

## RESEARCH ARTICLE

# UV-based dynamic control improves the robustness of multicolumn countercurrent solvent gradient purification of oligonucleotides

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**Abstract**

Therapeutic oligonucleotides (ONs) have great potential to treat many diseases due to their ability to regulate gene expression. However, the inefficiency of standard purification techniques to separate the target sequence from molecularly similar variants is hindering development of large scale ON manufacturing at a reasonable cost. Multicolumn Countercurrent Solvent Gradient Purification (MCSGP) is a valuable process able to bypass the purity-yield tradeoff typical of single-column operations, and hence to make the ON production more sustainable from both an economic and environmental point of view. However, operating close to the optimum of MCSGP can be challenging, resulting in unstable process performance and in a drift in product quality, especially when running a continuous process for extended periods where process parameters such as temperature are prone to variation. In this work, we demonstrate how greater process robustness is introduced in the design and execution of MCSGP for the purification of a 20mer single-stranded DNA sequence through the implementation of UV-based dynamic control. With this novel approach, the cyclic steady state was reached already in the third cycle and disturbances coming from fluctuations in the feed quality, loading amount and temperature were effectively compensated allowing a stable operation close to the optimum. In response to the perturbations, the controlled process kept the standard deviation on product recovery below 3.4%, while for the non-controlled process it increased up to 27.5%.

**KEYWORDS**

AutoPeak, chromatography, continuous chromatography, countercurrent, dynamic process control, MCSGP, oligonucleotides, simulated moving bed

## 1 | INTRODUCTION

Despite their great potential, only 18 oligonucleotide (ON)-based drugs are on the market at the time of writing and this partially reflects manufacturing challenges that still need to be addressed.<sup>[1–3]</sup> The production of ONs is mainly performed via phosphoramidite-based solid-phase synthesis, which often suffers from low yield and high

process mass intensity (PMI). In addition, the susceptibility of ONs to degradation by nucleases and poor pharmacokinetics require the incorporation of chemical modifications to the molecule backbones and the nucleobases for enhanced stability, enzymatic resistance, and cell internalization rate.<sup>[1]</sup> The synthesis generates a complex mixture of the target sequence and similar impurities (e.g., shortmers and longmers) that are difficult to separate at preparative scale.<sup>[4–7]</sup> In addition,

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stringent purity requirements make the downstream processing more challenging.<sup>[8]</sup>

The current benchmark for the purification of ONs is represented by liquid chromatography, due to its high resolution, versatility, and ease of scale up. Reversed-phase liquid chromatography is particularly efficient in the purification of the target ON sequence.<sup>[9–11]</sup> The main downside of “center-cut” batch chromatographic purifications is an intrinsic purity-yield trade-off. This trade-off exists for most single column operations in the downstream processing of biomolecules. One option to increase yield and purity simultaneously is to reduce the loading of the column to help resolve the product from impurities. However, this comes at the expense of higher eluent consumption, poorer resin utilization, and lower process throughput.

On the other side, Multicolumn Countercurrent Solvent Gradient Purification (MCSGP) is an effective and practical means to overcome the yield-purity trade-off for different biomolecules, including peptides<sup>[12,13]</sup> monoclonal antibodies<sup>[14–16]</sup> and PEGylated proteins<sup>[17–19]</sup> Over the past years, the twin-column embodiment of this continuous chromatography technology has become predominant. The interested reader is referred to the literature<sup>[20–22]</sup> and to the Supporting Information for a detailed description of MCSGP, with Figure S1 showing the different steps of the process.

While MCSGP has great potential to improve process efficiency, it also provides some new process development challenges because it runs in a semi-automated repetitive manner in a cyclic steady state. Therefore, process robustness is crucial to ensure consistent product quality. In addition, the disturbances that may be introduced over time by alterations in feed quality, column aging and fluctuations in the process parameters need to be promptly compensated.

## 2 | DYNAMIC PROCESS CONTROL FOR MCSGP (AUTOPEAK)

In this work, we demonstrate how the robustness of MCSGP can be improved through the implementation of AutoPeak, a UV-based dynamic process controller. AutoPeak automatically adjusts the duration of the recycling and collection windows in MCSGP in response to system perturbations and the consequent peak shifting based on the chromatogram recorded online and user-defined UV thresholds.

To start the design of MCSGP, the separation is first performed in a single column. The elution peak is fractionated and analyzed. Once the distribution of key impurities and the product has been confirmed by offline fraction analysis, the AutoPeak settings can be defined. MCSGP is traditionally designed by selecting fixed characteristic times for the borders of its three main phases. More specifically, the characteristic times mark the starts/ends of the weak impurities (W)/product (P) recycling phase, the product elution phase and P/strong impurities (S) recycling phases.<sup>[14–16,20]</sup> The transfer to MCSGP is done under the assumption that the MCSGP chromatogram and the batch design chromatogram are sufficiently similar in terms of elution time and shape, such that the characteristic times of the batch chromatogram lead to the desired phase start/end times also in MCSGP. This rigid definition of the phase borders based on time leaves MCSGP vulnerable

against perturbations affecting the position and shape of the elution peak. The AutoPeak control relies on the UV values corresponding to the characteristic times, rather than relying on the characteristic times themselves. In other words, AutoPeak implies that the phase borders are defined by UV thresholds, not by fixed times. Therefore, in case the elution peak shifts because of a parameter change (e.g., temperature), the process phase starts/ends are shifted along with the profile. As long as the parameter change does not significantly impact shape and height of the elution profile, this type of control is expected to deliver the same product concentration and quality as the original method using time-based settings.

While MCSGP is running, AutoPeak constantly compares the recorded UV signal against UV trigger criteria and modifies the MCSGP run protocol by terminating one phase and starting the next process phase based on a UV trigger. This corresponds to a flow path change in the chromatography system (e.g., a change from an interconnected state of the two columns into a parallel state).

