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## Tutorial Article

# Enrichment and purification of peptide impurities using twin-column continuous chromatography

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## ABSTRACT

N-Rich is a twin-column chromatography process that enriches target compounds relative to other components in a mixture, thereby facilitating their isolation and characterization. This study demonstrates the performance of N-Rich for isolation of Angiotensin II peptide impurities compared with standard analytical and preparative chromatography approaches. Peptides have diverse chemical properties and are produced using a wide range of methods, resulting in products with complex impurity profiles. The characterization of impurities for clinical development is essential but obtaining high purity samples in sufficient quantities is often a difficult task when using standard chromatographic techniques. In contrast, by using cyclic continuous chromatography with UV-based process control, N-Rich enables automatic on-column accumulation of target impurities while other compounds in the mixture are depleted. This has multiple advantages compared to standard techniques. Firstly, at the end of the cyclic accumulation phase the highly enriched target is eluted in one step with high purity and concentration. This means fewer fractions for analysis are generated and up-concentration steps are reduced. Secondly, the purification of target impurities using semi-preparative scale chromatography becomes viable, even if initial resolution is poor compared to analytical HPLC. This allows for very significant increases in productivity for purification of difficult to isolate impurities.

This study demonstrates two N-Rich strategies:

Example 1: Purification of  $\mu\text{g}$  quantities of multiple Angiotensin II impurities with a >9-fold increase in productivity compared to analytical HPLC.

Example 2: Specific isolation of 1 mg of a critical impurity at 88% purity. 79-fold increase in productivity and a 69-fold reduction in solvent consumption compared to analytical HPLC.

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## 1. Introduction

Therapeutic proteins, peptides and oligonucleotides are produced by chemical or biological synthesis. After synthesis, in addition to the active pharmaceutical ingredient, all drug products include product-related impurities, process-related impurities, and degradation products. According to ICH guidelines [1–3], impurities comprising >0.1% of the total drug product require identification and characterization as part of risk assessment regarding their impact on patient safety and product efficacy. Based on this assessment, a product specification is defined with strict limits for every impurity to guarantee product safety and efficacy. For complex molecules such as peptides, oligonucleotides and protein biologics, the number of impurities with abundance >0.1% can be

considerable. A bottleneck to completing impurity characterization is the lack of availability of highly purified material. Milligram amounts of such material are often required for structural confirmation by NMR or X-ray crystallography, and biological studies for toxicity, immunogenicity and pharmacokinetics can require even more [4–6].

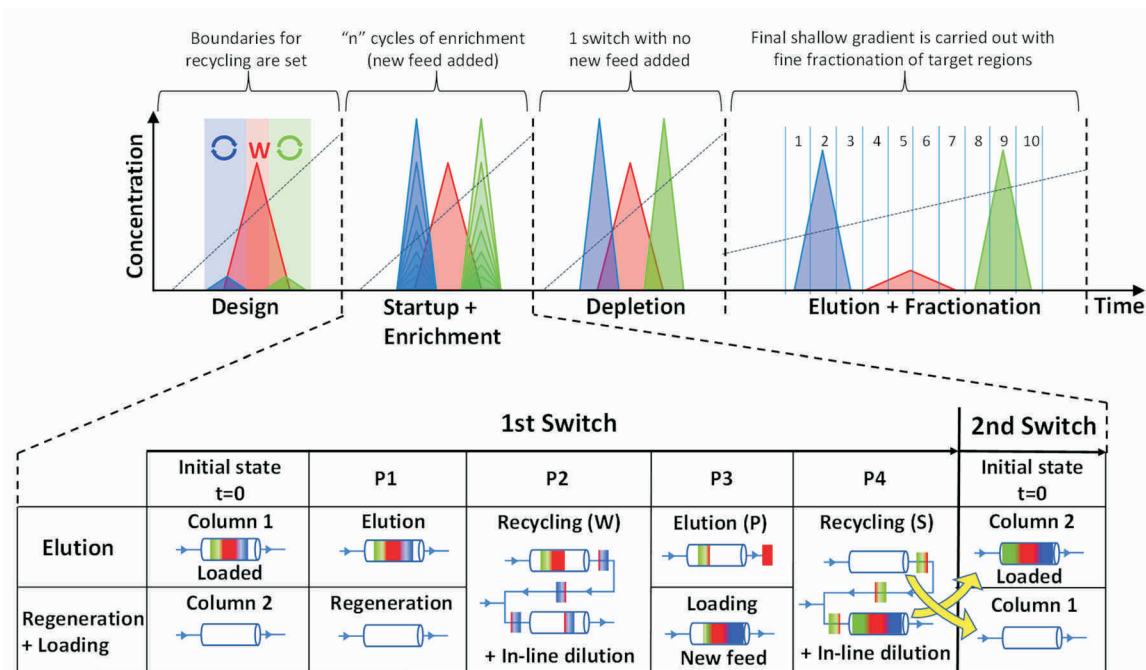
### 1.1. Standard isolation of impurities vs. N-Rich

To complete the task of impurity isolation, column chromatography is often the method of choice. However, even if a protocol is well optimized, standard chromatographic methods have technical limitations leading to a tradeoff between productivity and purity [7]. For example, standard HPLC techniques use stationary phases with very small particle diameters combined with a low sample load to maximize resolution and purity, but at the expense of productivity. This approach is suitable for tentative identification of impurities using very sensitive methods such as LC-MS but is

