Journal of Chromatography A 1737 (2024) 465440

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Integrated multidimensional chromatography on preparative scale for oligonucleotides purification

Chiara Nosengo [a], Desiree Bozza [a], Giulio Lievore [b], Sebastian Vogg [b], Martina Catani [a], Alberto Cavazzini [a,c], Thomas Müller-Späth [b,*], Chiara De Luca [a,*], Simona Felletti [d]

[a] Department of Chemical, Pharmaceutical and Agricultural Sciences, University of Ferrara, via L. Borsari 46, Ferrara 44121, Italy
[b] YMC ChromaCon, Technoparkstrasse 1, 8005 Zürich, Switzerland
[c] Council for Agricultural Research and Economics, Via Della Navicella 2/4, 00184, Rome, Italy
[d] Department of Environmental and Prevention Sciences, University of Ferrara, via L. Borsari 46, Ferrara 44121, Italy

ARTICLE INFO

Keywords:
Oligonucleotides
Multidimensional chromatography
Preparative chromatography
Reversed phase chromatography
Anion exchange chromatography
Active pharmaceutical ingredients

ABSTRACT

Therapeutic oligonucleotides represent a recent breakthrough in the pharmaceutical industry due to their ability to regulate gene expression with great specificity. This aspect allows treatment of a wide range of diseases. However, since oligonucleotides are used for therapeutic purposes, the Active Pharmaceutical Ingredient (API) must fulfill strict purity levels which require intensive purification steps. For oligonucleotides, and biomolecules in general, preparative liquid chromatography is the technique of choice to perform large scale purifications, typically in batch mode, i.e. using a single column. Specifically, since ONs are mainly large, hydrophilic and charged molecules, Anion Exchange chromatography (AEX) and Ion Pair Reversed Phase chromatography (IP-RP) are the preferred chromatographic modes for their downstream processing. Nevertheless, these approaches suffer from a purity-yield trade-off, and for this reason, more than one purification step is usually required. The two chromatographic modes can therefore be used consequently to remove different groups of impurities, thanks to their orthogonality.

In this work, a multidimensional and orthogonal approach on a (semi)preparative scale, namely "Integrated Batch process", was applied for the purification of a single-stranded DNA oligonucleotide. This process combines two chromatographic steps without any hold step, operator intervention or sampling of the first step. The performance parameters of the Integrated Batch were compared to those obtained in the single batch runs under different experimental conditions (chromatographic mode, eluent systems), showing the potential of this integrated approach. This proof-of-concept study illustrates how this technique can considerably reduce overall production time and how it allows to increase the robustness and reproducibility of the method, since the process is highly automated.

1. Introduction

Oligonucleotides are gaining increasing attention in the pharmaceutical industry as an emerging class of compounds with significant therapeutic potential. Their ability to modulate gene expression promotes them as promising candidates for addressing a broad spectrum of diseases [1,2]. Since unmodified oligonucleotide sequences are naturally vulnerable to degradation when introduced into biological systems, recent advances led to chemical modification to provide nuclease stability, establishing them on the market of novel biopharmaceuticals [3–5]. As of today, thirteen oligonucleotides have been approved by the Food and Drug Administration (FDA), and many of them are undergoing different stages of clinical trials, where the main application includes neurological disorders, ophthalmic diseases, cardiovascular and metabolic conditions [2,6–9].

These molecules are short nucleic acid chains that are mainly produced through solid phase synthesis, where nucleotide monomers are progressively added to the growing polymeric chain. This cyclic procedure for oligonucleotide manufacturing can be generally performed in four steps: deprotection, where the most used protective group is the 4,4′-dimethoxytrityl (DMT), coupling, oxidation/sulfurization, and capping. The interested reader is referred to literature [10,11] for more

* Corresponding authors.
E-mail addresses: thomas.mueller-spaeth@chromacon.com (T. Müller-Späth), chiara.deluca@unife.it (C. De Luca).

https://doi.org/10.1016/j.chroma.2024.465440
Received 30 July 2024; Received in revised form 10 October 2024; Accepted 14 October 2024
Available online 15 October 2024

details on the synthesis. However, this method leads to a great number of structurally similar impurities that can be classified as shortmers (n-1) or longmers ($n + 1$) and differ from the full-length oligonucleotide (n nucleotides) by the omission and addition respectively of one or more nucleotides. Other impurities are produced by depurination, failure sequences, and other side reactions [8,10,12–14]. Therefore, therapeutic oligonucleotides require extensive purification to fulfill the strict purity requirements to obtain high-quality API ensuring patient safety (> 90 %) [4]. The depletion of these impurities happens during the downstream process, which is the most challenging and expensive part of production.

Nowadays the most widely used purification technique for biopharmaceutical products is preparative liquid chromatography [15]. However, since the impurities usually have a similar structure compared to the product, their separation is often challenging, resulting in the coelution of the target product and unwanted impurities, as schematically reported in Fig. 1 [4,5]. This leads to the purity-yield trade-off typical of batch chromatography. Indeed, high product purity is obtained at the expense of yield, since in that case the window chosen to collect the product tends to be quite narrow. Basically, the windows where the product and impurities coelute are discarded or reprocessed in the following, and this is the main reason for the low recovery obtained at high purities. On the other hand, widening the collection window to increase the recovery results in a decrease of the total purity, since some unwanted impurities are also collected. For this reason, subsequent chromatographic steps are usually required to obtain a purity satisfying the requirements imposed. This results in higher production and analysis costs, also related to high solvent consumption, which decreases the overall sustainability of the process [16–20].

The most common purification methods for therapeutic oligonucleotides include Anion Exchange (AEX) and Ion Pair Reversed Phase (IP-RP) liquid chromatography. AEX takes advantage of the negative charge on the phosphate linkage on the oligonucleotide at a typical operating pH between 8 and 12. It allows a fine separation between the target oligo, shortmers and longmers and the purification can be conducted without the DMT protective group (DMT-off). The elution is generally performed with a sodium salt gradient, and, which yields the product already in its sodium form at the end of the process, requiring only a desalting step. On the other hand, IP-RP purification ensures to remove not only charge-based residues, thanks to the ion pairing agent, but also discriminates neutral lipophilic impurities [8]. Usually, in IP-RP-LC the purification is conducted using mobile phases consisting of a mixture of water and organic modifier, typically acetonitrile (ACN), while alkyl amines are the most common ion pair agents. Moreover, to increase the hydrophobicity of oligonucleotides, the DMT group is maintained (DMT-on) and removed either on column by acidic condition or after the purification. Besides, a salt exchange is required after the purification to remove the ion pair reagent [1,8,11,14,21].

The combination of two types of chromatography based on different interactions (e.g. AEX and RP) can be exploited to improve the separation. This approach is referred to as orthogonality [22–24]. Nevertheless, if the two chromatographic modes are not directly coupled, the manual intervention of the operator is required, leading to a significant amount of manual effort and time, since once the product is eluted from the first column, it needs to be collected and re-analyzed in the second column [9]. Coupling ("integrating") these two techniques, meaning that the effluent coming from the first column (using one separation mode) is directed to the second column (using a second separation mode), can save time, increasing the overall productivity of the process [16].