Two different types of UV triggers can be distinguished: absolute and relative. Absolute triggers are associated with a fixed UV value (for example “200 mAU”), while relative triggers are defined in reference to a peak maximum value (e.g., “80% of main peak maximum”). Relative triggers can be seen as very robust as they are independent of the actual main peak height. However, they require a clear main peak identification. Obviously, relative triggers can only be used for phase starts/ends occurring after the main peak elution. Main peak identification can be achieved by constantly evaluating the UV signal and confirming a +/- slope change over several data points.

To add more flexibility to the controller, a delay time can be added to the time that is associated with the reaching of a trigger such that the action of starting/ending a phase is delayed.

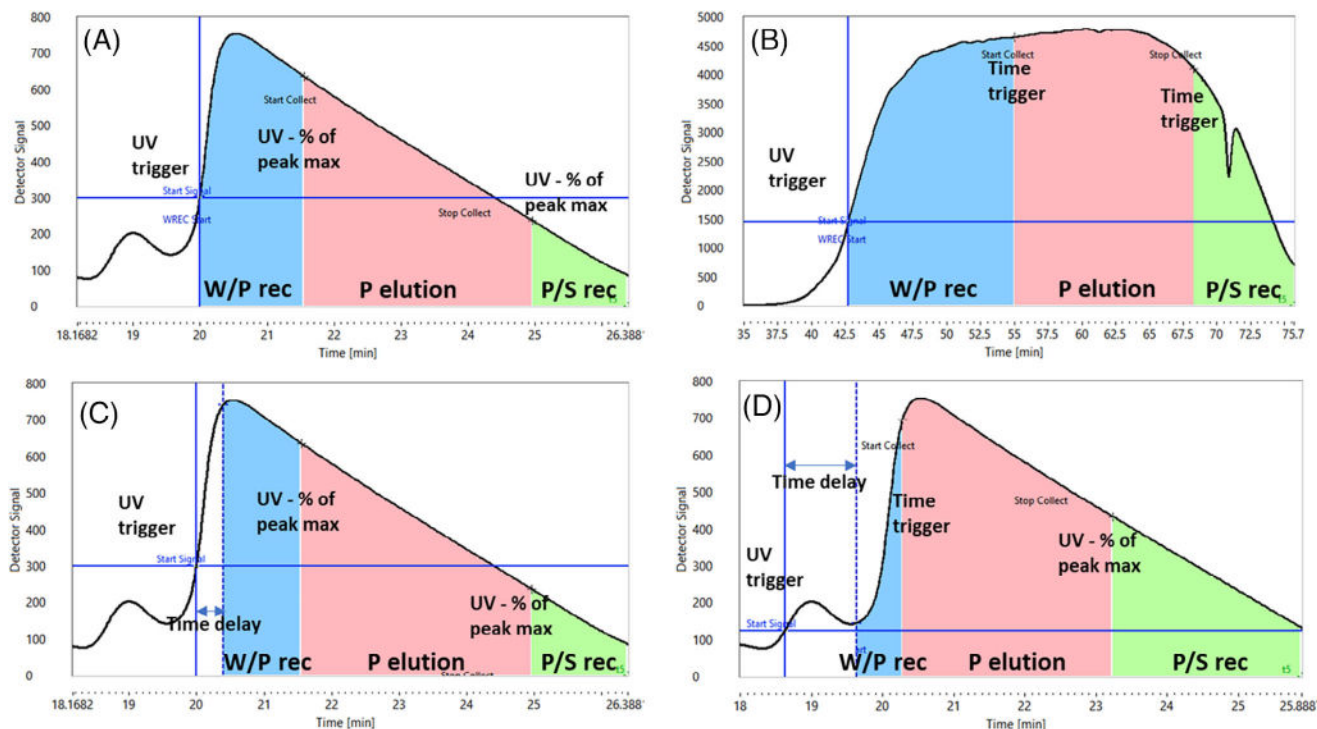
Moreover, depending on UV signal quality, AutoPeak can incorporate the use of “traditional” time-based starting/ending of phases or fixed phase durations.

While numerous combinations of triggers are possible, four examples of AutoPeak are provided in Figure 1, covering typical elution profiles observed in the purification of oligonucleotides.

The configuration reported in Figure 1A shows the start of the W/P recycling triggered by an absolute UV threshold, while the start and stop of the P collection are triggered by a relative UV threshold (percentage of peak maximum), respectively. This approach is typical for product elution profiles that have the highest purity behind the peak maximum. This AutoPeak configuration was investigated in this work.

The second configuration in Figure 1B is employed when the peak maximum cannot be easily identified because of a flat UV trend. In this case, the strategy is a simpler version of the previous one, with the W/P recycling triggered by an absolute UV threshold, while the start and the stop of the P collection window are controlled by time triggers.

The third example, in Figure 1C, shows the implementation of a time delay to start the W/P recycling phase. This time delay is counted starting from an absolute UV trigger, allowing to discard a larger amount of weakly adsorbing impurities, and preventing their accumulation during subsequent W/P recycling. Selecting a low absolute UV trigger value (which is certainly reached) in combination with a delay time reduces



**FIGURE 1** AutoPeak configuration according to different peak shapes and product/impurities distribution during the gradient elution. Four examples for automatic adjustment of product collection and product/impurities internal recycling cycle-by-cycle are shown: (A) W/P recycling triggered by absolute UV, P collection triggered by relative UV, (B) W/P recycling triggered by absolute UV, P collection triggered by times (C) W/P recycling triggered by absolute UV plus a delay time, P collection triggered by relative UV, (D) W/P recycling triggered by absolute UV of an impurity peak and started after a delay time, P collection start triggered by time, and P collection end triggered by relative UV. For clarity, “time trigger” refers to a fixed phase duration, not to a specific elution time.

the risk of missing a UV threshold, which can happen if a higher UV threshold value close to the peak apex is selected, and this value is not reached.

In the last AutoPeak configuration, reported in Figure 1D, product collection is ensured by adding a time-based collection to a UV-based trigger. More specifically, an initial UV-based trigger is combined with a delay time to discard a first impurity peak. After the delay time has passed, W/P recycling is started for a fixed time interval. As the desired product collection start is close to the peak apex, this fixed W/P time was chosen instead of a UV-based threshold to avoid the risk that product collection is not started in case the peak apex UV value is lower than a potential UV threshold value.