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**Fig. 1.** The N-Rich process is carried out as shown in the schematic. The black diagonal line indicates the steepness of the gradient (%B). Design: With the aid of software, the boundaries for target recycling (blue, green) are set using a batch chromatogram as template. Four N-Rich methods are generated: Startup – Column 1 is loaded with a full batch load. Enrichment – target compounds (blue, green) undergo “n” cycles of enrichment while undesirable compounds (red) are discarded as new feed is applied. Each cycle of enrichment consisted of 2 switches. Each “switch” is composed of four phases of elution, P1, P2, P3 & P4 as shown in Fig. 1 table. A full description of the enrichment process is detailed in the main text. Depletion – A single switch is carried out without loading new feed resulting in depletion of non-target compounds before the final elution step. Elution + Fractionation – Finally, enriched target impurities are eluted with fractionation. To maximize resolution the final method uses a shallow gradient, low flow rate, and double the bed height compared to the enrichment phase.

**Table 1**  
Materials – Analytical, semi-preparative batch and N-Rich chromatography.

Method	Material	Description
Analytical HPLC	Column	ACQUITY UPLC Oligonucleotide BEH C18 Column (130 Å, 1.7 µm, 2.1 mm x 50 mm)
	Mobile phase A	7 vol% acetonitrile (Merck, 1.00030) / 0.1 vol% TFA (VWR UN2699) in water
	Mobile phase B	90 vol% acetonitrile / 0.1 vol% TFA in water
Semi-Preparative Batch	Feed composition	1 mg/mL in Mobile phase A
	Feed purity	92%
	Column	YMC Triart Prep C18-S (150 × 4.6 mm ID, 5–10 µm, 12 nm)
	Mobile phase A	5 vol% acetonitrile / 0.1 vol% TFA in water
N-Rich	Mobile phase B	50 vol% acetonitrile / 0.1 vol% TFA in water
	Feed composition	2.1 mg/mL in Mobile phase A
	Feed purity	92%
	Column	2x YMC Triart Prep C18-S (150 × 4.6 mm ID, 5–10 µm, 12 nm)
N-Rich	Mobile phase A	5 vol% acetonitrile / 0.1 vol% TFA in water
	Mobile phase B	50 vol% acetonitrile / 0.1 vol% TFA in water
	In-line dilution buffer	1 vol% acetonitrile / 0.1 vol% TFA in water
	Feed composition	2.1 mg/mL in Mobile phase A
	Feed purity	92%

less suited for production of µg or mg quantities required for more detailed studies. To obtain larger quantities of target impurities for characterization the conventional solution is to pool impurity-containing fractions from multiple analytical HPLC runs until sufficient material has been accumulated. However, difficult to separate or low abundant impurities can require hundreds or thousands of analytical purifications over many weeks or months to generate enough material. Alternatively, the impurity may need to be chemically synthesized and purified [4]. By contrast, semi-preparative chromatography using stationary phases with larger particle diameter and higher loading capacities is ideal if an impurity is well resolved from neighboring compounds. However, semi-preparative chromatography often has insufficient resolution of critical target

impurities resulting in low purity fractions which leaves analytical HPLC as the only viable standard chromatographic option.

N-Rich is a twin-column chromatographic process with the potential to improve upon standard methods by facilitating the recycling and enrichment of target compounds on-column, and in a continuous manner. The process can be generally applied for the isolation of impurities from complex mixtures using standard gradient purification methods as a starting point. For example, N-Rich was successfully applied for monoclonal antibody (mAb) charge variant isolation [8,9] using ion-exchange chromatography showing a 30-fold decrease in the time required to obtain 10 mg compared to analytical HPLC [9]. Moreover, the purity obtainable with N-Rich was higher than the purity by analytical HPLC (95% vs. 85% purity).



**Table 2**

Semi-preparative batch run parameters.

Semi-Prep. Batch Method		Batch 1	Batch 2	Batch 3	Batch 4	N-Rich Example 1 – Design Batch	N-Rich Example 2 – Design Batch	
Column dimensions	[mL]	5 (4.6 mm ID x 30 cm)				2.5 (4.6 mm ID x 15 cm)		
Equilibration	[%B]		15				15	
Load	[g/L]	0.525	1.05	2.1	8.4	0.525	8.4	
Gradient start	[%B]		15				15	
Gradient end	[%B]		70				85	
Strip	[%B]		100				100	
Re-equilibration	[%B]		15				15	
Equilibration	[cm/h]		500				500	
Loading	[cm/h]		300				300	
Wash after load	[cm/h]		300				300	
Gradient	[cm/h]		100				300	
Strip	[cm/h]		500				500	
Re-equilibration	[cm/h]		500				500	
Equilibration	[CV]		3				3	
Load	[CV]	0.25	0.5	1	4	0.25	4	
Wash after load	[CV]		2				2	
Gradient duration	[CV]		22		25	8	10	
Strip	[CV]		2				2	
Re-equilibration	[CV]		3				3	
Run time	[h:min]	7:20	7:21	7:24	8:30	0:44	1:04	

**Table 3**

N-Rich run parameters.

