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# Enrichment and recovery of oligonucleotide impurities by N-Rich twin-column continuous chromatography

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ABSTRACT

N-Rich is a twin-column continuous chromatography technology well suited for small-scale isolation and the enrichment of product related impurities. For the first time, N-Rich was used for impurity isolation from a double-stranded RNA (dsRNA) therapeutic synthetic oligonucleotide (ON), produced by solid-phase synthesis. By employing the N-Rich process, where the desired impurities are recycled and selectively enriched, and interfering substances are depleted, it was possible to obtain substantial amounts of high purity marginal impurities with a reproducible, automatized, and productive method. The productivity-purity tradeoff inherent to traditional impurity isolation methods, i.e., analytical chromatography, was effectively alleviated. Using N-Rich, satisfactory purity values and mass recoveries of several low-concentrated impurities could be obtained simultaneously.

A performance comparison demonstrated an up to 15-fold increase for purity values and up to 20-fold mass impurity isolation and concentration with the N-Rich technology in comparison to conventional isolation procedures, drastically reducing processing times, manual handling, and waste production.

## 1. Introduction

Synthetic oligonucleotides (ONs) are short nucleic acid chains able to control the expression of target genes and accordingly modify expression of disease-related proteins that are normally inaccessible by small molecules and proteins. After overcoming some major hurdles (e.g., poor pharmacokinetics, ineffective target delivery, insufficient biological activity, off-target toxicity) therapeutic ONs are now going through a tremendous growth in the biopharmaceutical sector [1,2]. An increasing number of synthetic ONs are being developed for a wide range of research, diagnostic and therapeutic applications. To date, 13 ON-based therapeutics have been approved by the FDA and, as reported on ClinicalTrials.gov, more than 180 ON-based candidates have been approved for clinical trials for various applications [3,4].

Synthetic oligonucleotides comprise several classes of chemical entities differing by their chemistry and structure–activity relationship (SAR): small interfering RNA (siRNA), microRNA (miRNA), antisense oligonucleotides (ASO) and aptamers, to name a few [5]. These are typically generated using solid-phase synthesis, a cyclic approach, where the progressive addition of nucleotide monomers eventually results in a polymeric chain of predefined structure and length, $n$ [6]. Despite a high coupling yield, solid-phase synthesis generates many structurally closely related impurities, among which are shortmers (e.g., *n-1*, *n-2*), resulting from incomplete coupling reactions, longmers (e.g., $n + 1$, $n + 2$), and other by-products coming from deamination, depurination, oxidation and other side reactions [7]. Moreover, diastereomers are frequently formed due to the chirality of sulfur atoms presents on phosphorothioate oligonucleotides, the widest adopted backbone modification [3].

A mandatory aspect of the pre-clinical drug development of biopharmaceuticals is the isolation and characterization of impurities for further structural, biochemical, and toxicological elucidation, and cell or animal-based safety assays [8]. Currently regulatory agencies have yet to provide definitive reporting, qualification, and identification threshold values for ON impurities. However, a series of white papers from Capaldi et al. regarding the regulatory environment of oligonucleotides suggested the values of 0.2 % as reporting, 1 % as identification and 1.5 % as qualification limits [9,10,11]. However, it is anticipated that these limits will become more stringent as higher performing processing technologies are standardized.

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Prior to characterization, isolation of product related impurities is currently carried out using inefficient and time-consuming methods. A standard approach is the application of linear scale reversed phase (RP) or ion exchange (IEX) chromatographic methods, either using analytical U(H)PLC or preparative systems linked to a fraction collector [12,13]. The operator faces a tradeoff between productivity and purity, typical of single column chromatography [14–17]. These methods operate in the linear range of the adsorption isotherm, thereby achieving a high resolution and minimizing overlap between components, and thus obtaining high purity values. However, this comes at the cost of restricted injection amount per run (micrograms), very low final target concentration, and prolonged processing times due to the need to pool target material from multiple chromatographic runs.

Given the limitation of standard methods, employing the N-Rich process, a twin-column continuous chromatography technique, we can potentially alleviate bottlenecks with the result of providing larger amounts of high purity product-related impurities in a short operation time [18–20]. This technology was already successfully applied for the enrichment of antibody isoforms and peptide impurities [12,14]. This work describes the design and operation of the twin-column N-Rich process, applied for the first time to a therapeutic oligonucleotide, a 24-mer double-stranded RNA. Operating under reversed phase conditions, the process performance and product quality obtained with N-Rich and semi-preparative batch processes were evaluated and compared.