In recent years, multidimensional chromatographic techniques have been increasingly used, especially for analytical scale separations. The rationale behind these techniques is the direct injection of the eluted product from the first column (first dimension) into the second column (second dimension) with the goal of increasing peak capacity [25–28].In this study, a fully automated multidimensional approach, called "Integrated Batch process", was applied to a (semi)preparative scale purification of a single-stranded DNA oligonucleotide. More in detail, two orthogonal chromatographic techniques (AEX and IP-RP) were used. Differently from the common procedure, the process takes place in a single instrument, and thanks to a series of valves that facilitates the online sample dilution, the solvent eluting from the first dimension is made compatible with the stationary phase in the second dimension.

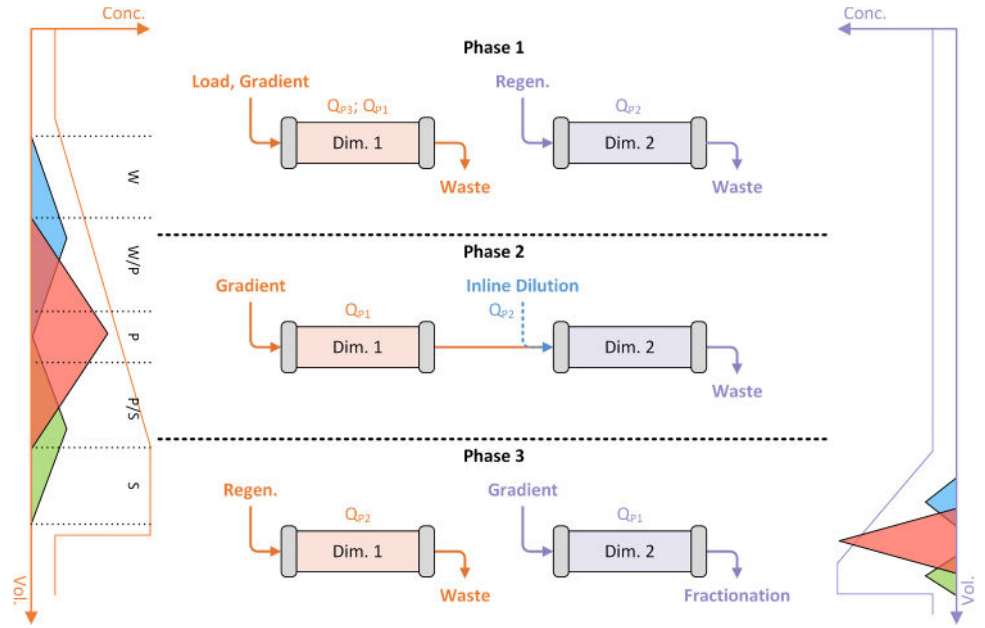

Fig. 1. Schematic Representation of the Integrated Batch process. Blue, red and green peaks are the schematic representation of weak impurities (W), product (P), and strong impurities (S), respectively; the zones W/P and P/S represent the overlapping regions between weak and strong impurities with the product. The first purification is reported on the left side (orange), while the second purification is reported on the right side (purple). This scheme shows how the two columns are working on the three steps of the process. In phases 1 and 3 the columns are operating in batch mode (in parallel), while in phase 2 they are interconnected (in series). The vertical axis, where volume is shown, is not on scale.

2. Integrated batch process

Generally, a single column (batch) process employed for the purification of oligonucleotides (and biomolecules in general) can be divided into five steps: equilibration, loading of the feed, wash, gradient elution, and regeneration, the last one including both the stripping of the column (with a high modifier content to remove strongly adsorbed impurities) and re-equilibration. In oligo purification, the elution is typically performed under gradient elution conditions since the feed, after the solid phase synthesis, contains many product-related impurities and oligonucleotide retention depends on the content of the organic modifier (RP) or salt (AEX) contained in the mobile phase. As previously stated, current approaches employed for oligonucleotides purification are based on discontinuous methods. This means that batch runs exploiting orthogonal selectivities are performed subsequently, either on the same or on a different instrument. This implies that the product collected after the first purification is reprocessed (diluted to lower salt concentration, adjusting pH) before being loaded onto the second column for the second purification, and that the instrument requires flushing with compatible mobile phases.

On the other hand, the advantage of the Integrated Batch process is that it combines two orthogonal chromatographic steps into a single process, since the product eluted from the first column is diluted inline and directly loaded onto the second column, without hold step or operator intervention. In order to establish a successful Integrated Batch procedure, multiple batch runs under different experimental conditions (IEX, RP with each counter ion) need to be performed. These will serve both as design tools for the interconnected process and as benchmarks.

This allows to determine the appropriate portion of the chromatogram to be transferred from the first to the second dimension, namely an elution "window" of satisfactory purity.

The whole process can be divided into three phases (Fig. 1), where the two columns can work either individually (*in parallel*, phases 1 and 3) or interconnected (*in series*, phase 2). In phase 1, the columns are operated in batch mode, meaning that they are disconnected: the first column is loaded with the feed, using the same amount used in the design batch method. After the loading phase, the gradient starts in the first column, and at the same time the second column is regenerated. When the window of interest of the chromatogram (chosen based on the fraction analysis of the design batch) starts eluting, a connection between the two columns is established by means of a system of column inlet and outlet valves, so that in phase 2 the columns are interconnected, and the selected portion of the chromatogram from the first column is inline diluted and transferred onto the second column. Before phase 3 starts, all relevant tubing of the system is flushed with water and then flushed back with the equilibration buffer of the second column to prevent incompatible solvents from entering the second column. Depending on the dilution factor chosen, an appropriate flow rate must be used for inline dilution. Lastly, during step 3 the columns work again in batch mode (disconnected) and, while the first column is regenerated, the gradient starts on the second column.

When using AEX and IP-RP elution modes, two sequences are possible: AEX-RP and RP-AEX. Since AEX makes use of mobile phases containing high percentages of salt, and usually extreme pH is used for oligonucleotide separation, AEX-RP sequence implies that it is necessary to dilute the product stream inline during the transfer. This is to decrease the pH and salt concentration before entering onto the RP column, in order for the recycling portion (meaning the portion to be transferred) to be compatible with the second dimension. At the end of the process, if the counterion is different from sodium, which is the form in which oligonucleotides are usually formulated, a salt exchange step is required. Conversely, with the opposite sequence (RP-AEX), the transfer can be directly performed from the hydrophobic to the anion exchange column, since in this case organic modifier content as well as pH and salt concentration are compatible with the features of the second dimension. At the end of the process, since the second purification is conducted with anion exchange chromatography, a desalting step is needed.

3. Materials and methods

3.1. Chemicals

Sodium Acetate Trihydrate (NaOAc), Sodium Hydroxide (NaOH), Sodium Chloride (NaCl), Acetic acid and HPLC grade Acetonitrile (ACN) and Methanol (MeOH) were purchased from Merck Millipore (Darmstadt, Germany). Triethylamine (TEA) ($\geq$ 99.5 %) was purchased from Sigma Aldrich (St. Luise, MO, USA.), while 1,1,1,3,3,3-Hexafluor-2-propanol (HFIP) ($\geq$99.5 %) was purchased from Thermo Fisher Scientific (Waltham, MA, USA.). The feed material was a 20-mer single stranded DNA oligonucleotide DMT-off (5′-ATA CCG ATT AAG CGA AGT TT-3′), synthesized by means of solid phase synthesis by NATIAS (Japan) with an initial purity of 51 %, determined by HPLC. For each purification, the feed was dissolved in the corresponding mobile phase-A to obtain a concentration of 10 g/L.