Procedure	N-Rich Example 1				N-Rich Example 2			
	Startup	Enrichment	Depletion	Elution	Startup	Enrichment	Depletion	Elution
Method								
Column dimensions	[mL]	2 × 2.5 (4.6 mm ID x 15 cm)						
Equilibration	–	IL Dil Buffer	–	–	–	IL Dil Buffer	–	–
Feed load	[g/L]	0.525	0.412	–	–	8.4	5.25	–
Wash after load	[%B]	15	–	–	–	15	–	–
Gradient start	[%B]	–	15	15	15	–	15	15
Gradient end	[%B]	–	85	85	70	–	59	55
In-line dilution	–	–	IL Dil Buffer	IL Dil Buffer	–	–	IL Dil Buffer	IL Dil Buffer
Strip	–	–	Mob pH-B	Mob pH-B	Mob pH-B	–	Mob pH-B	Mob pH-B
Re-equilibration	–	–	IL Dil Buffer	IL Dil Buffer	IL Dil Buffer	–	IL Dil Buffer	IL Dil Buffer
Equilibration	[cm/h]	600	–	–	–	600	–	–
Loading	[cm/h]	200	200	–	–	300	300	–
Wash after load	[cm/h]	200	–	–	–	300	–	–
Gradient flow rate (initial)	[cm/h]	–	300	300	100	–	300	100
Gradient flow rate (W-rec & P-collect)	[cm/h]	–	253	253	–	–	130	130
Gradient flow rate (S-Rec)	[cm/h]	–	199	199	–	–	216	216
Strip	[cm/h]	–	600	600	500	–	600	500
Re-equilibration	[cm/h]	–	600	600	500	–	600	500
In-line dilution flowrate (W-rec)	factor	–	2.57	2.57	–	–	–	–
In-line dilution flowrate (S-Rec)	factor	–	3.55	3.55	–	–	3	3
AutoPeak threshold	mAU	–	390	390	–	–	–	–
Equilibration	[CV]	3	–	–	–	3	–	–
Load	[CV]	0.25	0.196	–	–	4	2.5	–
Wash after load	[CV]	2	–	–	–	2	–	–
Gradient duration	[CV]	–	8	8	22	–	6.32	6.32
Strip	[CV]	–	2	2	2	–	2	2
Re-equilibration	[CV]	–	3	3	3	–	3	3
Cycles done	–	1	18	1	1	1	8	1
Feed processed	[mg]	1.32	36.96	0	0	21	210	0
Buffer consumption	[L]			3.03				1.08
Run time (Method)	[h:min]	0:11	19:21	0:37	7:06	0:26	7:11	0:27
Run time (Procedure)	[h:min]			27:15				16:04

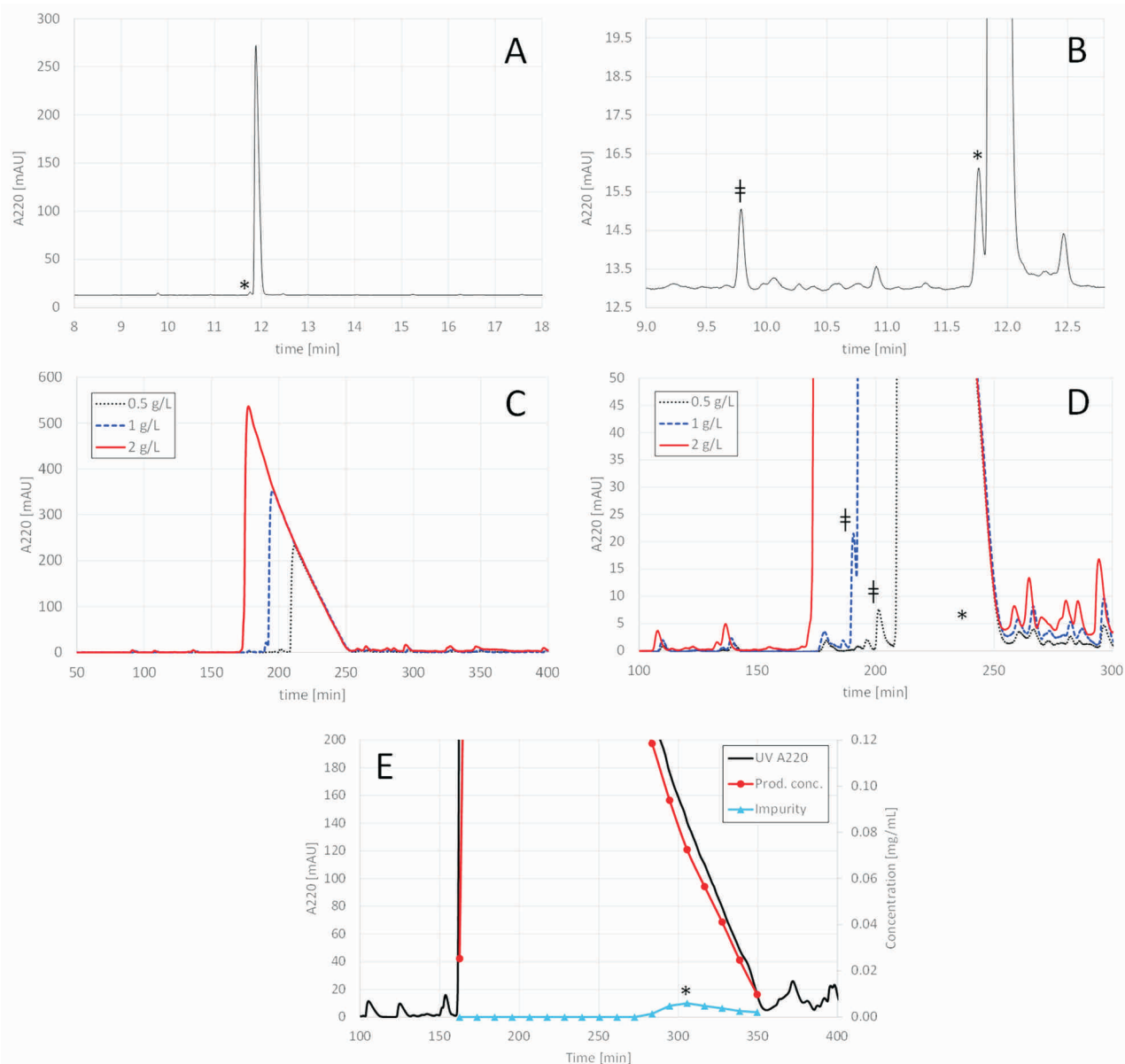
So given the high utility of N-Rich for antibody charge variant isolation, this study aims to evaluate the potential of N-Rich to obtain peptide impurities in a more productive way. In this study, reverse-phase chromatography was used to exploit displacement effects [10,11] which are less pronounced in ion-exchange chromatography, but are very useful to obtain target compounds with high purity. In contrast to other twin-column chromatographic processes such as Multicolumn Countercurrent Solvent Gradient Purification (MCSGP) [12] or CaptureSMB [13], the N-Rich process is not designed to reach a cyclic steady state with multiple product elutions for obtaining product with uniform concentration and purity. Instead, in N-Rich, the target compounds are accumulated from cycle to cycle.