In this way, AutoPeak adjusts the duration of the different phases cycle by cycle, making the process reactive to disturbances from different sources without requiring the development of a model of the operation, with the consequent uncertainties on the adsorption equilibria and mass transfer phenomena, as in other approaches.<sup>[23]</sup>

In this work, we investigated for the first time the design and implementation of AutoPeak in controlling reversed-phase MCSGP in oligonucleotide purification. We first designed a robust MCSGP by properly selecting the three UV thresholds highlighted in Figure 1A. Then, we performed a sensitivity analysis to quantify the robustness introduced by this UV-based dynamic process control compared side-by-side with an equivalent MCSGP with purely time-based control when fluctuations in the process parameters are introduced.

### 3 | MATERIALS AND METHODS

#### 3.1 | Materials

The ON employed in this study was a 20mer single-stranded DNA sequence produced via solid-phase synthesis and provided by YMC Japan. The buffer compositions exploited for both the analytical and the preparative chromatography are reported in the Supporting Information, together with a full characterization of the crude material via reversed-phase high-performance liquid chromatography (RP-HPLC, Table S1). Based on this analysis, three virtual key components have been identified in addition to the product, as detailed in the method description and in Figure S2. These comprise weakly adsorbing impurities 1 (W1) and 2 (W2), and strongly adsorbing impurities (S).

#### 3.2 | Single-column preparative method

Preparative chromatography runs in this study were performed on a Contichrom CUBE 30 (YMC ChromaCon), equipped with a prepacked YMC Triart C18, S–10 μm, 12 nm, 100 mm x 4.6 mm I.D. column from YMC Japan. The chromatogram was recorded at 300 nm with an external UV detector (BlueShadow UV/Vis Detector 40D Fiber Optics, Knauer, 0.5 mm flow cells). The specific operational conditions

employed for the single-column batch experiment used as a reference and for MCSGP design are shown in Table S2.

The evaluation of the process performance for this reference run was conducted assessing the purity and the yield of each fraction taken during the gradient using RP HPLC. The purity was determined according to Equation 1.

$$\text{Purity (\%)} = \frac{\text{Area}_{\text{target}}}{\text{Area}_{\text{total}}} \times 100 \quad (1)$$

where  $\text{Area}_{\text{target}}$  is the area under product peak in the analytical chromatogram of each fraction, while  $\text{Area}_{\text{total}}$  is the sum of all areas under the peaks observed in the analytical chromatogram of each fraction. The yield, which is measured with respect to the target component, can be determined by dividing the mass of the recovered product ( $m_{\text{recovered}}$ ) by the total amount of product initially loaded onto the column ( $m_{\text{total}}$ ), as indicated in Equation 2.

$$\text{Yield (\%)} = \frac{m_{\text{recovered}}}{m_{\text{total}}} \times 100 \quad (2)$$

### 3.3 | MCSGP operation and controller settings

MCSGP was implemented on a Contichrom CUBE 30 (YMC Chroma-Con), equipped with two pre-packed YMC Triart C18, S–10  $\mu\text{m}$ , 12 nm, 100 mm x 4.6 mm I.D. columns. The quantity of loaded crude was calculated to replace the product recovered within the collection window of the previous cycle. During the internal recycling phases, the concentration of organic modifier in the downstream column was adjusted through inline dilution (ID) to the value corresponding to the beginning of the gradient. Hence, the flow rate of the dilution buffer was adjusted to achieve the necessary dilution factor for the recycled stream coming from the upstream column, which maintained a constant linear velocity of 200  $\text{cm h}^{-1}$  throughout the entire elution phase.

Five different MCSGP runs were conducted by changing the dilution factor and the settings of the UV-based process controller in order to investigate their role in reaching and preserving the steady-state. The operating parameters for these five runs are summarized in Table 1.

The evaluation of MCSGP process performance across the different experimental runs is similar to the one for the batch mode. The overall purity and yield values are calculated according to Equations 1–2 over the different cycles from offline analysis of the product pool.

### 3.4 | Sensitivity analysis

The robustness of MCSGP was assessed through a sensitivity study. This analysis quantifies the change in a selected process output when perturbations to a specific input are introduced, leaving all the remaining parameters constant. In this work, we selected the product yield as the output tracked through the sensitivity analysis. Perturbations were applied in terms of: (1) loading duration, (2) feedstock purity, and (3) operating temperature. The Run 5 was used as reference process

(with loading = 15  $\text{g L}^{-1}$  resin, feedstock purity = 90.2%, and temperature = 50°C) and the extent of the perturbations applied to the system are reported in Table S3. This scheme was applied to the system with and without AutoPeak control and in each condition 5 cycles were run. The last three cycles of the operation were considered for performance evaluation.

The normalized sensitivity with respect to yield was calculated according to Equation 3.<sup>[24]</sup>

$$\text{Sensitivity (-)} = \frac{Y(\varphi + \Delta\varphi) - Y(\varphi)}{\Delta\varphi} \left( \frac{\varphi}{Y(\varphi)} \right) \quad (3)$$

where  $Y$  represents the process yield,  $\varphi$  corresponds to the input process parameters, as highlighted in Table S3, and  $\Delta\varphi$  represents the perturbation applied to the system for the different inputs.

## 4 | RESULTS AND DISCUSSION

### 4.1 | Reference single-column operation

First, the process parameters for the single-column preparative separation of ONs were optimized in dilute conditions, aiming at centering the product peak within the gradient boundaries. Then, a loading study led to a benchmark experiment with a loading of 15  $\text{g L}^{-1}$  resin. The resulting preparative chromatogram is shown in Figure 2A.

