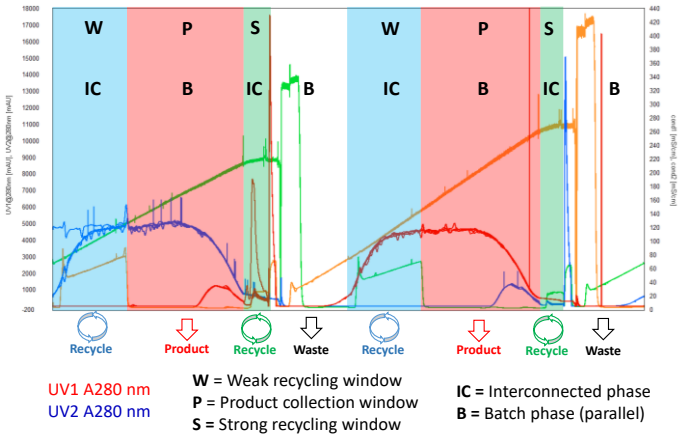


Fig. 12. Superposition of 5 cycles of a representative MCSGP run for purification of an oligonucleotide. It can be seen that the elution profiles change only slightly and that the product peaks are very similar. The first set of product elutions is from column 2 (blue), the second from column 1 (red).

Product was pooled on per cycle basis (i.e., two product elutions were pooled, one per column). Offline analytical evaluation confirmed that product amount, concentration and purity was constant from cycle to cycle. Product purity was evaluated using reverse phase HPLC. A superposition of the analytical chromatograms for five MCSGP cycles is shown in Fig. 13. Analytical HPLC results confirmed that purity exceeded the target threshold of 91% for all three MCSGP runs, 5 cycles per run, as seen in Table 4.

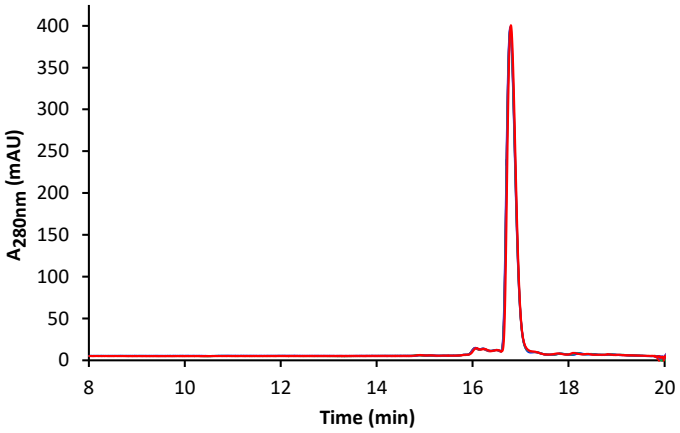


Fig. 13. Reverse phase analytical chromatogram of oligonucleotide product. 5 MCSGP cycles were superimposed showing consistent product quality. Results are shown for *MCSGP Run 150* and were similar for all 3 operating conditions tested.

Table 3 shows the process performance average over 5 cycles of run *MCSGP Run 300* in comparison to the prediction provided by the MCSGP Wizard during the process design procedure. The results are very similar, confirming that the wizard can serve as initial process performance prediction tool based on single column data.

Table 3. Process comparison of MCSGP Run 300 and wizard prediction.

	$c_{product}$ [mg/mL]	Purity [%]	Y_{cycle} [%]	Prod. [g/L/h]	B.C. [L/g]
Exp. Run	1.62	92.0%	91.2%	5.9	2.7
Prediction	1.84	90.5%	100%	6.6	3.0

Process comparison of single-column and MCSGP operation

The process performance for the single-column batch and twin-column MCSGP processes was calculated and compared in terms of yield, purity, productivity, product concentration and solvent/buffer consumption. MCSGP displays significant advantages over batch chromatography (Table 5).

The *Benchmark* experiment using a 20 cm bed height column was compared with two equivalent single-column experiments using shorter 10 cm columns (*Batch Run 150* and *Batch Run 300*). As could be expected, shorter columns lead to yields that were significantly lower than with the longer column, due to the reduced resolution. However, if yields were not of concern, shorter columns would be more desirable in terms of productivity especially when higher flow rates are used. At the cost of yield reduction from 60% to 55%, through use 10 cm bed height columns, a 3-fold productivity improvement was achievable compared to the *Benchmark* experiment.

The main advantage of MCSGP is that at the target purity of >91%, product yields are greatly improved compared to all single-column experiments. Depending on the tested flow rate, 50-57% more product was recovered at 92% purity compared to the *Benchmark* single column experiment as seen in Table 5, Fig. 12 and Fig. 14. The advantages in yield were even greater when compared to the 10 cm column batch experiments.