## 1.2. The N-Rich process principle

A step-by-step depiction of the N-Rich process principle is outlined in Fig. 1. The N-Rich design procedure is described below in section 4.2. N-Rich is composed of four steps run as an uninterrupted procedure. The enrichment principle is detailed in the table in Fig. 1. The table shows the activities of column 1 and column 2 during a single N-Rich switch:

Initial state – At the very beginning of an N-Rich run before the enrichment phase begins, column 1 is pre-loaded in a startup step while column 2 is clean. This pre-load allows non-target compounds to reach a cyclical steady state quickly which facilitates their removal using UV-based dynamic process control. During the





**Fig. 2.** Chromatograms of crude Angiotensin II (92% purity) comparing analytical HPLC and semi-preparative chromatography. † = “easy” to isolate impurity; (\*) = “difficult” to isolate impurity. **A** - Analytical reversed phase (C18) HPLC. **B** - “Zoom in” of A. **C** - Semi-preparative (C18) chromatography comparing 3 different loads. **D** - “Zoom in” of C. **E** - A fraction analysis of an Angiotensin II preparative batch chromatogram (8.4 g/L load). Critical target impurity (\*) is obtainable at a purity of 8%.

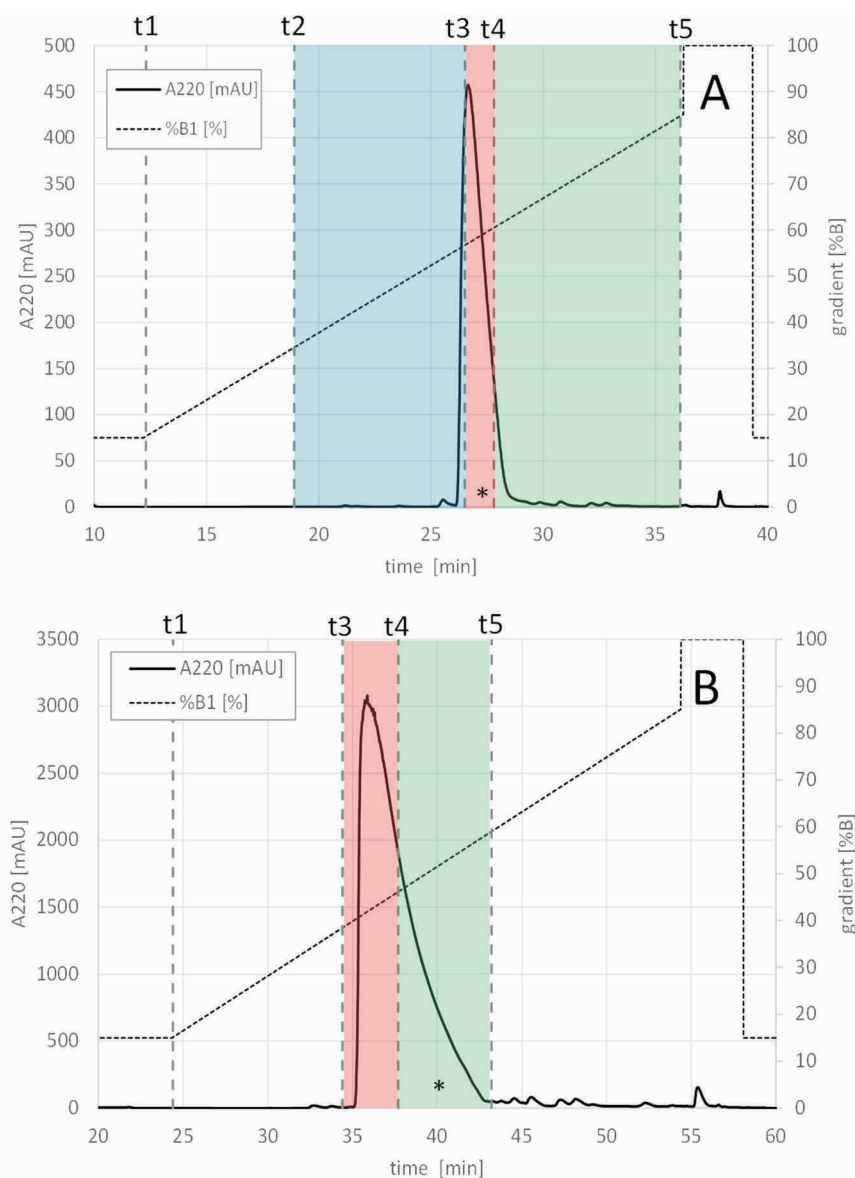
cyclical enrichment phase, column 1 is always fully loaded at the completion of the prior switch, while column 2 requires cleaning and regeneration to remove strongly adsorbing non-target impurities.