### 1.1. The N-Rich rationale

Continuous chromatography is increasingly used by industrial separation specialists as a scalable manufacturing technology capable of reducing bottlenecks in downstream processing of biopharmaceuticals in capture and polishing applications [21–24]. In contrast, application of the technology in product development for reducing bottlenecks in the generation of impurity standards or other difficult-to-purify or low abundant compounds, is relatively new. N-Rich is an automated process using two identical columns to enrich and purify a desired compound from a complex mixture. Conventional resin material can be used for separations with N-Rich, such as ion exchange (IEX), hydrophobic interaction (HIC) or reversed phase (e.g., C18). N-Rich can be set up to target a single compound, or a region of the chromatogram containing several compounds. The N-Rich process is composed of four steps (Fig. 1). The first method (Startup) begins by loading feed material onto the first column and performing a linear gradient elution. During the second method (Enrichment), a region of the chromatogram containing target impurities is transferred from the first column, with in-line dilution, and re-adsorbed to the second column. All fractions not intended for recycling, including main product and other side-impurities, can be either discarded in waste or, if valuable, separately collected for other purposes. In the meantime, fresh feed is loaded onto the second column in addition to the recycled target. This step leads to an enrichment of the target molecules relative to other compounds in the mixture. The process step is repeated in a cyclic fashion between the two columns, progressively increasing the concentration of the target impurities. Phase three (Depletion) is a single switch without addition of new feed. This step depletes non-target compounds while internally recycling the accumulated target impurities before the final elution step. The depletion step greatly improves the final purity obtained for impurities that are closely eluting with the product peak. Finally, in the fourth phase (Final Fractionation), the enriched target material is eluted with a shallow gradient over two columns in-series and the target material is collected performing a fine fractionation. This strategy maximizes the resolution of the enriched compounds, and the pure target material is recovered at a higher concentration than with batch methodology.

The advantages of N-Rich over analytical and batch-wise elution include reduced processing times and consumables, in addition to increased final resolution, recovered mass, and purities. Furthermore, the automatization of the recycling and elution processes requires fewer operator activities, e.g., pooling, analyzing, merging, and reprocessing fractions, and consequently reduced operational footprint and qualified personnel time consuming [16,17].

## 2. Materials and methods

### 2.1. Chemicals

HPLC-grade acetonitrile (ACN) and methanol (MeOH) from Merck Millipore (Darmstadt, Germany) in addition to deionized water from a Milli-Q Advantage A10 were employed as mobile phases. An ion-pairing system TEA/HFIP was added, prepared with triethylamine (≥99.5 %) and Hexafluor-2-propanol (≥99.5 %), both purchased from Sigma-Aldrich (St. Louis, MO, USA). The feed material was a 24-mer double-stranded RNA oligonucleotide, synthetized by means of solid-phase synthesis, solubilized in 30 % ammonium hydroxide at the concentration of 44.8 g/L with a chromatographic purity of 75.6 %. The feed was diluted to 0.5 g/L with preparative chromatography buffer A.

### 2.2. Preparative chromatography conditions

Batch-wise and N-Rich chromatographic experiments were performed on a Contichrom® CUBE 30 continuous chromatography system (ChromaCon AG, A YMC Company, Zurich, Switzerland). The system was equipped with a fraction collector. Mobile phases were kept at a constant temperature of 60 °C using an AZURA® CT 2.1 column thermostat from Knauer (Berlin, Germany). The solvents used for preparative chromatography were as follows: 0.2 % ACN, 0.2 % TEA, 1 % HFIP (Buffer A); 1.66 % MeOH, 15 % ACN (Buffer B); a stripping buffer of 5 % MeOH, 45 % ACN (Buffer C). Two Triart Prep C18-S, 12 nm-10 μm 4.6 mm i.D. × 150 mm columns from YMC (Kyoto, Japan) were used as stationary phases. The UV signals were monitored at 280 nm.

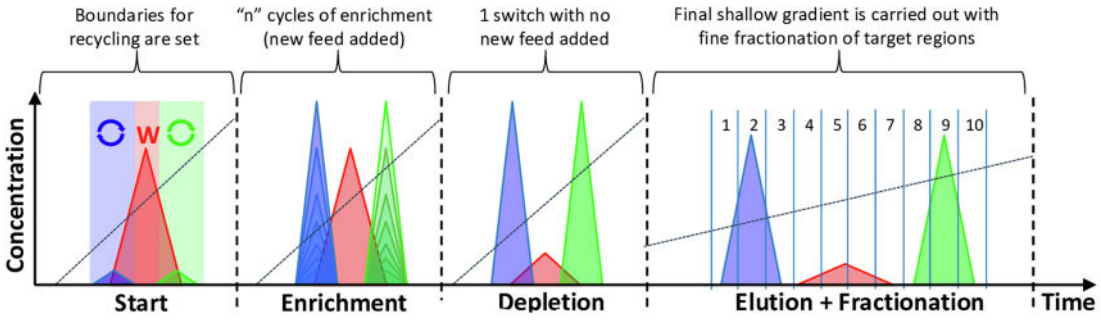


**Fig. 1.** The N-Rich process automatically enriches and isolates compounds in fractions that are initially highly dilute. This is done in four methods: 1) Start; 2) Enrichment of target compounds (blue, green); 3) depletion of interfering compounds (red); 4) Final elution with fractionation.

### 2.3. Offline analytics

Feed analytical characterization and pooled fraction evaluations were conducted by injecting 2 µL sample volume on a 1290 Infinity II LC System (Agilent, Santa Clara, CA, USA) equipped with an Acquity UPLC Oligonucleotide BEH C18, 1.7 µm, 2.1 mm i.D. × 50 mm column (Waters Corporation, Milford, MA, USA). Mobile phase A () was constituted of 0.2 % TEA, 1 % HFIP in water whilst mobile phase B () was a solution of 10 % MeOH and 90 % ACN. The mobile phase flow was set at 0.3 mL/min, the signal recorded at UV 260 nm wavelength and the column thermostat set to 62 °C. The analytical protocol began with a one-minute initial equilibration at 1 %B, followed by a four-minute gradient from 1 %B to 6.5 %B and a ten-minute gradient from 6.5 %B to 8.5 %B. Finally, after three minutes of stripping the stationary phase with 30 %B, the percentage of was decreased to the initial value.