Concerning productivity, MCSGP has the advantage of facilitating higher flow rates during elution than the *Benchmark* single column experiment because the column bed height is halved. This means that productivity was doubled compared to the *Benchmark* run while yield was still above 90% (Fig. 15). When comparing MCSGP directly to the 10 cm batch runs, the two batch runs have better productivity. Firstly, like MCSGP, the 10 cm columns support the higher flow rates. Secondly, unlike MCSGP, there are no interconnected steps that require a lower flow rate. Finally, due to the recycling intrinsic to MCSGP, only the quantity of material that was removed from the system is fed each subsequent cycle, which in this case was ≈50% of the load per mL resin compared to the batch experiments. In terms of productivity, the large increase in yield in MCSGP was not enough to compensate for these other factors. However, the modest advantage in productivity of the 10 cm batch experiments comes at the great price of an even lower yield than the *Benchmark* run. For many oligonucleotide productions at scale, it is economically more beneficial to have a 5% increase in yield than to improve productivity 3-fold (see Table 5 *Benchmark* vs. *Batch Run 300*). This is due to the very high synthesis costs. Indeed, the advantages in yield are so great in MCSGP that the economic savings are expected to offset rapidly any additional cost for the equipment needed to carry out the more specialized process (see Müller-Späth, T., & Bavand, M. (2019). Purification of Synthetic Peptides by Countercurrent Chromatography (MCSGP)-Economic Evaluation. *Pharmaceutical Engineering*, *39*(2), 68–77). Therefore, yield improvement was the main objective in this study.

Table 4. Cycle by cycle comparison of MCSGP results for 3 different operating points.

MCSGP RUN 150	Volume	Mass $_{product}$	Purity	Yield $_{cycle}$	Productivity	Load	Buffer Consumption
	[mL]	[mg]	[%]	[%]	[g/L/h]	[g/L]	[L/g]
Feed input (All cycles)	29.89	978.4	73.1%			20.57	
Cycle 1	106	184.0	92.0%	94.0%	3.76	4.20	0.23
Cycle 2	106	186.4	92.3%	95.2%	3.81	4.20	0.23
Cycle 3	106	185.5	91.8%	94.8%	3.80	4.20	0.23
Cycle 4	106	184.9	91.6%	94.5%	3.78	4.20	0.23
Cycle 5	106	183.1	91.8%	93.6%	3.75	4.20	0.24

MCSGP RUN 300	Volume	Mass $_{product}$	Purity	Yield $_{cycle}$	Productivity	Load	Buffer Consumption
	[mL]	[mg]	[%]	[%]	[g/L/h]	[g/L]	[L/g]
Feed input (All cycles)	27.16	889.0	73.1%			18.69	
Cycle 1	100	167.4	91.9%	94.2%	6.08	3.82	0.26
Cycle 2	100	156.5	91.9%	88.0%	5.68	3.82	0.28
Cycle 3	100	161.6	92.0%	90.9%	5.86	3.82	0.27
Cycle 4	100	161.5	92.0%	90.8%	5.86	3.82	0.27
Cycle 5	100	163.8	92.0%	92.1%	5.94	3.82	0.27

MCSGP RUN 450	Volume	Mass $_{product}$	Purity	Yield $_{cycle}$	Productivity	Load	Buffer Consumption
	[mL]	[mg]	[%]	[%]	[g/L/h]	[g/L]	[L/g]
Feed input (All cycles)	27.11	887.4	73.1%			18.66	
Cycle 1	100	156.5	91.7%	88.2%	8.14	3.81	0.28
Cycle 2	100	159.4	91.5%	89.8%	8.29	3.81	0.27
Cycle 3	100	161.6	91.7%	91.0%	8.40	3.81	0.27
Cycle 4	100	161.1	91.8%	90.8%	8.37	3.81	0.27
Cycle 5	100	161.3	91.9%	90.9%	8.38	3.81	0.27

Table 5. Process comparison of batch vs. MCSGP runs.

		Benchmark	Batch Run 300	MCSGP Run 150	MCSGP Run 300	MCSGP Run 450
Bed height	[cm]	20	10	2x 10	2x 10	2x 10
Loading Flow rate	[cm/h]	240	120	150	150	150
Elution flow rate	[cm/h]	150	300	150	300	450
Washing, cleaning flow rate	[cm/h]	240	480	480	480	480
Pool Purity	[%]	91.9%	91.6%	91.9%	91.9%	91.7%
Pool Yield	[%]	60.2%	55.7%	94.4%	91.2%	90.1%
Pool Conc	g/L	1.81	1.70	1.7	1.6	1.6
Mass balance	[%]	80.8%	84.0%	94.4%	91.2%	90.1%
Productivity	[g/L/h]	3.7	11.9	3.78	5.89	8.32
Load	[g/L]	32.3	32.8	20.6	18.7	18.7
Buffer cons.	[L/g]	2.4	2.6	2.3	2.7	2.7