3.2. Offline analytics

An Agilent UPLC 1290 Infinity (Agilent, Santa Clara, CA, USA) equipped with a diode array detector (DAD) was used for the offline analysis of the collected fractions and feed. The injection volume was 1μL for each sample. The flowrate was set at 0.4 mL/min, the column thermostat was set to 60 °C and the wavelength to 260 nm. A YMC-Triart Bio C18, 150 × 2.1 mm, 1.9 μm was used as the stationary phase. The mobile phase A (MP-A) consisted of 0.1 M HFIP + 4 mM TEA, while the mobile phase B (MP-B) consisted of 100 % MeOH. The linear gradient ranged from 10 % to 18 % MP-B in 33 min, then 2 min of stripping at 90 % MP-B was performed, followed by 3 min of re-equilibration at the initial condition.

3.3. Batch operating conditions

Batch purification and Integrated Batch experiments were conducted on a Contichrom® CUBE 30 (YMC ChromaCon, Zurich, Switzerland) equipped with a fraction collector Foxy R1. The instrument was also equipped with two buffer selection valves and two column bypass valves, to be able to switch eluents between the purification and to bypass the column during the wash of all tubing in the Integrated Batch method.

For AEX and RP purifications a screening of mobile phases and different temperatures was performed. Concerning AEX, the best performance was obtained with 25 mM NaOH as Buffer A (to favor adsorption) and 25 mM NaOH, 1.5 M NaCl as Buffer B (to promote the elution of the compounds), both with a pH equal to 12, and a temperature of 25 °C. A Tricorn column 100 × 5 mm packed with BioPro IEX SmartSep Q30 resin was used as stationary phase. For RP chromatography, two different buffers with different ion pair agents were screened. In one case, 99/1 % 200 mM NaOAc/ACN (MP-A) and 80/20 % 200 mM NaOAc/ACN (MP-B) were used. Even if NaOAc is not an ion pair agent per se, it has shown suitable separation results [4]. In the other case, 99/1 % 100 mM Triethylammonium Acetate (TEAA)/ACN (MP-A) and 75/25 % 100 mM TEAA/ACN (MP-B) were mixed for the gradient, while 20/80 % 100 mM TEAA/ACN (MP-C) was employed as a stripping buffer. All mobile phases used for the IP-RP-LC had a pH of 8.5. A YMC-Triart Prep C18-S column, 100 × 4.6 mm, 10 μm from YMC (Kyoto, Japan) was used as a stationary phase. Also, the RP column was kept at a constant temperature of 50 °C for the first buffer system and 60 °C for the second one, by means of a column thermostat AZURA® CT 2.1 from Knauer (Berlin, Germany). UV detection was performed at 300nm.

For each batch, the column was first equilibrated with 3 column volumes (CV), which represents the geometrical volume of a cylindrical column, of mobile phase A with a flowrate of 600 cm/h. The feed was then loaded at 40 g/L_{column} both for AEX and the second IP-RP batches,

while for the RP purification using NaOAc the loading was 35 g/L_{column} at 600 cm/h, to avoid breakthrough. After that, the column was washed with 5 CV of MP-A at 600 cm/h in all runs. The linear gradient ranges were chosen to perform the elution of the target compound at a comparable retention: from 5 % to 80 % MP-B for AEX, from 5 % to 60 % MP-B for RP using NaOAc, and from 5 to 65 % MP-B for IP-RP using TEAA. During the gradient elution, the flowrate was kept at 300 cm/h, and its duration was 10 CV in all cases. During the elution step, a fractionation was performed with a frequency of 1 fraction/min. In the end, a stripping phase with 100 % MP-B (or MP-C in the case of the second IP-RP batch) and a re-equilibration at 100 % MP-A, both 3 CV long, were performed.

3.4. Integrated batch operating conditions

The integrated Batch process was developed starting from the separate design of the batch runs, to determine the appropriate quantity of product to be transferred to the second dimension. Indeed, it is both undesirable to obtain a highly pure product at low yield or to transfer most of the impurities to the second dimension having high product yield; therefore, it was decided for each sequence (AEX-RP, RP-AEX) to transfer two different portions of the chromatogram onto the second dimension.

In the first transfer, it was decided to transfer about 90 % of the product based on the fraction analysis, even though part of the weakly adsorbed impurities was also transferred. The beginning of the portion to be transferred was set 8.5 min after the beginning of the gradient for the sequence AEX-RP with both ion pairs, 10.0 min for the sequence RP (NaOAc)-AEX, and 11.0 min for the sequence RP (TEAA)-AEX.

In the second transfer, about 85 % of the product was transferred to the second dimension, almost completely cutting out the maximum of the weak impurity peak (Fig. 2). The time for the beginning of the transfer was set to 9.5, 11.0 and 12.0 min after the start of the gradient, respectively, for the sequence AEX-RP, RP(NaOAc)-AEX and RP(TEAA)-AEX. For both setpoints, the transfer was stopped at a UV signal value corresponding to 37 % of the peak maximum of the chromatogram. Since during product transfer from the first to the second dimension for the AEX-RP sequence, in-line dilution is required to lower pH and salt concentration, several offline dilution tests were conducted using the mobile phase A of the second purification. As the dilution factor would have been greater than 10 to obtain a pH level compatible with the stationary phase of the RP dimension, it was decided to add 25 mM acetic acid to mobile phase A. Therefore, for the sequence using NaOAc, a 1:1 dilution was carried out with 99/1 % 0.2 M NaOAc/ACN and 25 mM Acetic Acid (pH 5.8), and for the sequence using TEAA the inline dilution was 1:1 with 99/1 % 200 mM TEAA/ACN and 25 mM Acetic Acid (pH 5.6). Since the flowrate of the gradient is 300 cm/h for both batch and integrated batch, and the dilution factor was imposed to be 2, the flowrate set for the inline dilution is 300 cm/h as well. Therefore, since the flowrates are cumulative, it results that the product is loaded onto the second column with the same velocity as for the batch. This is however not a requirement when designing Integrated Batch processes.

As mentioned in Section 2, between operating the first and second dimensions all relevant tubing of the chromatography system was flushed with water and equilibration buffer of the second purification method to ensure that only compatible eluent entered the second column. The same operation is done also at the end of the process but purging the tubing with the equilibration of the first dimension instead, so that both columns are equilibrated with their respective equilibration buffer.

3.5. Process performance parameters

Process performance was evaluated using the four fundamental parameters for preparative chromatography: purity, yield, productivity, and eluent consumption.

The first parameter to consider is purity, which is expressed as the ratio of the area of the product peak (A_{target}) to the sum of the areas of all peaks (A_{total}) in the chromatogram in HPLC analytics, and it is expressed as a percentage.

$$Purity\ [\%] = \frac{A_{target}}{A_{total}} \cdot 100 \tag{1}$$

Yield or recovery, on the other hand, is expressed as the ratio of the mass of the product collected ($m_{target\ collected}$) in a given pool or fraction to the mass of product that has been injected ($m_{target\ injected}$) into the column. This parameter is also expressed as a percentage.

$$Yield\ [\%] = \frac{m_{target\ \ collected}}{m_{target\ \ injected}} \cdot 100 \tag{2}$$

As for productivity, it represents the amount of target product collected ($m_{pool\ collected}$) in a fraction or pool per unit of time and column volume (CV). It is expressed as $g_{product}/L_{column}/h$.

$$Productivity\ [g/L/h] = \frac{m_{pool\ \ collected}}{CVtime} \tag{3}$$

Finally, the last parameter to consider is the eluent consumption and it is typically expressed as the total volume of eluent required to purify a defined mass of target product ($m_{target\ collected}$). It is expressed as $L_{eluent}/g_{product}$.

$$Eluent\ Consumption\ [L/g] = \frac{Total\ Volume}{m_{target\ \ collected}} \tag{4}$$

As for the Integrated Batch runs, in the productivity and eluent consumption the CV is the sum of the CVs of the two columns, while the time and the buffer volume are intended for the duration of the entire method, and the target collected is the product collected from the second dimension.