### 2.4. Design batch chromatogram

The first step to design a continuous enrichment process is to select a single column batch "design chromatogram". The batch protocol, in this case serving both the purpose of representing the single column benchmark and the reference method for the continuous chromatography setup, included a 3 column volumes (CVs) equilibration with Buffer A, a 0.2 CV feed load (0.25 g per liters of resin load) and a 2 CVs wash after load with Buffer A performed at 300 cm/h. Thereafter, a 200 cm/h flow rate was used to perform a linear gradient elution from 15 % to 70 % of Buffer B in 12 CVs. Eventually, a resin regeneration step with strip Buffer C and a re-equilibration procedure with Buffer A were applied for 2 and 3 CVs, respectively, at the increased flow rate of 600 cm/h. During the elution stage, a fine fractionation was performed to both characterize the impurities in order to establish suitable recycling windows for the N-Rich process.

### 2.5. N-Rich operating parameters

The N-Rich process uses the same column, solvents, and elution protocols as the single column preparative batch process. By means of the N-Rich design software (N-Rich wizard) included with the ChromIQ® operating software of the Contichrom® CUBE system, the previously obtained batch "design chromatogram" is imported and used for process design. At this stage, based on the findings from the analytical evaluations of the batch experiment, the recycling windows for the impurities to be accumulated are selected. Therefore, the chromatogram is divided into 4 zones (see example in Fig. 2) using five section borders (-). They correspond to the switching times that compose the N-Rich elution protocol. In particular, corresponds to the modifier gradient starting time and it is employed by the wizard to set dilution parameters, is the trigger point where the elution of the weakly adsorbing impurities begins that are internally recycled to the second column. The window defined by and corresponds to a region of nearly pure main compound, which undergoes depletion at each switch. However, it is worth noting that the pure product region could have been also collected instead of discarded and used for other purposes. Eventually, in the to interval, strongly adsorbing impurities are eluted and inline diluted before entering the second column. Inline dilution ensures re-adsorption of the target compound in the downstream column by lowering the modifier concentration. Simultaneously, corresponds to the end of the elution stage and the begin of the stripping procedure. In specific, the operational switching times were as follows: $t_1$=14.8 min, $t_2$=31.3 min, $t_3$=52.8 min, $t_4$=54.3 min and $t_5$=64.3 min.

Once section borders for impurity recycling and product removal are configured, the wizard automatically determines the load per switch, the in-line dilution factors for the recycling phases, and the gradient start and end concentrations of each section to be operated by the CUBE system pumps. For each switch, the elution sections enclosed between and (weak recycling region) and and (strong recycling region) were inline diluted and transferred to the relative downstream column.

The wizard computes the inline dilution flow rates for this operation. However, taking into consideration bed height and pressure constraints, the operator can autonomously optimize this feature to reduce processing times and thus increase productivity. Selected values were respectively 1.17 mL/min and 1.22 mL/min for W and S compounds.

Moreover, with the aid of the Dynamic Process Control, i.e., a UV-based software control part of the ChromIQ® operating system, it is