Here, it is evident how W2 is predominant with respect to the other two impurities and, moreover, it is almost completely co-eluting with P. This represents the most challenging impurity group to be separated from the product. While the front of W2 is sharp, its tailing is significant, even exceeding the elution time of the P peak maximum. This constitutes a relevant challenge also for the design of MCSGP. The co-elution of P with W2 and S introduces the trade-off between yield and purity typical for single-column operations. To visualize this trade-off, a Pareto curve was plotted starting with the yield/purity values of the purest fraction of the batch run, and then simulating different collection windows by pooling together contiguous fractions, and recalculating their purity and yield values. The results are reported in Figure 2B (black squares), which highlights the Pareto front purity versus yield, showing that one of the two parameters can be improved only by sacrificing the other. The maximum purity achieved with this single-column operation was 96.7% at a yield of < 10%. On the other hand, by introducing a purity specification of 96.0%, the achievable product yield was 56%. This batch set point was then considered as the reference experiment to design the MCSGP, keeping the purity specification at 96.0%.

### 4.2 | MCSGP operation

The MCSGP process was designed from the batch reference run implementing the UV-based AutoPeak control strategy. This required the definition of an absolute UV threshold to start the W/P recycle, a

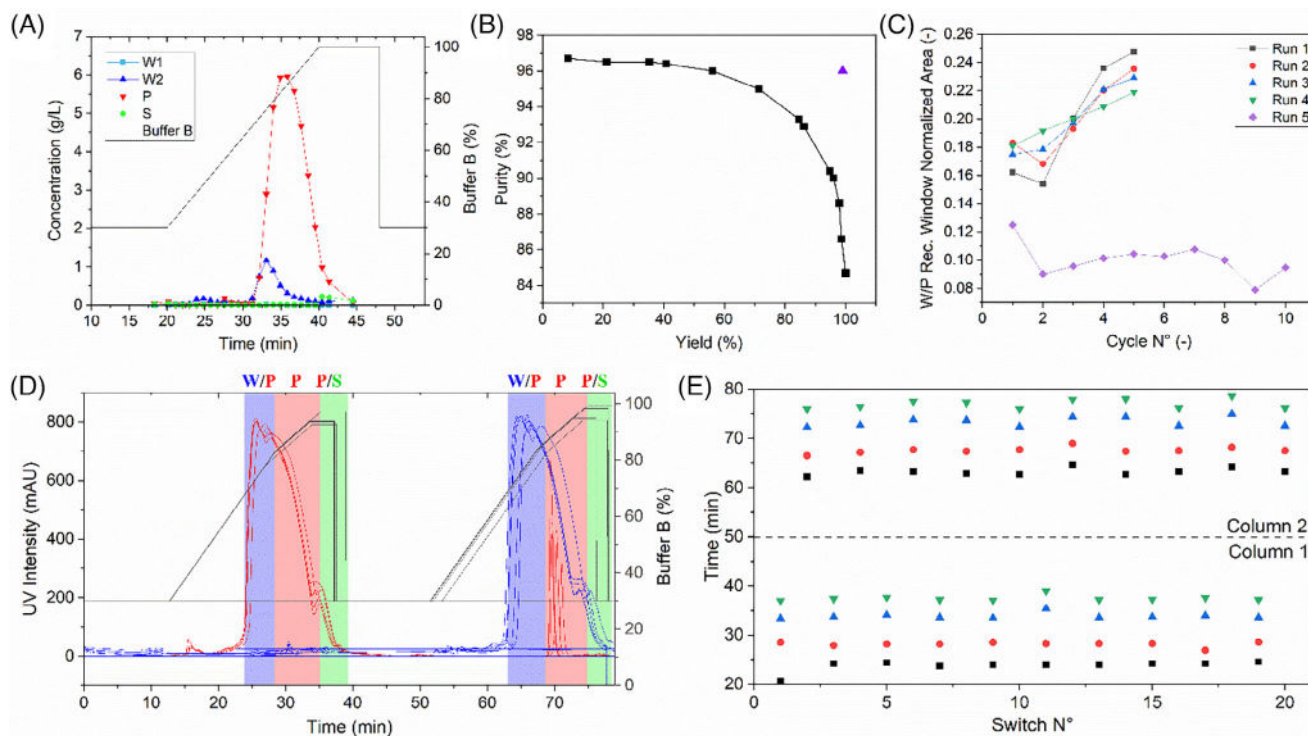
**TABLE 1** operating conditions of MCSGP experiments performed with UV-based dynamic process control.

|                                    |                                       | Run 1   | Run 2 | Run 3 | Run 4 | Run 5 |
|------------------------------------|---------------------------------------|---|-------|-------|-------|-------|
| Column dimension                   | [mL]                                  | 1.7 (100 mm x 4.6 mm)   |       |       |       |       |
| Resin                              |                                       | YMC Triart C18, S–10 μm, 12 nm  |       |       |       |       |
| Buffers                            |                                       | Equilibration (A): 99% 0.2 M sodium acetate + 1% acetonitrile<br>Elution (B): 90% 0.2 M sodium acetate + 10% acetonitrile |       |       |       |       |
| Startup resin loading              | [g L <sup>-1</sup> <sub>resin</sub> ] | 15  |       |       |       |       |
| Number of cycles                   | [-]                                   | 5   | 5     | 5     | 5     | 10    |
| Cycle duration                     | [min]                                 | 75.9  | 77.0  | 78.3  | 76.5  | 78.6  |
| Inline dilution factor W/P         | [-]                                   | 1.67  | 1.67  | 1.67  | 1.67  | -     |
| Feed concentration                 | [g L <sup>-1</sup> ]                  | 4.0   |       |       |       |       |
| Startup loading duration           | [CV]                                  | 3.77  |       |       |       |       |
| Loading duration per cycle         | [CV]                                  | 2.18  |       |       |       |       |
| Loading velocity                   | [cm h <sup>-1</sup> ]                 | 300   |       |       |       |       |
| Pre-load wash duration             | [CV]                                  | 0.5   |       |       |       |       |
| Pre-load wash velocity             | [cm h <sup>-1</sup> ]                 | 600   |       |       |       |       |
| Post-load wash duration            | [CV]                                  | 1.5   |       |       |       |       |
| Post-load wash velocity            | [cm h <sup>-1</sup> ]                 | 600   |       |       |       |       |
| Gradient boundaries                | [%B]                                  | 30-100  |       |       |       |       |
| Gradient velocity                  | [cm h <sup>-1</sup> ]                 | 200   |       |       |       |       |
| CIP (100% B) duration              | [CV]                                  | 2   |       |       |       |       |
| CIP velocity                       | [cm h <sup>-1</sup> ]                 | 150   |       |       |       |       |
| Re-eq. (100% A) duration           | [CV]                                  | 3   |       |       |       |       |
| Re-eq. velocity                    | [cm h <sup>-1</sup> ]                 | 400   |       |       |       |       |
| UV trigger for W/P recycling start | [mAU]                                 | 100   |       |       |       |       |
| UV trigger for P collection start  | [% of peak maximum]                   | 93  | 96    | 93    | 90    | 93    |
| UV trigger for P collection stop   | [% of peak maximum]                   | 47  | 30    | 30    | 30    | 30    |