The trade-off between purity and yield in a batch process can be represented through the Pareto curve. In the batch process, fractions are collected periodically. Then, they are analyzed offline by means of HPLC to determine the mass of both target and impurities for each. By beginning with the purest fraction (which contains a small product recovery) and progressively adding adjacent fractions, purity and recovery are calculated for all cumulative fractions, called pool. By enlarging the product window, yield increases at the expense of purity, since also the regions where the product overlaps with impurities are collected. These hypothetical pools are then plotted on a graph, forming a curve of optimal points that demonstrates how increasing recovery can only be achieved by accepting a lower level of purity, and vice versa (Pareto curve) [16].

4. Discussion

4.1. Batch results

As reported in Section 3.1, the feed material had an initial purity of 51 %. For the purification of the oligonucleotide, three methods have been used, one using AEX chromatography and the other with IP-RP with TEAA and RP with NaOAc.

From both the preparative chromatograms and the fraction analysis of the three batches (Fig. 2), it can be noted that AEX (Fig. 2A) leads to a different elution behavior of weakly adsorbing impurities; indeed, part of these elutes during the wash after loading, while for the IP-RP (Fig. 2B-C) only one peak is obtained. Another difference that can be seen from the chromatograms is the absorbance, which is higher in the purification using IP-RP compared to the AEX purification. This is due to the difference in the pH of the mobile phases. As said in section 3.3, the pH of anion exchange mobile phase is 12, while the mobile phases used for both IP-RP purification have a pH of 8.5. At high pH level the DNA bases are totally deprotonated, and this probably affects the UV absorbance properties. To confirm this hypothesis the UV absorbance was also

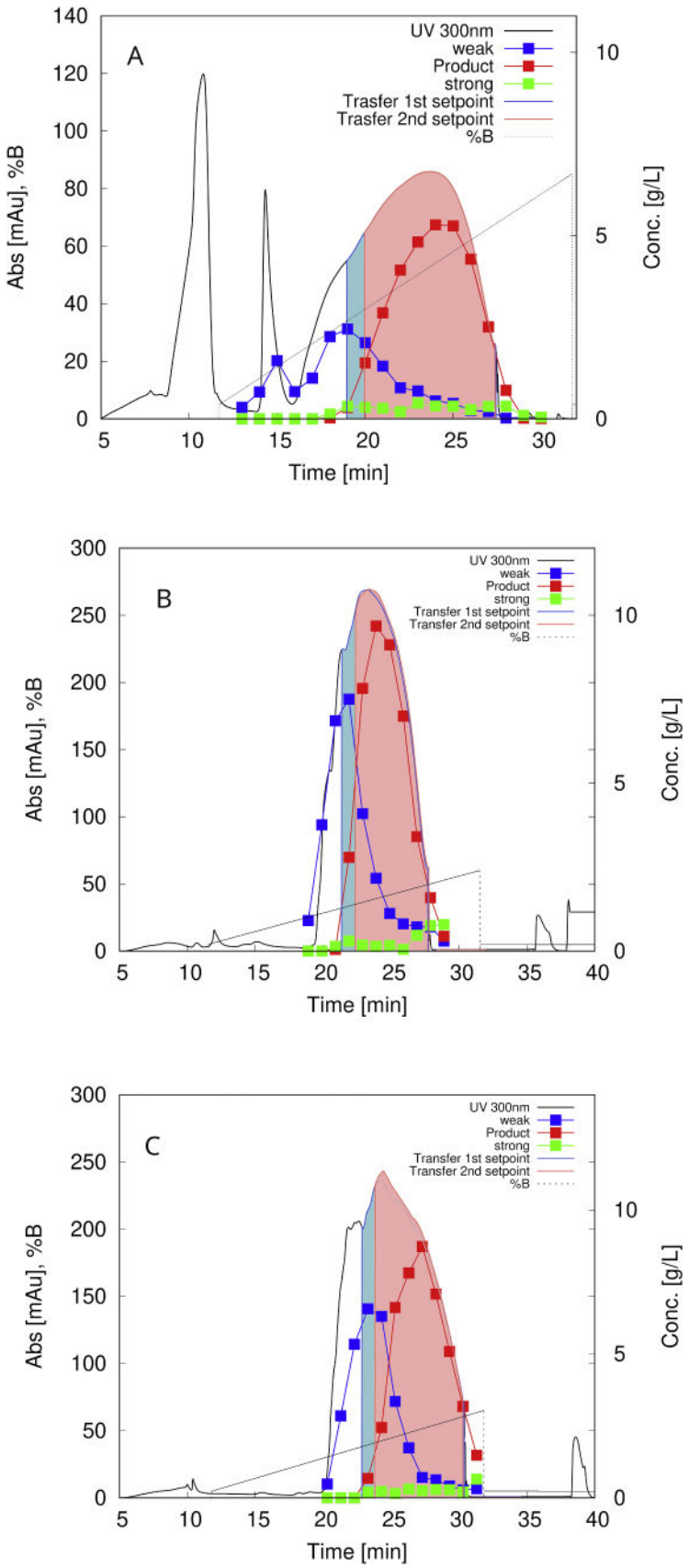

Fig. 2. Preparative chromatograms of the three batch methods. A) AEX purification; B) RP purification using NaOAc; C) RP purification using TEAA as ion pair. Blue, red, and green dotted lines represent the concentration profile of Weak impurities, Product, and Strong impurities, respectively. Red zone corresponds to 85 % of the mass of the product; red and blue zones correspond to 90 % of the mass of the product.

measured by using a Nanodrop lite Spectrophotometer (see supplementary information Section 1). Using Sodium Acetate the absorbance at 260 nm resulted in 8.03 AU. For the second and third samples where the range of pH was about the same (pH ~ 8), the absorbance values measured at 260 nm were 7.03 AU and 7.74 AU. For the sample dissolved in Buffer A (pH = 12) the absorbance at 260 nm was 2.08 AU, explaining the significantly smaller UV intensity in the AEX chromatograms.

Results of fraction analysis are reported as Pareto curves in Fig. 4A. From this figure it can be noted that a maximum purity of around 90 % was achieved for all the three batch methods, with different recoveries: 13.2 % for AEX, 16.4 % for NaOAc-RP and 20.8 % for TEAA-RP. These data clearly indicate that a single batch method for oligonucleotide purification is not sufficient to achieve the optimal desired product purity under the selected gradient and load condition. In this context, the combination of two different elution modes is usually advantageous. While not required for Integrated Batch process design, but to comprehend the full potential and orthogonality of the combinations AEX-RP and RP-AEX, as well as to correctly set the conditions for Integrated Batch runs, a complete tracking of all impurities present in the purest fraction of the batch runs was performed. The concentration profile for each single impurity was overlaid with the concentration profile