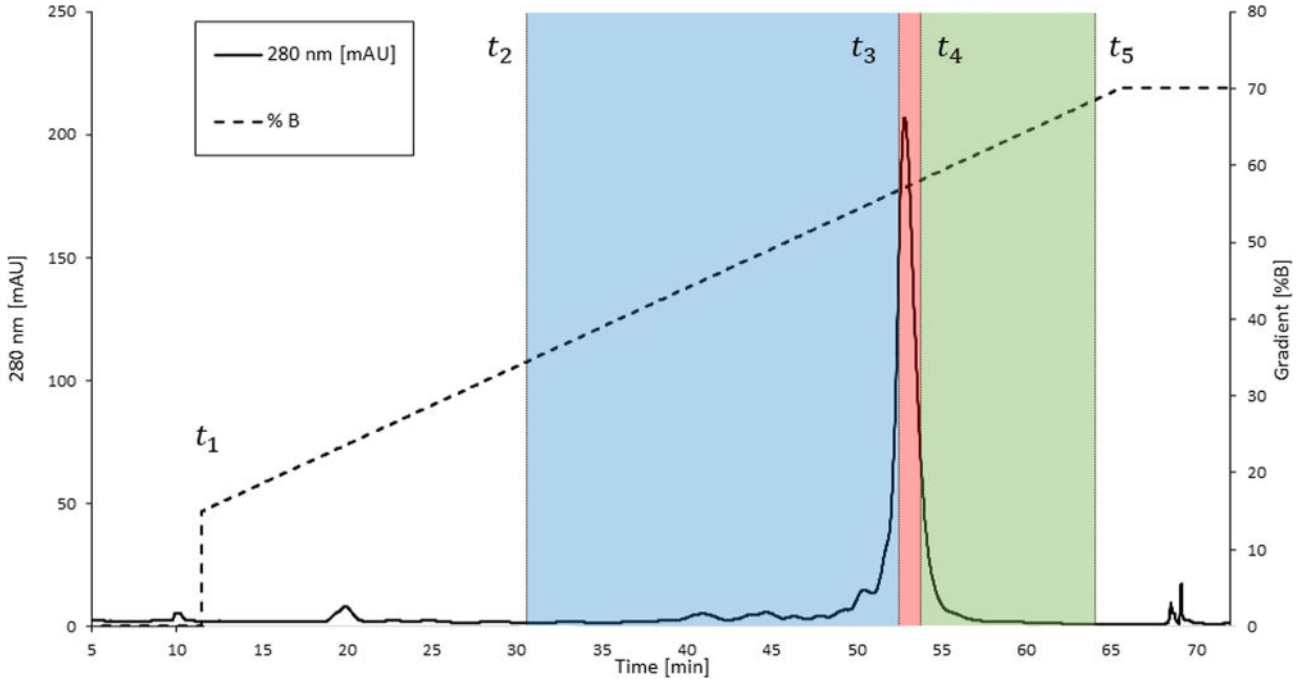


**Fig. 2.** Batch chromatogram. The colored regions are determined with the selection of section borders ($t_1$-$t_5$): in blue the weakly adsorbing impurities region, in red the region containing the pure product and in green the strongly adsorbing impurities area.

possible to establish a threshold intensity value which triggers product removal by UV threshold. This feature is introduced so that any shift in product retention time due to column aging, buffer variabilities, and other possible inconsistencies over multiple cycles, would be compensated for and have no impact on process performance. In this way, consistent product quality and yields are ensured. To achieve maximal reproducibility, product removal was triggered with UV-based dynamic process control, at an intensity value of 200 mAU. The accumulation step was performed for a total of 10 cycles. Upon finalization of the design parameters, the N-Rich wizard automatically creates the operating methods described in the N-Rich rationale section (Startup, Accumulation, Depletion & Elution). [11].

## 3. Results

### 3.1. Batch chromatography

In the investigated case, the starting material of a preparative chromatographic polishing process (AIEX) contained 22 % impurities which were reduced to 7 % by the polishing step. Despite the well optimized process, several impurities exceeded reporting thresholds, and therefore qualify for further characterization according to regulatory guidance. In this current example, N-Rich was designed with the aim to accumulate not only a single compound, e.g., a closely eluting impurity, but rather a heterogeneous group of impurities above the threshold, for further characterization.

As a precursor to N-Rich design, the first step was to generate a single column batch chromatogram with sufficient resolution using the Contichrom CUBE (Fig. 2). In the current drug development process, impurities are pooled from multiple high-resolution batch runs for further characterization. For this reason, the preparative batch chromatogram, besides being a template for the N-Rich experimental design, was also used as performance benchmark. Batch chromatography conditions are described in section "2.2 Preparative Chromatography Conditions" and the batch chromatogram is shown in Fig. 2. Fractions generated during the experiment were evaluated with analytical HPLC in order to qualify the separation capabilities of single column chromatography and to highly resolve and identify the contained species, generally pure oligonucleotide chain and structurally similar species. Close-eluting compounds, including shortmers, longmers and other structurally related impurities, require deep characterization and understanding.

Therefore, based on analytical evaluations and on relative retention times and purity values, two different recycling zones of the design chromatogram, comprising large amounts of close-eluting species, were defined by means of the ChromIQ® software, as illustrated in Fig. 2. A weakly adsorbing impurity zone (W), highlighted in blue and enclosed between min 31.3 - 52.8 min ($t_2$-$t_5$), containing more weakly adsorbing compounds than the pure oligonucleotide chain. A strongly adsorbing impurity zone (S), highlighted in green and enclosed between 54.3 - 64.3 min ($t_4$-$t_5$), conversely populated by impurities more strongly adsorbed on the stationary phase. Finally, a zone (P) is shown in red, where the analytical evaluations delivered the highest purity values for the main oligonucleotide product. During the continuous chromatography run development, such recycling zones were selected across a wide temporal range for both weak and strong adsorbing impurities, with the final intent to collect in a single run as much as possible enriched byproducts species.

### 3.2. Continuous chromatography

The N-Rich process was developed starting from the design batch chromatogram and the relative offline analytics data. The triggering