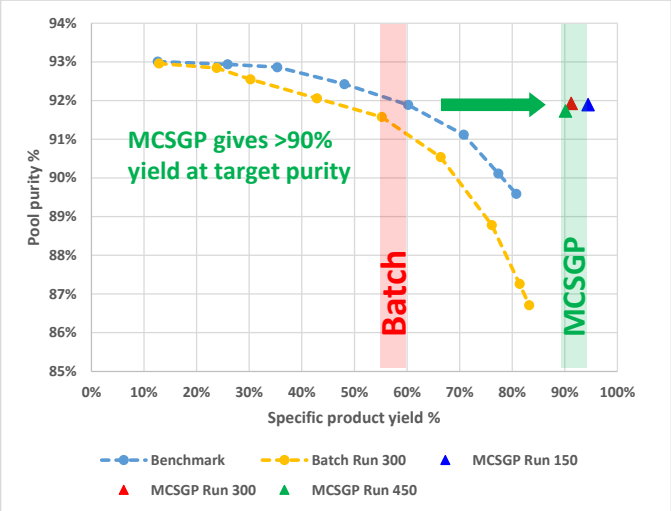


Fig. 14. Pareto curve of the MCSGP (triangles) and the single-column batch reference runs (circles). The individual data points represent different options for pooling product-containing fractions. MCSGP performance shows that the yield/purity tradeoff of the batch runs is overcome.

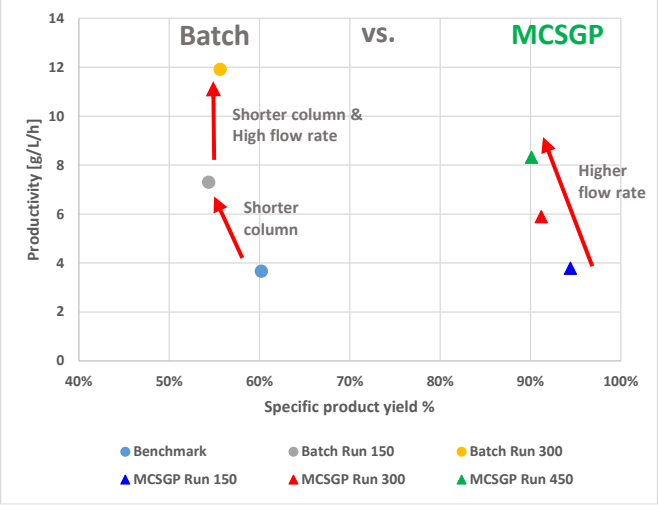


Fig. 15. Yield-productivity chart showing experimental results of MCSGP (triangles) and the single-column batch reference runs (circles).

Summary

MCSGP offers significant advantages over single-column chromatography in the purification of oligonucleotides. The advantages include:

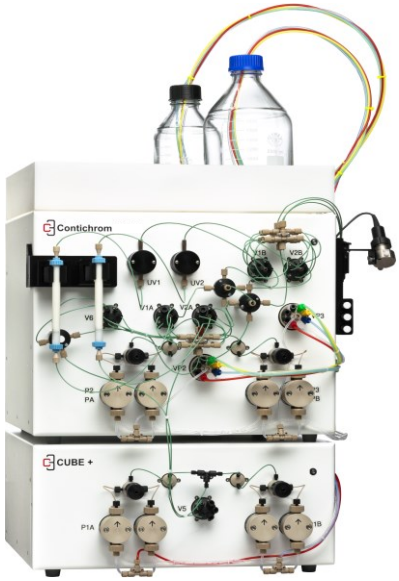
- Oligonucleotide yields were increased by at least 50%, leading to over 90% recovery at 92% purity (target purity was >91%)
- For single-column chromatography, the recovery was 60% at 92% purity
- Increased yield allows massive scale-down of oligonucleotide synthesis upstream to obtain required oligo amounts
- The productivity was increased 2-fold compared to the *Benchmark* run
- A massive reduction in the number of samples for analytical characterization, especially if re-chromatography of waste fractions is carried out. (Only one sample is generated per MCSGP cycle for two feed injections, while multiple fractions per injection are generated in single-column chromatography)
- The high yield of MCSGP makes re-chromatography obsolete
- All other process parameters were comparable to the batch runs

The increase in productivity by MCSGP allows multiple manufacturing options:

- The same target amount can be produced within the same time with smaller columns
- A specified target amount can be produced in a shorter period with columns of the same size
- More target compound can be produced per total column volume in the same amounts of time

Contichrom® CUBE

The MCSGP process 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.



Contichrom CUBE 30/100 System Specifications	
Flow rate range	0.1 – 36 / 0.1 – 100 mL/min
Pressure rating	100 bar (1450 psi)
Number of columns	1-2
Number of buffers / solvents	Up to 18
Fractionation	3 fractions (valve), optional fraction collector
UV Detectors	4-Channel external variable wavelength detectors with 190-500 nm wavelength
Conductivity / pH detectors	1 conductivity behind each column 1 pH included in system
Optional Column Thermostats	Up to 80°C

Contichrom® Twin HPLC scale-up systems

With the Contichrom Twin HPLC series from YMC Process Technologies (YPT), MCSGP is available for manufacturing under GMP conditions. The twin-column scale-up systems have been co-developed by YPT and YMC ChromaCon to ensure transferability through the scales.

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

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