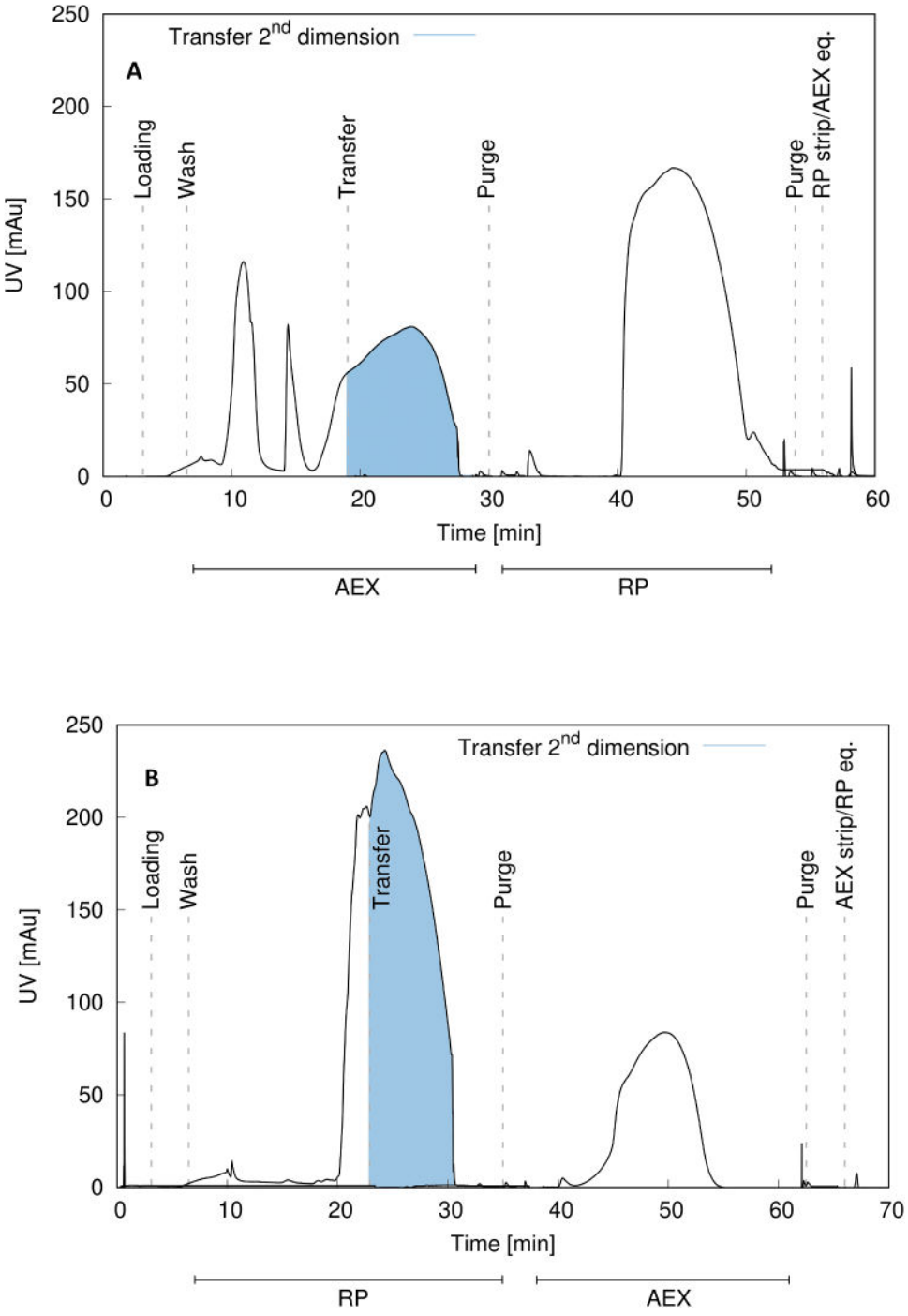

Fig. 3. Preparative chromatograms of the two sequences for Integrated Batch process. The sequence AEX-RP (A); the sequence RP-AEX (B) using TEEA as ion pair. The blue area represents the product (90 % of yield) that has been transferred to the second dimension. For Purge is intended the flush of the tubing first with water and then with the mobile phase used in the second column. Note the UV intensities are affected by operating pH.

obtained for the product (see Figs S1–2).

Common impurity peaks to both the AEX purification and the two RP purifications were identified, with the aim of understanding which impurities can be more easily removed during the transfer of the product to the second dimension. The criterion to determine the better chromatographic sequence was the achievement of the highest final purity.

As it can be seen from Fig. S1, comparing AEX purification and RP purification with NaOAc, for the latter one, the common impurities overlap with the product peak to a smaller extent, which makes them easier to remove and thus making the sequence RP-AEX preferable. On the other hand, regarding the comparison of common impurities between AEX and RP purification with TEAA as the ion pair (Fig. S2), in both cases the impurity profiles overlap with the entire concentration profile of the product, making it more difficult to separate them during the transfer of the product.

4.2. Integrated batch results

Four sequences were tested for the Integrated Batch runs: i) AEX-NaOAc RP, ii) AEX-TEAA RP, iii) NaOAc RP-AEX and iv) TEAA RP-AEX. Moreover, based on the results of fraction analysis and impurity tracking of the three design batch runs (Fig. 2 and S1–2), two different portions of the target peak were chosen to be transferred to the second dimension, i.e. 90 and 85 % of the target recovery, resulting in a total of 8 purifications. For simplicity, Integrated Batch profiles of only two sequences (AEX-RP and RP-AEX using TEEA as ion pair) are shown in Fig. 3.

For the sequence where the AEX purification is in the first dimension and RP with both ion pair agents in the second dimension, inline dilution is needed during the transfer of the product, as previously mentioned in Section 3.4. This is necessary to ensure an adequate pH level and salt concentration while the product eluting from the first column enters the RP column. On the other hand, for the opposite sequence (RP-AEX), since the AEX column characteristics are compatible with pH, salt and organic solvent concentration typical of RP conditions, no inline dilution is needed. Moreover, this sequence, having the AEX purification as a second dimension, allows to obtain the oligo in its sodium form without an additional step of salt exchange.

The Pareto curves for the 8 purifications are shown in Fig. 4B-C. In general, all sequences resulted in an increase of the maximum purity, thanks to the combination of the two orthogonal separation mechanisms; nevertheless, it is well-known that a high purity is usually associated with a low yield. Interestingly, when considering the same purity level reached in batches, a considerable gain in yield can be obtained by combining two techniques in an integrated approach, as reported in Table 1. This result is clearly observed in Fig. 4B and 4C, where the Pareto curves of all Integrated Batch runs are located above the Pareto curves of the three batch methods, since they show the yield of the second step. This means that, at a given purity, the higher the curve, the greater the recovery. In case of TEAA as ion-pair reagent, for purities below 85 %, the curves of Integrated Batch and single column batch cross, hence single column operation would be more favorable than integrated batch chromatography (Fig 4C).

More in detail, for both sequences (AEX-RP and RP-AEX) with NaOAc, the use of the second setpoint (85 % of target product transferred) leads to a better performance in terms of purity if compared to the first setpoint (90 % transferred). As previously hypothesized from impurity tracking (Fig. S1), the sequence RP-AEX showed superior separation capabilities with respect to AEX-RP, being able to reach a purity $>$ 95 %. Conversely, the use of TEAA as ion pair leads to a less marked improvement in purity between all the Integrated Batch runs, with maximum achieved purity in the range 93–94 %. In this case, the transfer of 85 % of the product to the second dimension did not lead to an overall better performance. Indeed, at high yield the pareto curves for the first setpoint (90 % transferred) show better results in terms of purity.