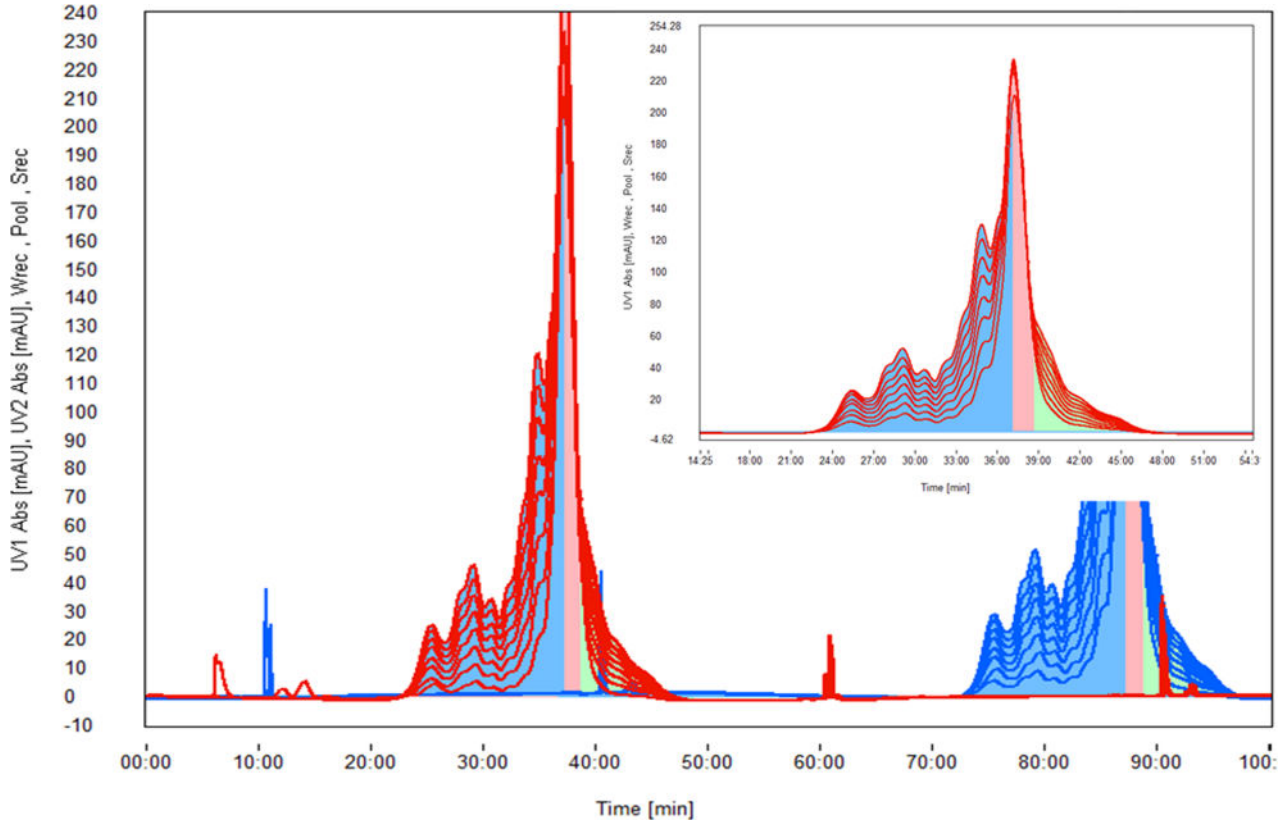


**Fig. 3.** Accumulation phase in N-Rich. The signals of column 1 are shown in red, with a zoomed perspective on the upper right corner, and the signals of column 2 are shown in blue. A progressive and gradual increase of the concentration of the target compounds from cycle to cycle is demonstrated. Interfering substances are instead constantly depleted and reduced in the overall percentage.

times for recycling and the rationale behind their selection are introduced in the previous section. Fig. 3 shows the outcomes of the accumulation phase: a nearly perfect match in elution profiles and progressive growth of all impurities chromatographic profile in the recycling portions was achieved. Meanwhile, the main compound region, devoid of critical impurities, was successfully removed at each switch and consequently resulted at the same intensity values of a single column batch run. In other words, cycle after cycle, the undesired substance (pure oligonucleotide chain) was depleted from the system, whereas targeted substances (impurities) were isolated and loaded, previous inline dilution, on the respective downstream column. The steady removal of the main compound prevents non-linear effects, such as displacement effects, which could negatively impact the isolation. Once the "Accumulation" cycles were completed, the "Depletion" method was applied. This method corresponds to a switch without loading new feed and thus leads to further depletion of any excess non-target compounds that would otherwise contaminate the desired target impurities. Consequently, a major increase of the target compounds purities and their relative enrichment was obtained.

As illustrated in Fig. 4, the conclusive "Final Fractionation" phase comprising final elution and fine fractionation was executed. The elution gradient was designed to boost separation capacities, exploiting the whole stationary resin. Indeed, the two columns employed in the continuous recycling of components were positioned in-series and operated with a mobile phase flowrate of 100 cm/h, performing a 20 % to 60 % of in 25 CVs shallow gradient. Fractions were collected and their analytical characterization allowed to identify those containing impurities of a sufficiently high quality and to establish a comparison between the batch and continuous experiment outcomes.

## 4. Discussion

To compare N-Rich vs batch processes we measured the purity and the productivity of each approach. Purity is a major consideration in impurity collection since optimal characterization requires a high degree of purity for each compound. The purity value was calculated from the analytical chromatographic profiles of all the pooled fractions obtained with the preparative system. The second parameter measured was the process productivity, i.e., the overall amount of time and chromatographic resources to achieve an adequate impurity mass recovery. In fact, not only a satisfactory degree of purity is required, but also enough material to fully characterize with multiple techniques.

A graphical evaluation of the N-Rich outcome is shown in Fig. 5 where the analytical chromatogram of the feed material is presented (A) followed by its overlay with the chromatograms obtained injecting equal amount of material evaluating several fractions obtained during the final elution phase containing the enriched impurities (B). In the latter graph, singular peaks of high intensity, each representing an isolated target impurity, can be identified both on the weakly and strongly adsorbing regions of the chromatogram. This graphical comparison clarifies the enrichment capabilities of the continuous chromatography approach. The throughput of linear isotherm range HPLC separation (Fig. 5A), with minor substances barely reaching detection limits, is outperformed by the N-Rich technology which progressively accumulated targeted substances and allowed their final separation and collection. In this regard, the reader should note as well how the regions outside the recycling windows were not subjected to peak intensity grow, demonstrating the technique selectivity and precision.