- P1 (columns in parallel) - column 1 undergoes elution of weakly adsorbing, non-target compounds to waste; column 2 simultaneously undergoes regeneration ready for the loading steps in P2, P3 and P4.
- P2 (columns interconnected for target recycling) - column 1 undergoes elution of weakly adsorbing target impurities (W), recycled with in-line dilution directly to column 2. The in-line dilution ensures re-adsorption of the target impurities on column 2.
- P3 (columns in parallel) - column 1 undergoes elution of non-target compounds (P) to waste; column 2 is simultaneously loaded with new feed. This step allows enrichment of the tar-

get compounds relative to non-target compounds in the mixture. To maintain a switch-to-switch steady state, the quantity of new feed applied is in equilibrium with non-target products removed. The load required to achieve this is calculated with the aid of software, based on the batch chromatogram and fraction analysis.

- P4 - (columns interconnected for target recycling) - column 1 undergoes elution of strongly adsorbing target impurities (S), recycled with in-line dilution directly to column 2. Again, the in-line dilution ensures re-adsorption of the target impurities on column 2.

At the conclusion of switch 1, both columns are immediately ready for switch 2. Column 2 is now fully loaded and column 1 requires cleaning and regeneration. P1 - P4 are repeated as described above, but with column positions interchanged (Fig. 1 table - yellow arrows). By alternating the position of column 1 and column 2



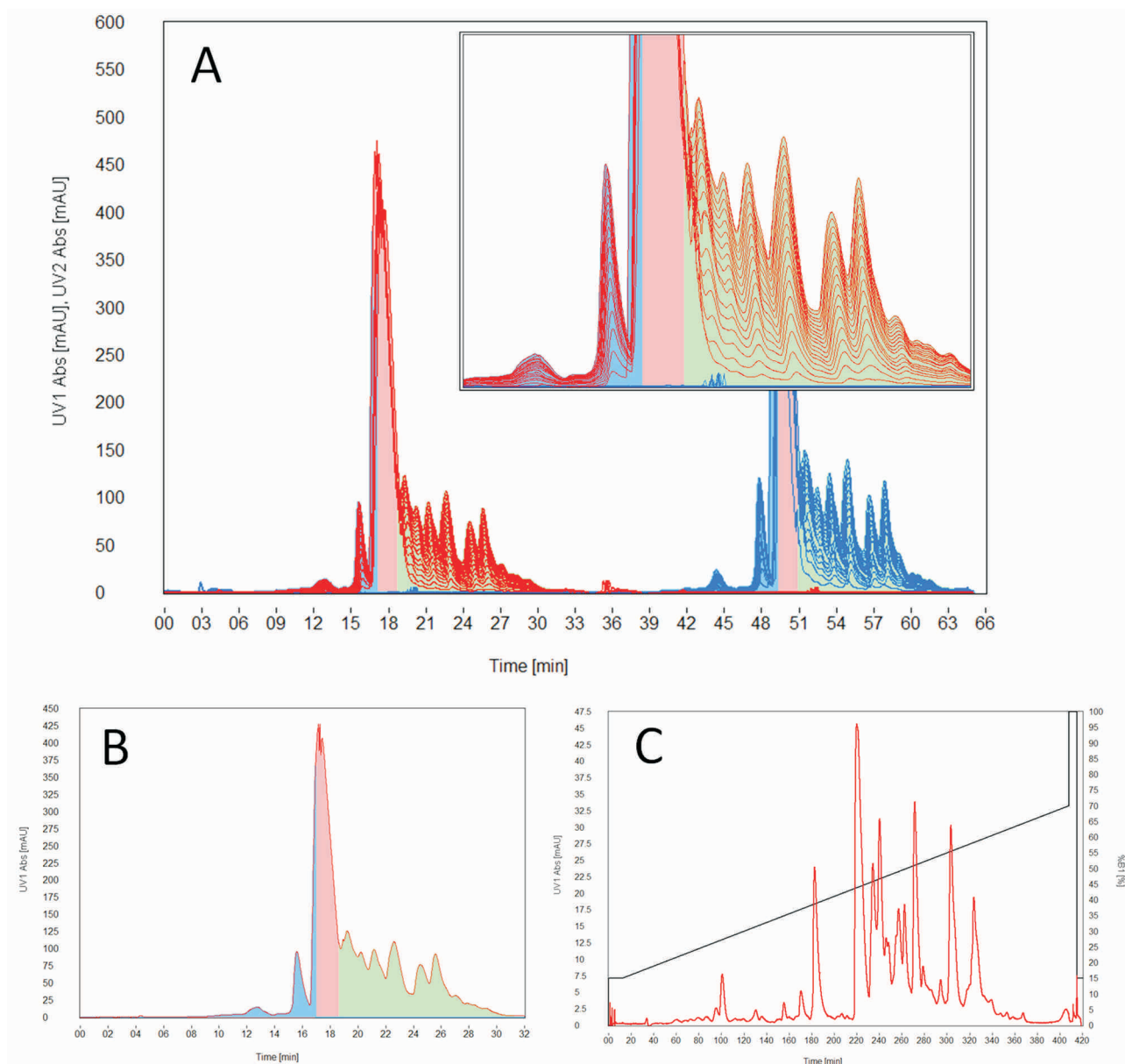
**Fig. 3.** A batch chromatogram is the starting point of N-Rich design. **A** – Example 1 uses a low load (0.5 g/L) and a resulting chromatogram with resolution of all impurities except one species (\*) that co-elutes with the main compound. Two enrichment windows were applied covering the early eluting (blue) and late eluting (green) target impurities. **B** - N-Rich example 2 was designed using a high load (8.4 g/L). Whilst overall resolution of impurities was reduced, the high load is advantageous for the specific enrichment of target impurity (\*) that co-elutes with the tail of the main compound peak (green). The higher load increased the area of the main compound peak (red) that contains no target impurity (\*) thus allowing a higher load during the enrichment phase and higher productivity.