relative UV trigger to stop this interconnected phase and to start P collection and a second relative threshold to stop the P collection and initiate the internal recycling of the P/S overlap. In general, the P collection start/end thresholds are adjusted such that the product pool reaches the required purity specification. The W/P start threshold instead is set to internally recycle the out-of-specification product in the front of the peak. Five different runs were conducted to study the effect of changing the UV thresholds for the product collection window (Figure S3-S6 and Table S4-S7). In the first run, P collection was started when the UV signal decreased to 93% of the peak maximum value and was stopped at 47% of the peak maximum. With this run, the purity specification of 96.0% was fulfilled, with a yield of 95%. This is a remarkable improvement compared to the single-column operation,

where the same purity could only be achieved at a yield of 56%. To further improve the yield of the process, the product collection window was extended in the second run from start at 96% to a stop of collection at 30% of peak maximum. Indeed, with this operation, the purity was maintained > 96.0%, with the yield increasing to 99%. However, this improvement came at the cost of an accumulation of W2 in the W/P overlap, as confirmed by an area increase under the chromatograms in the W/P overlap recycling phase from cycle to cycle, as shown in Figure 2C (red circles).

With the aim of preventing this weakly adsorbing impurity accumulation, the P collection window was progressively narrowed by moving the left boundary to lower UV thresholds (93% of peak maximum for Run 3 and 90% of peak maximum for Run 4). Although the yield could



**FIGURE 2** (A) Batch chromatogram at  $15 \text{ g L}^{-1}$  resin loading used as reference. (B) Pareto front purity versus yield for the batch chromatogram with  $15 \text{ g L}^{-1}$  resin loading (black squares) compared to the MCSGP Run 5 obtained with the UV-based control strategy (violet triangle). (C) Normalized area under curve of the W/P recycling window throughout the different cycles of the five MCSGP runs. (D) UV signals versus time for the MCSGP Run 5. The signals are recorded for the two column positions: the UV signal represented in red is related to Column 1 and the UV signal in blue is related to Column 2. In the second y-axis the percentage of the elution buffer (buffer B, black line) is shown, representing the linear gradient. (E) Modulation of the five characteristic times of the MCSGP Run 5 operated by AutoPeak shown for the 20 switches (10 cycles): (■)  $t_2$  (the start of the W/P recycling step), (●)  $t_3$  (the end of the W/P recycling phase and the start of P collection), (▲)  $t_4$  (the end of P collection and the start of the P/S recycling phase), (▼)  $t_5$  (the end of the P/S recycling step).

be maintained very high ( $> 99\%$ ), the process still experienced W2 accumulation in the W/P recycling window, although with a decreasing slope (Figure 2C).

Since a cyclic steady state was not achieved after 5 cycles in Runs 1 – 4 due to accumulation of W impurities, Run 5 was conducted without inline dilution for the W/P recycling stream to reduce W adsorption. Looking at the corresponding chromatogram in Figure 2D, this approach allowed to remove a portion of the weakly adsorbing impurities in the flow-through, visible especially for Column 1 where peaks in the UV signal can be observed at ca. 70 min. Through this approach, Run 5 achieved a cyclic steady state with the AutoPeak control parameters that had been previously used in Run 3. These results demonstrate that despite different degrees of accumulation of W and different peak retention times, the UV-based control is capable of keeping the run at high yield and purity. This is facilitated by the circumstance that product collection is on the tail side of the elution peak, while W accumulation occurs on the front side of the peak.

The cyclic steady state of Run 5 was confirmed by constant areas of the W/P overlap from cycle to cycle (Figure 2C), by the good overlay of the MCSGP chromatograms over the 10 cycles (Figure 2D), and

by the consistent product pool composition and volume, as determined by offline analyses (Table S8). With the exception of cycle nine considered an outlier, the weakly adsorbing impurities are consistently maintained at a concentration below  $0.04 \text{ g L}^{-1}$  throughout the entire run, while the strongly adsorbing impurities are absent in each product pool collected (Table S8). Moreover, the target product is collected at a consistent concentration ( $3.93 \pm 0.54 \text{ g L}^{-1}$ ) and pool volume ( $3.83 \pm 0.34 \text{ mL}$ ), highlighting once again the robustness of the MCSGP controlled by AutoPeak.

The effect of the UV-based controller is evident by looking at the different gradient durations in Figure 2D. These are due to the modulation of the characteristic times of MCSGP ( $t_2 - t_5$ ) by AutoPeak, based on the UV signal of the elution peak, as shown in Figure 2E. Here it is possible to appreciate the important intervention on  $t_2$  in the first switch, initially low (i.e., 20.3 min) due to the startup loading applied to the upstream column and then adjusted to 24.1 min. This intervention is crucial to speed up the achievement of the cyclic steady state, obtained already from cycle 3 onwards (see Table S8). On the other hand, as expected, once the cyclic steady state is reached, only slight adjustments are made by the controller, within a range of 1–2 min. This dynamic modulation of the characteristic times is crucial to com-

pensate peak shifts due to process parameter variations during the MCSGP run. In fact, AutoPeak allows the process to evolve towards new steady states whenever variations in critical parameters are introduced, preserving the required purity and yield. This would not be possible instead with fixed characteristic times, as demonstrated later on. Considering the cyclic steady state, MCSGP alleviated the trade-off observed in the single column purification of this ON sequence by achieving high yield (> 99%) and purity (> 96.0%) simultaneously, as shown in Figure 2B. Given the performance and stability of Run 5, this run was used as the benchmark for the sensitivity analysis discussed in the following.