For the numerical comparison eight impurities isolated both batch-wise and towards continuous chromatography were chosen. Table 1 shows their retention time and relative values obtained from purity and mass recovery assessments. For each listed compound, identified by its retention time, the highest purity values obtained via HPLC fraction analysis were selected as representative of batch or N-Rich isolation capabilities. Once selected the fractions containing the highest value of purity of each of the eight compounds, we then proceeded estimating their concentration, i.e., the mass of material dissolved in the pooled eluate. To do so, the intensity of the peak obtained in the analytical evaluation was introduced in the calibration line previously built with a standard of the feed material. The linearity characteristics in the

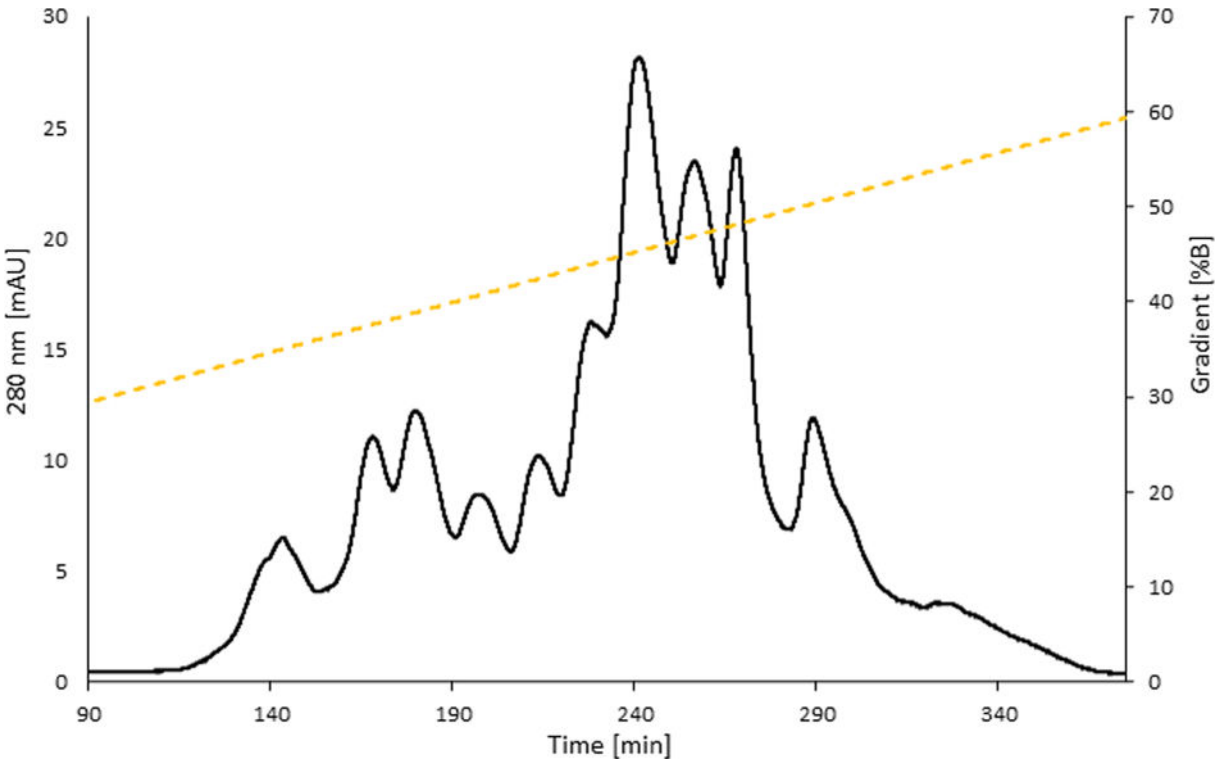


**Fig. 4.** Final Fractionation phase. The enriched regions are eluted using a shallow gradient over both the column in series applying a shallow gradient and a fine fractionation.