switch-to-switch, N-Rich can operate continuously resulting in the incremental enrichment of the target impurities inside the system. A single cycle of N-Rich is defined as 2 switches that includes 1 elution from each column and the enrichment phase is continued for “n” cycles, until sufficient feed is processed.

Following completion of the enrichment step a depletion step is carried out. This consists of a single switch without loading new feed so that only the enriched target compounds are transferred to the next column before starting the final elution step. This greatly reduces the amount of non-target compounds present in the final elution step. Finally, the enriched target material is eluted with a very shallow gradient over two columns in-series and the target material is collected with a fine fractionation. Thus, a high resolution of the enriched compounds is obtained, and the pure target material is collected by pooling suffi-

ciently pure fractions only once. Another benefit of the enrichment process is that the impurity concentration in the collected fractions is much higher than compared to standard chromatographic methods.

As a model for impurity isolation, we used crude Angiotensin II as starting material. Angiotensin II is a human hormone which functions as a vasoconstrictor and can be used as a medication to treat hypotension. The therapeutic version is a synthetic 8-amino acid peptide marketed under the brand name Giapreza®. Since the peptide is relatively short, it is produced by solid phase peptide synthesis (SPPS). In general, SPPS is a highly efficient process (97% coupling reaction efficiency), but with many coupling steps and just as many deprotections steps, it is common to generate many impurities which differ from the target peptide in a minor way, for example by missing a single intermediary amino acid. This study



**Fig. 4.** N-Rich Example 1. Absorbance at 220 nm; column 1 – red line, column 2 – blue line; Regions of enrichment – blue & green shading, region of depletion – red shading. **A** – Enrichment step – “Zoom out” – 18 Cycles enrichment phase. Inset – “Zoom in” of column 1 enrichment profile. **B** – Depletion step – 1 switch with no new feed. **C** – Final elution step – 2 columns in series (2x bed height, 2x CV gradient, 0.5x flow rate compared to enrichment phase).

evaluates the twin-column N-Rich process compared to single column chromatography for generating large quantities of low abundant impurities from crude Angiotensin II obtained by SPPS.