### 4.3 | Process robustness achieved by the UV-based dynamic control AutoPeak

To investigate the effectiveness of the UV-based dynamic process controller in compensating the impact of fluctuations in the process parameters on the MCSGP performance, a sensitivity analysis was conducted. The crude purity, loading duration and temperature were chosen as the process parameters to be artificially perturbed with respect to the benchmark process following the scheme reported in Table S3. The effect of these perturbations on the yield was studied experimentally by running MCSGP, maintaining the design settings of the reference experiment and conducting the process for five cycles for each altered process parameter. Each MCSGP run was performed twice, first with activated dynamic process control (same controller settings as for the benchmark Run 5), and second, using standard time-based product collection (without dynamic process control) as reference (Figure S7-S19 and Table S9-S21). The normalized sensitivity on the yield was then calculated based on Equation 3 for both the controlled and non-controlled MCSGP. The side-by-side comparison for the two processes is shown in Figure 3.

Although most of the runs performed with and without AutoPeak control satisfied the purity specification of 96.0%, Figure 3 shows that the process is much less sensitive to the introduced perturbations when the UV-based controller is activated. The fluctuations introduced to the input process parameters may in fact cause peak shifts, altering the chromatogram position compared to the reference process. When the control is not activated, the recycling and collection windows are defined by fixed time triggers and therefore any shift in the chromatogram leads to a drift of the product pool quality as product collection is taking place at a wrong position. In this specific case, the product yield, considered as the output variable, is significantly impacted, as confirmed by the high sensitivity shown in Figure 3 (black empty circles) for the three reference inputs. On the other hand, AutoPeak can modulate the collection and recycling windows cycle-by-cycle, adapting them to the chromatogram shifts caused by the applied perturbations. This ensures that the quality of the product pool can be preserved, as shown by the low sensitivities to all the perturbed process parameters in Figure 3 (red squares).

The beneficial effect of the UV-based control on the MCSGP can be appreciated from the process performance values reported in Table 2.

Specifically, when reducing the loading duration by 30%, the mean retention time for the product is not significantly impacted. This means that even with fixed recycling and collection windows (no control), high purity and yield can be preserved (see Figure S9 and Table S11). The yield increases to 99.0% with respect to the one achieved with the non-controlled process (89.3% yield) at the reference loading of  $15 \text{ g L}^{-1}_{\text{resin}}$ . Comparing these results, both runs are satisfying the purity specification of 96.0%. The yield improvement is due to the fact that with  $-30\%$  loading the peak becomes slimmer and more product ends up in the P collection window while less product is lost outside the W/P recycling and P/S recycling phases.

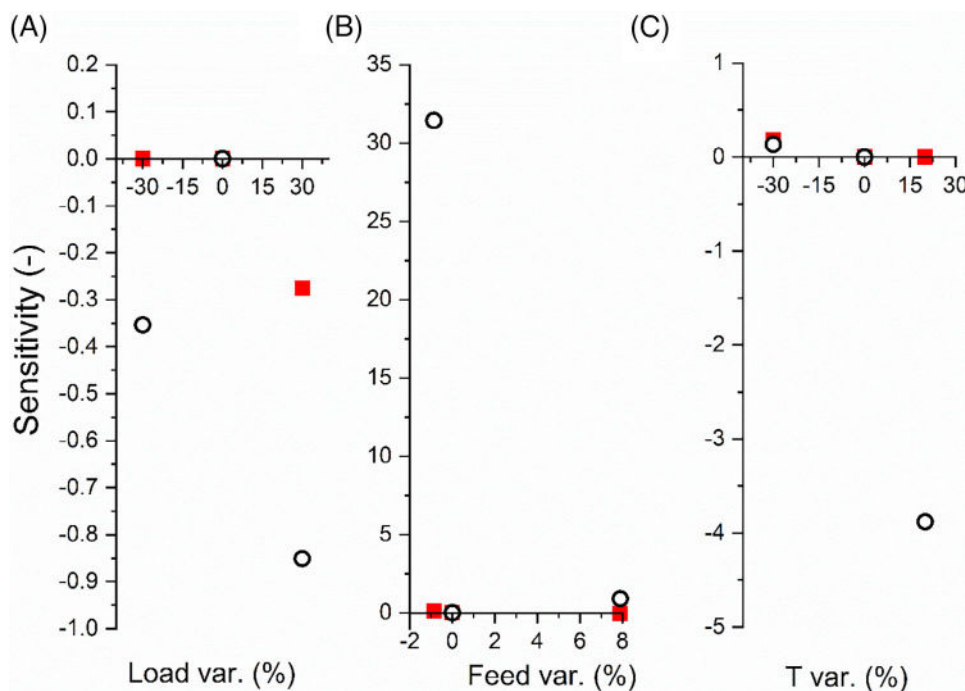
On the other hand, an increase in the loading by 30% leads to an earlier elution of the peak front and a more pronounced tailing (see Figure S11), as expected for a Langmuir adsorption behavior. With a fixed collection window (no control), the product eluting in this tail cannot be collected, which leads to a drop in the yield to 66.5%. On the other hand, with UV-based dynamic control in operation, the collection window is automatically adjusted to include this tail (see Figure S10), allowing to preserve high yield equal to 90.8% while satisfying the purity specification.