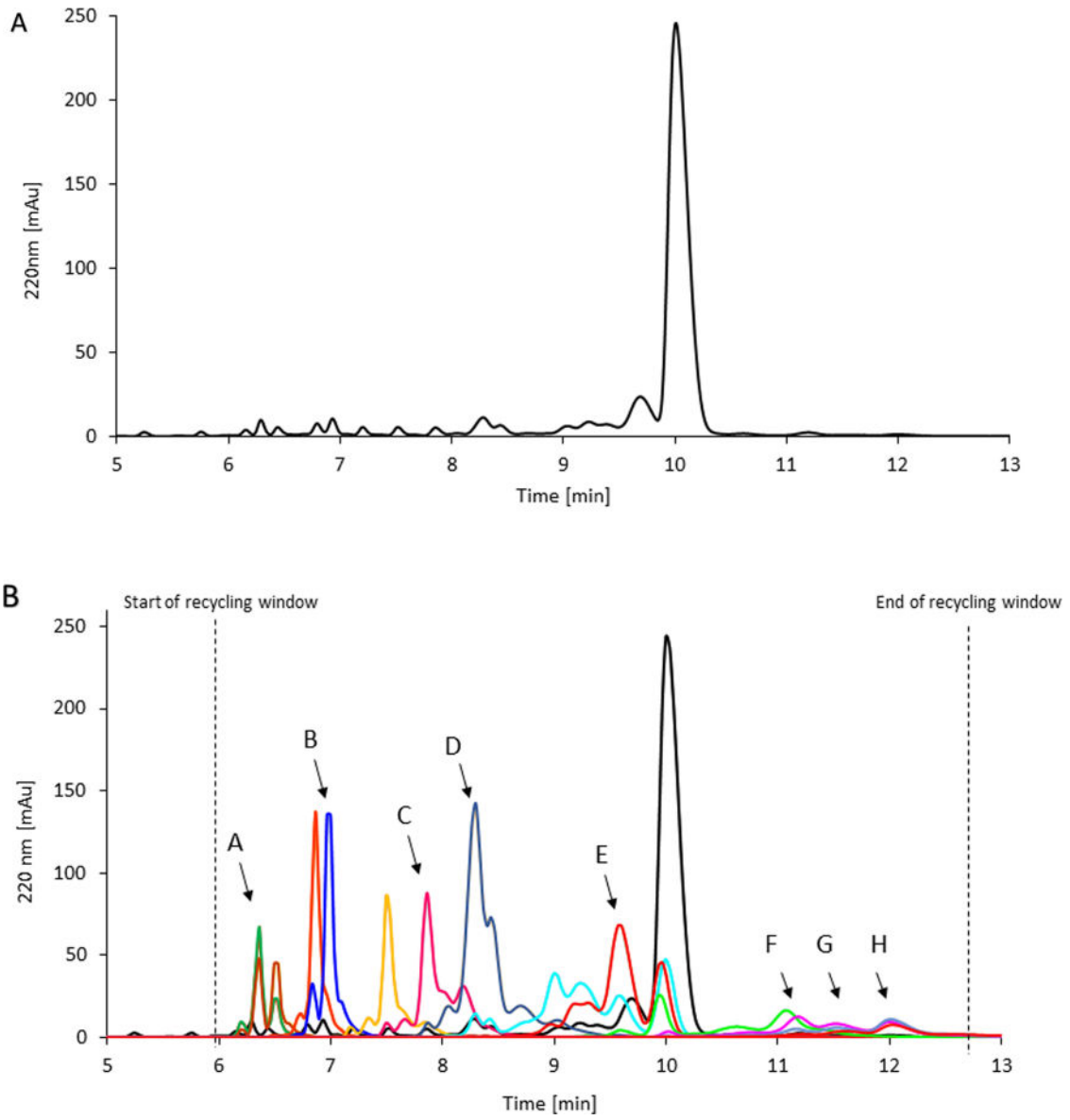


**Fig. 5.** A) Analytical chromatogram of the 24-mer oligonucleotide feed B) Overlay of the feed analytical chromatograms (black) and of the enriched impurities chromatograms.

**Table 1**
Purity and concentration performance of batch and N-Rich processes carried out on the CUBE system with the same stationary resin and mobile phase buffers.

| Compound | Retention time (min) | Batch | | N-Rich | |
|---|---|---|---|---|---|
| | | Purity (%) | Concentration (μg/mL) | Purity (%) | Concentration (μg/mL) |
| A | 6.3 | 25.2 | 1.85 | 60.8 | 6.58 |
| B | 7.2 | 11.9 | 0.46 | 59.9 | 6.81 |
| C | 7.7 | 20.9 | 1.81 | 59.8 | 17.02 |
| D | 8.2 | 12.9 | 1.11 | 53.7 | 21.57 |
| E | 9.5 | 21.1 | 15.61 | 49.3 | 19.38 |
| F | 11.3 | 3.6 | 1.27 | 49.5 | 5.74 |
| G | 11.6 | 1.5 | 0.15 | 22.8 | 2.48 |
| H | 12.2 | 4.6 | 0.46 | 38.3 | 3.69 |

analytical range of different byproducts were approximated to be constant. Fractions obtained by means of continuous chromatography were characterized by higher purity values. For instance, Compound A (6.3 min.) and Compound B (7.2 min), both weakly adsorbing impurities, were respectively pooled with purities 2.5-fold and 5-fold higher using continuous chromatography. Even superior outcomes were achieved with Compound G (11.6 min.), a minor strongly adsorbing impurity, which was obtained 15-fold purer in comparison with Compound G fractions from batch runs. Indeed, the molecule chromatographic profile completely overlaps with the main product and other strongly adsorbed impurities to the point that with normal batch processing the highest purity achieved was only 1.5 %. This value was increased to 22.8 % by using N-Rich. Although it was not carried out in this study, it is conceivable that a more targeted N-Rich experimental set-up on this specific molecule would result in higher purity outcomes. Using a restricted recycling zone would result in a progressive enrichment of this singular compound with the simultaneous depletion of the other closely eluting impurities. The absence of these interfering substances would eventually boost the purity values in the "Final Fractionation" method for the specific impurity.

The comparison of impurities obtained between single column batch and N-Rich also demonstrates fraction concentrations up to 19-fold higher in N-Rich (Table 1). In this respect, the purest fraction containing Compound C was obtained batchwise at a concentration of 1.81 μg/