Yield, productivity and eluent consumption were calculated and compared for the single column batches and for the Integrated Batch runs and are shown in Fig. 5. These values were calculated at two fixed purity levels: at 89 % purity, a value common to all batch methods, and at 91 % purity, a value reached only with Integrated Batch runs. It must be stated that for oligonucleotides the purity required for a product of clinical grade is generally $>$ 90 % [4].

Regarding the yield calculated at 89 % purity (Fig. 5A), RP using TEAA showed the best performance among the three single batches. Nevertheless, Integrated Batch sequences with both ion pairing agents lead to an increase in yield when compared to single batches.

As shown in Fig. 5A and Table 1 productivity and eluent consumption were best with the RP batch method using TEAA, despite the rather low yield of 48.7 %, outperforming also Integrated Batch sequences using the same ion pair agent. Instead, AEX and RP with Sodium Acetate had worse productivity and eluent consumption values than integrated batch chromatography, which can be attributed to their low yields of 22.9 % and 16.4 %, respectively. Additionally, in the mathematical calculation for these two parameters, it is important to consider that, as mentioned in Section 3.5, the CV is the sum of the CVs of the two columns, while the time and volume are intended for the entire duration of the method.

Upon comparing the two different sequences, AEX-RP and RP-AEX, it was observed that for both productivity and eluent consumption parameters there is a slight overall gain when using the AEX-RP sequence. This is due to the different column size in first dimension, which results in a different absolute load, moreover, as already said in Section 3.3, the loading for the two IP-RP purification is different, for the one using TEAA the loading is 40 g/ L_{column}, while for the NaOAc it is 35 g/L_{column}.

As for the process comparison calculated at 91 % purity (Fig. 5B), none of the three batches reached that value. Even in this case, by using the second set point (85 % transferred) for the product transfer to the second dimension, a generally better performance in yield, productivity, and eluent consumption can be obtained.

5. Conclusions

The Integrated Batch process has proven to be a promising alternative method for the purification of oligonucleotides when compared to individual batches, since it allows to significantly reduce analysis times and to completely automate the purification process. This has several advantages not only in terms of productivity, but also in the fact that there is no need to offline analyze the fractions between one dimension and the next one nor to perform any sample preparation step before injecting the feed into the second column. This allows to keep the QC demand the same as for the single-column batch process, while improving process performance parameters. It must be mentioned that in this work sequential batches were not run as a comparison, since similar performance parameter and purity results as the Integrated Batch would be expected.

In this way, once the experimental conditions are defined for the individual batch steps, this technique allows to directly combine two chromatographic steps, by automating the transfer of the selected portion of the chromatogram from the first column to the second one, thus increasing the reproducibility of the process while saving time.

Regarding the two possible sequences, AEX-RP and RP-AEX, they both led to quite a significant improvement in yield at fixed values of purity, with respect to the corresponding batch. Nevertheless, even if from a mere practical consideration, the RP-AEX sequence would seem the best choice, at least for the specific experimental conditions and target oligonucleotide used in this work, since there is no need of inline dilution and sodium is the counterion, which is the form in which oligonucleotides are usually formulated.

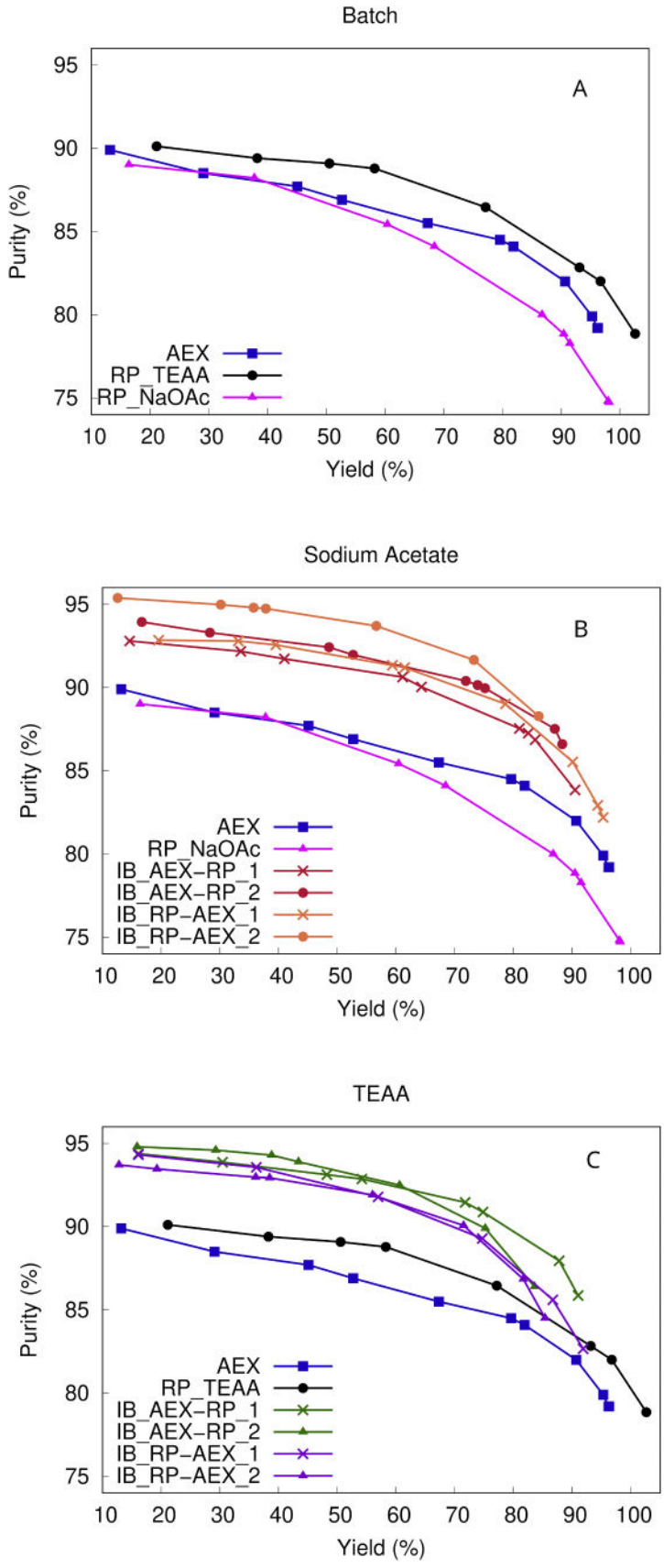

Fig. 4. A) Pareto curves of the three batches. Pink: RP using NaOAc; blue: AEX; Black: RP using TEAA as ion pair. B) Pareto curve of the integrated Batch (IB) runs using Sodium Acetate compared to the batch methods. C) Pareto curve of the Integrated Batch runs using TEAA as ion pair agent compared to the batch methods. 1 and 2 refer to the wide (90 % recovery) and narrow (85 % of recovery) transfers.

Table 1
Process performance improvement (in terms of Yield, Productivity and Eluent consumption) between the three batches and the Integrated Batch runs calculated at 89 % of purity.