Regarding feed quality, both the controlled and non-controlled runs delivered product in specification when the feed purity was increased by  $+7.9\%$ , which is not surprising. On the other hand, the results showed that MCSGP is very sensitive to decreasing feed purity, if operated without dynamic process control. Even for slightly reduced feed purity from 90.2% to 89.4%, the purity specification of 96.0% was violated, and product purity dropped to only 90.9%. This is due to the increasing amount of weakly adsorbing impurities present in the product fraction (see Table S17). In contrast, the MCSGP run operated with dynamic process control delivered product in specification with a yield of 98.9% as the controller adjusted the start of product collection to exclude weakly adsorbing impurities from the product pool (see Table S16).

The set of experiments performed with the temperature perturbation is particularly useful to visualize the online adjustments made by AutoPeak with respect to the non-controlled runs. Temperature significantly impacts the adsorption equilibrium and, in turn, the mean retention times of the different species. Looking at the MCSGP chromatograms recorded at the three investigated temperatures (i.e., 35, 50, and 60°C) in Figure 4, it is evident how an increase in the process temperature drastically reduces the adsorption, causing a shift in the chromatograms to lower retention times.

For the non-controlled MCSGP, this shift in the retention time is particularly dramatic. Since the collection window is fixed according to the process design operated at 50°C, most of the product could not be collected when increasing the temperature to 60°C and the yield dropped to 20% (see Figure S19 and Table S21). Also, at 35°C, the fixed time-based collection interval did not lead to collection of the desired part of the elution peak, and the obtained product was not in specification ( $< 96.0\%$  purity) as the peak eluted later.

On the other hand, when utilizing the UV-based controller AutoPeak, the product collection window was automatically adjusted in response to peak shifts caused by the temperature variations. This is



**FIGURE 3** Sensitivity versus input variation in percentage for: (A) loading duration, (B) feed stock purity, (C) temperature. The results of the controlled process (red squares) are compared with the corresponding process with no control (black empty circles).

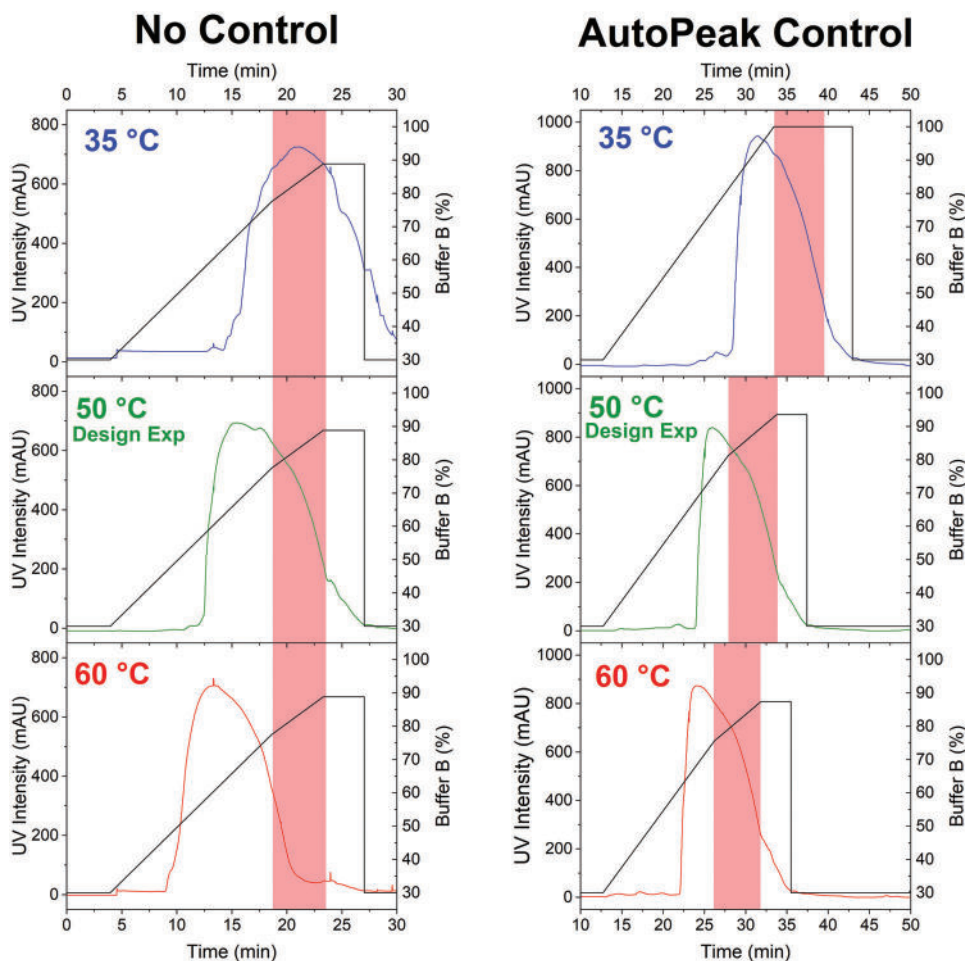
**TABLE 2** Yield, purity and sensitivity values calculated for each perturbation applied to the experimental runs with and without the control strategy. The parameters for the reference center point were: Loading =  $15.0 \text{ g L}^{-1} \text{ resin}$ , Temperature =  $50^\circ\text{C}$ , Feed purity =  $90.2\%$ .