mL, while the corresponding fraction delivered by N-Rich contained 17.02 μg/mL. The available amount for molecule characterization was almost tenfold increased. A productivity scenario for the collection of 1 mg of an impurity is presented in Fig. 6. The times (days) provided were calculated considering the experimentally obtained throughput (mg) and the required duration to run each individual method. These factors were then normalized per column volume of stationary resin (CV). Since continuous chromatography is a scalable process, therefore feasible to be operated with larger stationary resin volumes consequently diminishing the required processing time per mg of compound without losing chromatographic resolution, it was possible to consider a scale-up scenario from 0.46 cm (CV = 2.5 mL) to 1.0 cm i.D. (CV = 11.8 mL) columns. With this normalization it was computed the number of runs, and consequently of time, required in order to separate and concentrate 1 mg of compound D (8.2 min). The N-Rich process would require 8 days of processing time. On the other hand, obtaining the same amount of material using semi-preparative batch chromatography and analytical chromatography would result in an operational time of 10 and 87 days, respectively. Additionally, for the same compound, the purity values obtained batch-wise were 4 times lower than those obtained with N-Rich, thanks to the progressive depletion of interfering substances at each cycle and to the final two column in series elution method. In theory, similar purity values could be achieved batch-wise with a single column or two columns in series process applying very shallow gradient or reducing the injected mass. However, this scenario would be counterbalanced by an exponential increase of the processing times and solvents requirement that would result in a drastically reduced the productivity. Purities reported in the graph are shown according to experimental data. For UHPLC, this value was omitted as it was not possible to collect the compound in question (or any other) with a sufficient concentration for re-analysis.

Another advantage of N-Rich that should be noted is the ability to control for process variability (e.g., column deterioration, variation in buffer composition, etc.) by means of the Dynamic Process Control ("Autopeak") supported by the Contichrom CUBE® system. Shifts in retention time are compensated by means of UV-triggered valve switching so that the main, non-target compound is consistently removed at the correct time. In contrast, the process variabilities can have a significant impact on the retention time of single column batch chromatograms resulting in the need to carefully pool fractions from individual runs.

In this study, impurity isolation and their relative purity assessment were not investigated with U(H)PLC. Despite the potential for purity values that could be in the range of those obtained with N-Rich or higher, the enormous processing time that this methodology would require to achieve a reasonable amount of product casts shadows on the actual implementation of U(H)PLC for production of impurity standards. In addition, the solvents requirements for the production of 1 mg compound G with N-Rich and U(H)PLC were calculated, and for the continuous set-up, 57 L of mobile phases would be required, while the hypothetical projection to produce 1 mg with U(H)PLC was approximatively 178 L of solvents, corresponding to a more than 3-fold increase.

## 5. Conclusion

In this research paper the advantages of continuous chromatography over semi-preparative batch and analytical scale chromatography concerning biotherapeutic impurities separation and collection for analytical characterization were presented. The innovative impurities enrichment method N-Rich was applied to a 24-mer double-stranded RNA (dsRNA) therapeutic oligonucleotide (ONs) and its outcomes compared with traditional impurities collection methods. The process was designed as a proof of concept aiming to recycle, amplify and collect a broad range of minor side-compounds through a completely automatized procedure.

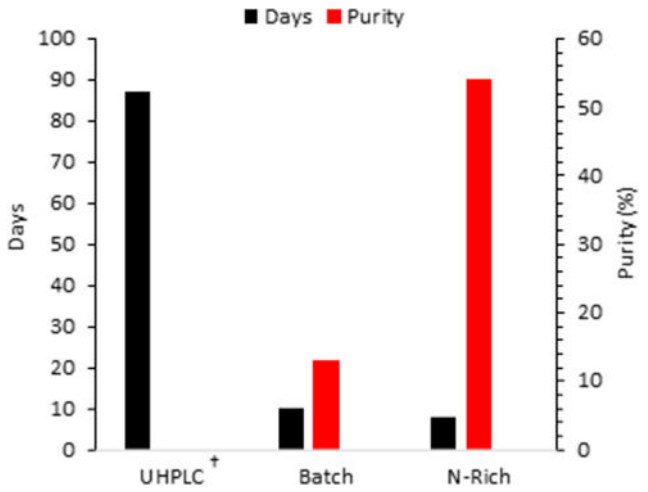


**Fig. 6.** Comparison of processing times (days), considering a scale-up scenario for batch and continuous experiments, from 0.46 cm i.D. to 1 cm i.D. columns, and achieved purity values (%) obtained through U(H)PLC, batch single column chromatography and continuous chromatography (N-Rich). The time was calculated for producing 1 mg of the impurity compound D. (see Table 1). † Purity values were not assessed for the U(H)PLC technique, in consideration of the extremely unfavorable processing times.

N-Rich was proven able to deliver analytical impurity standards with greater efficiency than traditional techniques optimizing crude material processing, and minimizing the number of analytical evaluations, expensive and long concentration steps and reducing waste production in terms of solvents and consumables (e.g., valuable feed material, collection materials). Furthermore, the chromatographic resins are preserved due to decreased processing times and the reduced number of harsh cleaning procedures. A remarkable reduction of time requirements in order to isolate the impurity standard compounds was demonstrated. Eventually, this technique leads to a positive effect on the constant need for qualified operators in carrying out time-consuming repetitive experiments. Moreover, it significantly reduces the need for manual handling, pooling, and sample storage and management. In the constant demand for a more efficient, effective, and greener biopharmaceutical production, continuous chromatography and the N-Rich process offer a powerful and robust tool.

## CRediT authorship contribution statement

**Giulio Lievore:** Investigation, Data curation, Writing – original draft. **Richard Weldon:** Conceptualization, Resources, Writing – review & editing. **Martina Catani:** Data curation, Validation. **Alberto Cavazzini:** Formal analysis, Funding acquisition. **Thomas Müller-Späth:** Writing – review & editing, Supervision, Funding acquisition, Project administration.

## 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.

## Data availability

Data will be made available on request.

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