	Yield [%] vs TEAA	Yield [%] vs NaOAc	Yield [%] vs AEX	Productivity [g/L/h] vs TEAA	Productivity [g/L/h] vs NaOAc	Productivity [g/L/h] **vs AEX**	Eluent cons. [L/g] vs TEAA	Eluent cons. [L/g] vs NaOAc	Eluent cons. [L/g] vs AEX
AEX-NaOAc_1	–	+333 %	+210 %	–	+54 %	+40 %	–	−34 %	−31 %
AEX-NaOAc_2	–	+388 %	+249 %	–	+75 %	+59 %	–	−40 %	−38 %
AEX-TEAA_1	+72 %	–	+265 %	−40 %	–	+55 %	+108 %	–	−36 %
AEX-TEAA_2	+60 %	–	+241 %	−45 %	–	+44 %	+85 %	–	−44 %
NaOAc-AEX_1	–	+379 %	+243 %	–	+40 %	+27 %	–	−25 %	−21 %
NaOAc-AEX_2	–	+403 %	+260 %	–	+46 %	+33 %	–	−28 %	−25 %
TEAA-AEX_1	+56 %	–	+233 %	−53 %	–	+23 %	+123 %	–	−32 %
TEAA-AEX_2	+56 %	–	+231 %	−53 %	–	+21 %	+123 %	–	−32 %

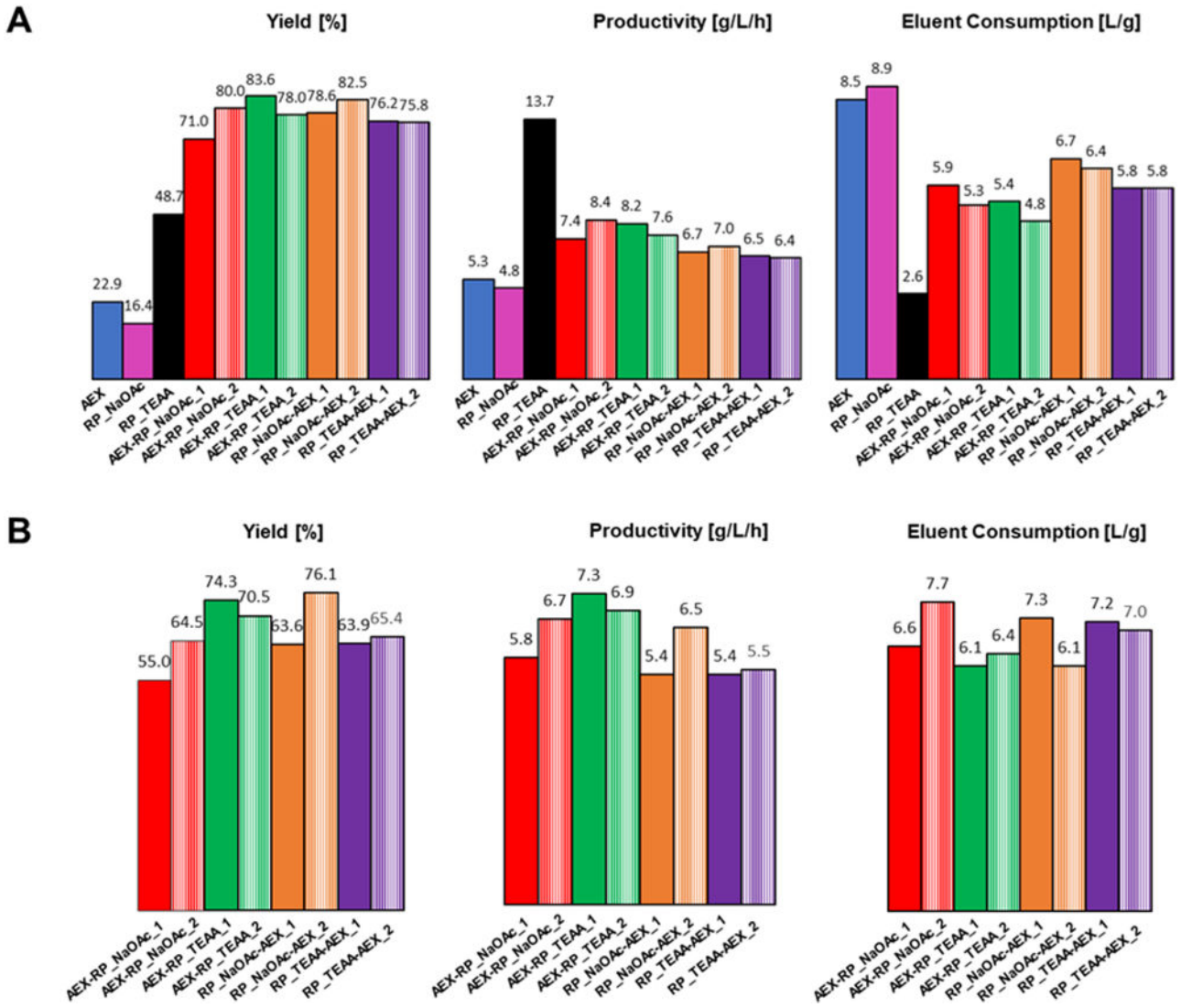

Fig. 5. Histograms showing the process performance (yield [%], productivity[g/L/h], and eluent consumption [L/g]) of the three batch methods and of the Integrated Batch sequences. A) Process comparison calculated at 89 % purity; B) process comparison calculated at 91 % purity.

CRediT authorship contribution statement

Chiara Nosengo: Writing – original draft, Investigation, Formal analysis. **Desiree Bozza:** Writing – original draft, Data curation. **Giulio Lievore:** Validation, Investigation. **Sebastian Vogg:** Supervision, Methodology, Data curation. **Martina Catani:** Validation, Data

curation. **Alberto Cavazzini:** Funding acquisition, Data curation. **Thomas Müller-Späth:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Chiara De Luca:** Writing – review & editing, Supervision. **Simona Felletti:** Visualization, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to thank the National Recovery and Resilience Plan (NRRP), Mission 04 Component 2 Investment 1.5 - NextGenerationEU, Call for tender n. 3277 dated December 30, 2021, Award Number: 0001052 dated June 23, 2022.

The authors would also like to thank the Italian University and Scientific Research Ministry (grant P2022PTYWP, title: "Design of high-pRofit fostEring bioActive coMpounds through integral valorization of seaWEEDs infesting the MEditerranean sea (DreamWEEDme)".

Chiara De Luca and Chiara Nosengo benefit from funding FSE REACT-EU, within the program PON "Research and Innovation" 2014–2020 (PON R&I), Action IV.6 "Contratti di ricerca su Tematiche Green" and Action IV.5 "Borse di Studio di Dottorato di Ricerca su Tematiche Green," respectively.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2024.465440.

Data availability

The data that has been used is confidential.

References

[1] A. Zimmermann, R. Greco, I. Walker, J. Horak, A. Cavazzini, M. Lämmerhofer, Synthetic oligonucleotide separations by mixed-mode reversed-phase/weak anion-exchange liquid chromatography, J. Chromatogr. A 1354 (2014) 43–55, https://doi.org/10.1016/j.chroma.2014.05.048.

[2] W. Brad Wan, P.P. Seth, The medicinal chemistry of therapeutic oligonucleotides, J. Med. Chem. 59 (21) (2016) 9645–9667, https://doi.org/10.1021/acs.jmedchem.6b00551.

[3] R. Kole, A.R. Krainer, S. Altman, RNA therapeutics: beyond RNA interference and antisense oligonucleotides, Nat. Rev. Drug Discov. 11 (2) (2012) 125–140, https://doi.org/10.1038/nrd3625.

[4] R. Weldon, J. Lill, M. Olbrich, P. Schmidt, T. Müller-Späth, Purification of a GalNAc-cluster-conjugated oligonucleotide by reversed-phase twin-column continuous chromatography, J. Chromatogr. A 1663 (2022), https://doi.org/10.1016/j.chroma.2021.462734.