|             |                              | Input value                           | Yield [%] | Purity [%] | Sensitivity [–] |
|-------------|------------------------------|---------------------------------------|-----------|------------|-----------------|
| Loading     | Reference (AutoPeak control) |                                       | 99.0      | > 96.0     | 0.00            |
|             | Reference (no control)       |                                       | 89.3      | > 96.0     | 0.00            |
|             | + 30% (AutoPeak control)     | $19.5 \text{ g L}^{-1} \text{ resin}$ | 90.8      | > 96.0     | -0.28           |
|             | + 30% (no control)           | $19.5 \text{ g L}^{-1} \text{ resin}$ | 66.5      | > 96.0     | -0.85           |
|             | – 30% (AutoPeak control)     | $10.5 \text{ g L}^{-1} \text{ resin}$ | 99.0      | > 96.0     | 0.00            |
|             | – 30% (no control)           | $10.5 \text{ g L}^{-1} \text{ resin}$ | 99.0      | > 96.0     | -0.36           |
| Feed purity | + 7.9% (AutoPeak control)    | 97.3%                                 | 98.4      | > 96.0     | -0.08           |
|             | + 7.9% (no control)          | 97.3%                                 | 95.6      | > 96.0     | 0.90            |
|             | – 0.87% (AutoPeak control)   | 89.4%                                 | 98.9      | > 96.0     | 0.11            |
|             | – 0.87% (no control)*        | 89.4%                                 | 64.4      | 90.9       | 31.44           |
| Temperature | + 20% (AutoPeak control)     | $60^\circ\text{C}$                    | 99.0      | > 96.0     | 0.00            |
|             | + 20% (no control)           | $60^\circ\text{C}$                    | 20.0      | > 96.0     | -3.88           |
|             | – 30% (AutoPeak control)     | $35^\circ\text{C}$                    | 93.7      | > 96.0     | 0.18            |
|             | – 30% (no control)*          | $35^\circ\text{C}$                    | 85.7      | 88.6       | 0.14            |

\*Purity out of specification.

visualized by the shift of the red shaded area in the chromatograms and of the gradient durations for the different temperature perturbations. The drift in the retention times due to the temperature alteration is captured by the AutoPeak thanks to the UV thresholds previously selected during the design of MCSGP. The controller, for each cycle, adjusted the durations of the recycling and collection windows provid-

ing the corrected timing for switching the valves, and thus ensuring the maintenance of the cyclic steady state throughout the entire experiment duration. For this particular case, this automatic adjustment allowed to preserve a stable steady state for both  $35^\circ\text{C}$  and  $60^\circ\text{C}$  (see Figure S16 and S18, respectively) and deliver product in specification in both cases (> 96.0% purity) at yields of 93.7% ( $35^\circ\text{C}$ )



**FIGURE 4** Chromatogram of the 5<sup>th</sup> cycle of the MCSGP corresponding to the outlet of Column 1 at the different tested temperatures (i.e. 35, 50, and 60°C) for non-controlled runs and AutoPeak-controlled runs. In all cases, the process parameters were identical to those used at 50°C. On the left, chromatograms for the non-controlled MCSGP runs are shown. The product collection window highlighted in red is defined by time triggers and remains constant for the three runs. The chromatograms on the right refer to the experiments performed with AutoPeak using the same parameters as in the initial process design (Run 5). In this case, the product collection window (red area) was dynamically adjusted to compensate for peak shifting. The black line shows the linear gradient expressed in terms of elution buffer volume fraction over time.

and > 99.0% (60°C), respectively. Overall, the results clearly demonstrate the enhanced robustness, consistent product quality, and high process performance, provided by the UV-based AutoPeak control strategy, which is able to compensate for disturbances from different sources.

## 5 | SUMMARY AND CONCLUSIONS

In this work, the novel dynamic process control strategy (AutoPeak) for MCSGP was investigated for the purification of an oligonucleotide with respect to process robustness.

Firstly, MCSGP was designed based on a single column batch run. Through a series of MCSGP screening runs, MCSGP phase boundaries and the corresponding AutoPeak settings were selected such that MCSGP was operated in cyclic steady state over a 10-cycle run (20 product elutions), producing product in specification (purity > 96.0%). More specifically, relative UV triggers (product collection from “93% of

peak maximum” to “30% of peak maximum”) were used. These AutoPeak settings were then applied to a set of MCSGP runs to assess the robustness of the benchmark process parameters under significantly different starting conditions, exploring temperature, feed stock purity and load variations.

As expected, under benchmark conditions MCSGP could be operated without UV-based dynamic control and meet the purity specification. However, when process parameter variations did occur, significantly lower yield (down to 20%) was observed for the non-controlled runs. In contrast, in MCSGP runs where AutoPeak was applied, the dynamic process control fully compensated for the process perturbations, delivering product in specification in all cases with high yield (> 90%). This enhanced stability for the controlled process is reflected in a standard deviation below 3.4%, calculated for the different yields. On the other side, the greater sensitivity to the perturbations for the non-controlled operation led to a standard deviation on yield of 27.5%, more than 8 times larger than for the AutoPeak controlled process.

The control strategy investigated in this work is fully adherent to the process analytical technology (PAT) guidelines.<sup>[25]</sup> In fact, it allows prompt intervention on the critical process parameters (i.e., switching times) to maintain the critical quality attributes in specification, based on in-line monitoring (in this case through UV spectroscopy). In this way, any source of variability is managed directly by the process control, without the need for human intervention, reducing the risk of lost production lots. Furthermore, this strategy ensures a more consistent product quality and, in turn, more safety for these pharmaceuticals.<sup>[26–27]</sup>

It is worth pointing out that the UV-based control strategy AutoPeak has a great potential to be implemented in MCSGP of any biomolecule. AutoPeak shifts the definition of the phase boundaries of MCSGP from the time to the UV domain and is not constrained by a certain chromatogram shape. Hence, we expect that all case studies reported so far for MCSGP could potentially be converted to this UV dynamic controller, provided that UV absorbance is in the linear range of the detector.

Finally, the UV-based control strategy AutoPeak is a critical part of MCSGP scale-up as it also compensates for differences in elution times originating from system hold-up volumes and other equipment-related sources, besides compensating for operating parameter variations. This eliminates the need for costly engineering runs on the large scale MCSGP chromatography systems. In addition, AutoPeak enables stable cyclic steady state operation for extended periods, minimizing the process monitoring effort required at scale.

## AUTHOR CONTRIBUTIONS

**Ismaele Fioretti:** Data curation (lead); investigation (lead); writing—original draft (lead). **Thomas Müller-Späth:** Funding acquisition (equal); supervision (supporting); writing—review and editing (equal). **Lars Aumann:** Validation (equal); writing—review and editing (equal). **Mattia Sponchioni:** Funding acquisition (equal); supervision (lead); writing—review and editing (equal).

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interests for this work.

## DATA AVAILABILITY STATEMENT

Data available within the article or its supplementary materials

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