## 2. Methods

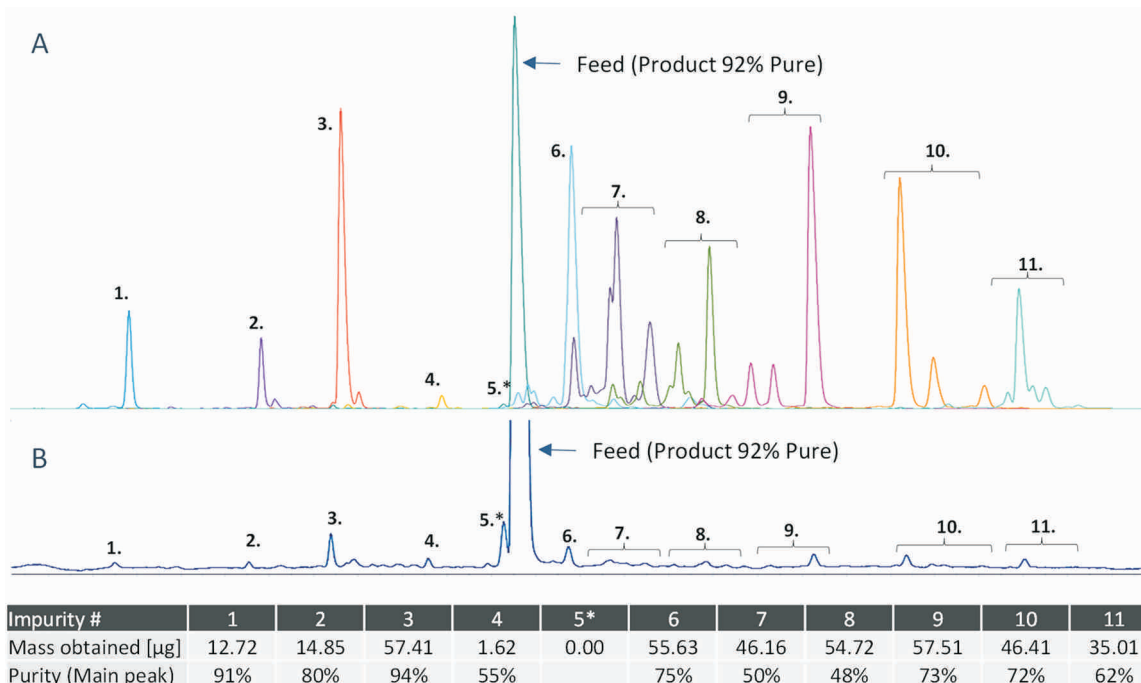
### 2.1. Analytics

Analytical HPLC chromatography was carried out using an Agilent 1290 series system set at 60 °C. Table 1 shows the column, solvents and feed used in the method. The gradient parameters and flow rate are detailed in Supplemental Table 1. Samples from the Angiotensin II feed, the batch fractions & N-Rich fractions were prepared for HPLC analysis by diluting 1:1 with water. Samples were analyzed at a wavelength of A220 nm. Fraction concentrations were also calculated using the area under the curve (AUC) measured from analytical HPLC.

### 2.2. Batch and N-Rich chromatography

Semi-preparative batch methods were carried out for comparison with N-Rich as well as to serve as the process design template for N-Rich. Batch and continuous chromatography were carried out using the Contichrom CUBE 30, a lab-scale twin column system for continuous chromatography (ChromaCon AG, A YMC Company). UV absorbance at A220 nm was recorded by external UV detectors (2x Knauer AZURA UVD2.1S, ADA00 + 0.5 mm flow cells, A4069) located directly after each column outlet (UV1@220 nm and UV2@220 nm respectively) and connected to the Contichrom CUBE system. Table 1 details the column characteristics, buffer composition and feed composition used for semi-preparative batch chromatography and N-Rich runs. Table 2 gives an overview of the method parameters used for semi-preparative batch runs and N-Rich design runs (Batch 1, 2 & 3 = Fig. 2C and 2D. Batch 4 = Fig. 2E, N-Rich Design Batch = Fig. 3A and 3B). Table 3 gives





**Fig. 5.** N-Rich Example 1 – Analytical Evaluation. **A** – 11 enriched fractions were analyzed by HPLC, and chromatograms were overlaid with the feed profile. **B** – “Zoom in” of the feed profile shows the level of impurities in the feed relative to the main Angiotensin II peak. The failure to enrich the early eluting impurity (\*) that co-elutes with the main compound was notable. This impurity was targeted separately in N-Rich example 2. The table at the bottom of Fig. 5 indicates the total mass obtained per 1.5 mL fraction.

an overview of the method parameters used for two N-Rich example runs shown in Fig. 4 and Fig. 6.

### 3. Results and discussion

#### 3.1. Limitations of standard chromatography for impurity isolation

A high resolution analytical HPLC chromatogram of crude Angiotensin II purified using an analytical reversed phase C18 column is shown in Fig. 2A and 2B. The purity of the crude material is 92% and there are multiple low-level impurities present on either side of the main compound peak. Whilst analytical HPLC provides excellent resolution of impurities and main compound, the very low loading capacity of the analytical stationary phase severely limits the productivity of HPLC for impurity production. Purification of µg quantities of these impurities would require pooling of hundreds of chromatographic runs, taking weeks of run time. As an alternative to analytical HPLC, semi-preparative chromatographic methods give worse resolution due to the larger stationary phase particle diameters (10 µm compared to 1.7 µm). However, if sufficient resolution of impurities is achievable, this is a preferable option for impurity isolation as semi-preparative stationary phases are cheaper, have a higher loading capacity and can run at high flow rates with lower backpressure. Thus, the semi-preparative scale allows for far higher productivity, provided that the target purity can be achieved.

To explore the viability of semi-preparative chromatography for Angiotensin II impurity isolation a high resolution batch method was carried out. To maximize resolution, the method utilized a very shallow gradient, a low flow rate and longer columns than analytical HPLC (Fig. 2C and 2D). While a generally good resolution was achievable with a load of 0.5 g/L (the lower limit at which impurities remained detectable), compared to analytical HPLC, a critical target impurity (\*) completely co-eluted with the main compound peak (Fig. 2B vs. 2D). This impurity, which was well resolved in analytical HPLC, had a resulting purity of only 8% in fractions

from a semi-preparative batch method (Fig. 2E). Note that, when comparing analytical and semi-preparative scale, selectivity reversal of impurity (\*) was seen.