[5] M. Catani, C. De Luca, J. Medeiros Garcia Alcântara, N. Manfredini, D. Perrone, E. Marchesi, R. Weldon, T. Müller-Späth, A. Cavazzini, M. Morbidelli, M. Sponchioni, Oligonucleotides: current trends and innovative applications in the synthesis, characterization, and purification, Biotechnol. J. 15 (8) (2020), https://doi.org/10.1002/biot.201900226.

[6] T.C. Roberts, R. Langer, M.J.A. Wood, Advances in oligonucleotide drug delivery, Nat. Rev. Drug Discov. 19 (10) (2020) 673–694, https://doi.org/10.1038/s41573-020-0075-7.

[7] D. Collotta, I. Bertocchi, E. Chiapello, M. Collino, Antisense oligonucleotides: a novel Frontier in pharmacological strategy, Front. Pharmacol. 14 (2023), https://doi.org/10.3389/fphar.2023.1304342.

[8] E. Paredes, V. Aduda, K.L. Ackley, H. Cramer, Manufacturing of oligonucleotides, in: Comprehensive Medicinal Chemistry III, Elsevier, 2017, pp. 233–279, https://doi.org/10.1016/b978-0-12-409547-2.12423-0.

[9] O. Al Musaimi, D. Al Shaer, F. Albericio, B.G. de la Torre, 2022 FDA TIDES (Peptides and Oligonucleotides) harvest, Pharmaceuticals 16 (3) (2023), https://doi.org/10.3390/ph16030336.

[10] R. Eritja, Solid-phase synthesis of modified oligonucleotides, Int. J. Pept. Res. Ther. 13 (1–2) (2007) 53–68, https://doi.org/10.1007/s10989-006-9053-0.

[11] Y.S. Sanghvi, CHAPTER 19: large-scale automated synthesis of therapeutic oligonucleotides: a status update. RSC Drug Discovery Series, Royal Society of Chemistry, 2019, pp. 453–473, https://doi.org/10.1039/9781788015714-00453, 2019-January, no. 68.

[12] D. Capaldi, A. Teasdale, S. Henry, N. Akhtar, C. Den Besten, S. Gao-Sheridan, M. Kretschmer, N. Sharpe, B. Andrews, B. Burm, J. Foy, Impurities in oligonucleotide drug substances and drug products, Nucleic Acid. Ther. 27 (6) (2017) 309–322, https://doi.org/10.1089/nat.2017.0691.

[13] T. Fornstedt, M. Enmark, Separation of therapeutic oligonucleotides using ion-pair reversed-phase chromatography based on fundamental separation science, J. Chromatogr. Open 3 (2023), https://doi.org/10.1016/j.jcoa.2023.100079.

[14] N. Tugcu, R.R. Deshmukh, Y.S. Sanghvi, J.A. Moore, S.M. Cramer, Purification of an oligonucleotide at high column loading by high affinity, low-molecular-mass displacers, J Chromatogr A 923 (1–2) (2001) 65–73, https://doi.org/10.1016/s0021-9673(01)00954-2.

[15] G. Guiochon, Preparative liquid chromatography, J. Chromatogr. A 965 (2002) 129–161, https://doi.org/10.1016/S0021-9673(01)01471-6.

[16] C. De Luca, S. Felletti, G. Lievore, T. Chenet, M. Morbidelli, M. Sponchioni, A. Cavazzini, M. Catani, Modern trends in downstream processing of biotherapeutics through continuous chromatography: the potential of multicolumn countercurrent solvent gradient purification, TrAC Trends Anal. Chem. 132 (2020), https://doi.org/10.1016/j.trac.2020.116051.

[17] I. Fioretti, T. Müller-Späth, R. Weldon, S. Vogg, M. Morbidelli, M. Sponchioni, Continuous countercurrent chromatographic twin-column purification of oligonucleotides: the role of the displacement effect, Biotechnol. Bioeng. 119 (7) (2022) 1861–1872, https://doi.org/10.1002/bit.28093.

[18] C. De Luca, S. Felletti, G. Lievore, A. Buratti, S. Vogg, M. Morbidelli, A. Cavazzini, M. Catani, M. Macis, A. Ricci, W. Cabri, From batch to continuous chromatographic purification of a therapeutic peptide through multicolumn countercurrent solvent gradient purification, J. Chromatogr. A 1625 (2020), https://doi.org/10.1016/j.chroma.2020.461304.

[19] A.S. Rathore, N. Kateja, D. Kumar, Process integration and control in continuous bioprocessing, Curr. Opin. Chem. Eng. 22 (2018) 18–25, https://doi.org/10.1016/j.coche.2018.08.005.

[20] G. Lievore, R. Weldon, M. Catani, A. Cavazzini, T. Müller-Späth, Enrichment and recovery of oligonucleotide impurities by N-Rich twin-column continuous chromatography, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 1209 (2022), https://doi.org/10.1016/j.jchromb.2022.123439.

[21] M. Enmark, J. Bagge, L. Thuberg, E. Örnskov, H. Leek, F. Limé, T. Fornstedt, Analytical and preparative separation of phosphorothioated oligonucleotides: columns and ion-pair reagents, Anal. Bioanal. Chem. 412 (2) (2020) 299–309, https://doi.org/10.1007/s00216-019-02236-9.

[22] C. De Luca, G. Lievore, D. Bozza, A. Buratti, A. Cavazzini, A. Ricci, M. Macis, W. Cabri, S. Felletti, M. Catani, Downstream processing of therapeutic peptides by means of preparative liquid chromatography, Molecules. 26 (15) (2021), https://doi.org/10.3390/molecules26154688.

[23] G. Lievore, D. Bozza, M. Catani, A. Cavazzini, T. Chenet, L. Pasti, L. Ferrazzano, W. Cabri M. Macis, A. Ricci, C. De Luca, S. Felletti, Benefits of a mixed-mode stationary phase to address the challenging purification of an industrially relevant peptide: a proof-of-concept study, Separations. 9 (5) (2022), https://doi.org/10.3390/separations9050125.

[24] A.A. Kazarian, W. Barnhart, J. Long, K. Sham, B. Wu, J.K. Murray, Purification of N-acetylgalactosamine-modified-oligonucleotides using orthogonal anion-exchange and mixed-mode chromatography approaches, J. Chromatogr. A 1661 (2022), https://doi.org/10.1016/j.chroma.2021.462679.

[25] D.R. Stoll, P.W. Carr, Two-dimensional liquid chromatography: a state of the art tutorial, Anal. Chem. 89 (1) (2017) 519–531, https://doi.org/10.1021/acs.analchem.6b03506.

[26] F. Li, X. Su, S. Bäurer, M. Lämmerhofer, Multiple heart-cutting mixed-mode chromatography-reversed-phase 2D-liquid chromatography method for separation and mass spectrometric characterization of synthetic oligonucleotides, J. Chromatogr. A 1625 (2020), https://doi.org/10.1016/j.chroma.2020.461338.

[27] S.G. Roussis, I. Cedillo, C. Rentel, Two-dimensional liquid chromatography-mass spectrometry for the characterization of modified oligonucleotide impurities, Anal. Biochem. 556 (2018) 45–52, https://doi.org/10.1016/j.ab.2018.06.019.

[28] A. Goyon, K. Zhang, Characterization of antisense oligonucleotide impurities by ion-pairing reversed-phase and anion exchange chromatography coupled to hydrophilic interaction liquid chromatography/mass spectrometry using a versatile two-dimensional liquid chromatography setup, Anal. Chem. 92 (8) (2020) 5944–5951, https://doi.org/10.1021/acs.analchem.0c00114.