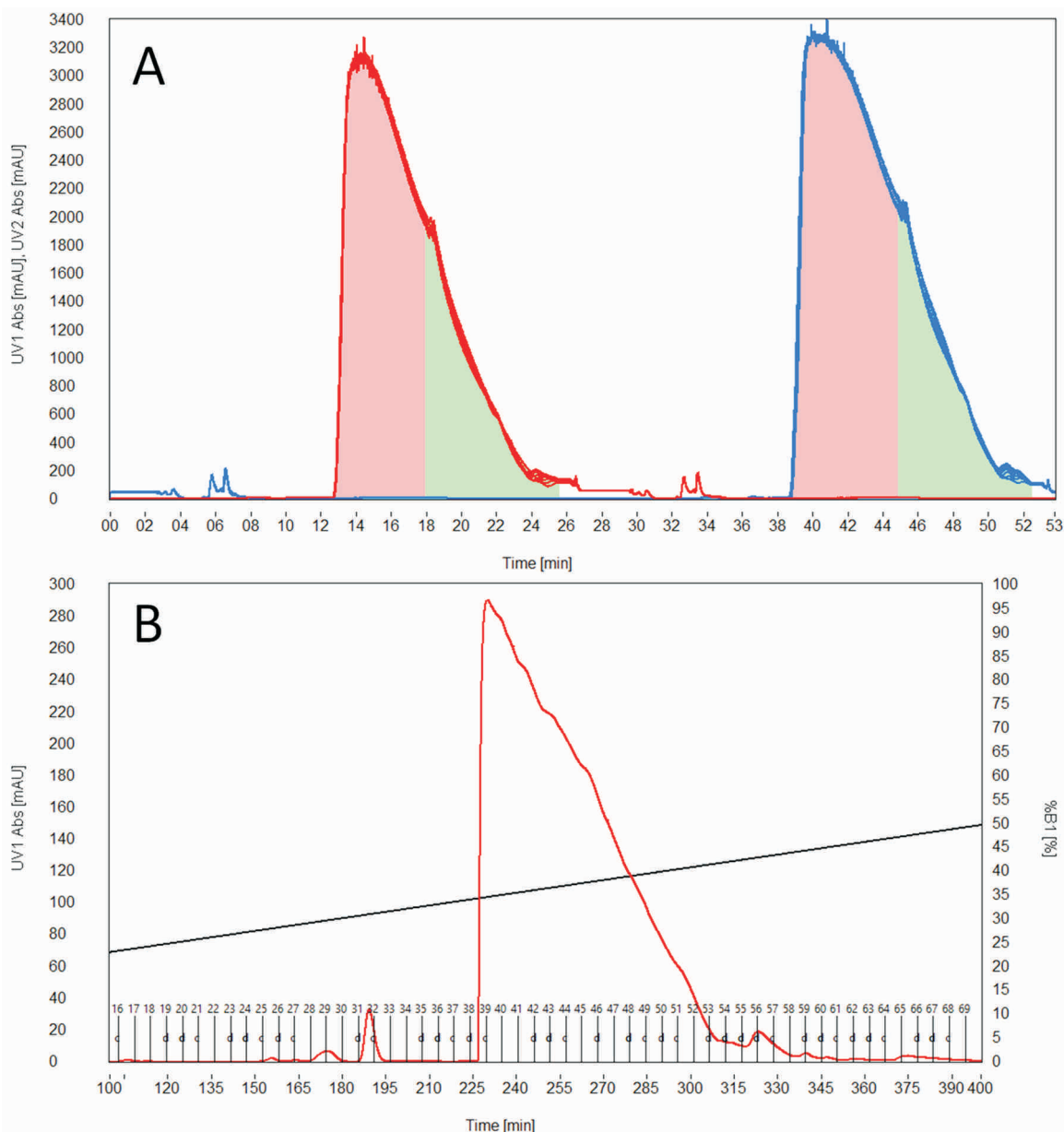
As loading was increased from 0.5 g/L to 1.0 and 2.0 g/L resin, it was also observed that additional early eluting impurities (†, Fig. 2D) were no longer resolved from the main compound peak as the Langmuirian peak profile broadened to the left. This further limits the utility of batch methods with a higher load than 0.5 g/L. In conclusion, compared to analytical HPLC, semi-preparative batch chromatography is limited due to a lack of resolution of early eluting target impurities (\*, †). For later eluting impurities, loads up to 2 g/L did not severely impact the separation performance (Fig. 2C and 2D, 250 – 350 min). For these late eluting impurities semi-preparative batch chromatography could be a good alternative to analytical HPLC.

Given the limitations of these standard chromatographic approaches, N-Rich was tested as an alternative process for impurity production.

#### 3.2. N-Rich design procedure

Two complementary N-Rich designs were tested. N-Rich was firstly used to purify a broad spectrum of Angiotensin II peptide impurities simultaneously and then used in a more targeted way to accumulate one difficult to isolate impurity (\*).

A pre-requisite to design N-Rich is to generate a single column batch “design” chromatogram using the Contichrom CUBE system. An N-Rich design method serves as a template for the cyclical enrichment step and is typically optimized for productivity rather than very high resolution, since high resolution is only advantageous in the final elution and fractionation step and not required in the impurity accumulation step of the process. After running the design method, the resulting chromatogram (Fig. 3A and 3B) was imported into the N-Rich design software (N-Rich wizard, a module within the Contichrom CUBE operating software). Using the wizard, the gradient (15%B to 85%B) of the chromatogram was then



**Fig. 6.** N-Rich Example 2. Absorbance at 220 nm; Column 1 – red line, Column 2 – blue line; Regions of enrichment – green shading, Region of depletion – red shading. **A** – 8 Cycle enrichment phase. **B** – Final elution through 2 columns in series (2x bed height, 2x CV gradient, 0.5x flow rate compared to enrichment phase).

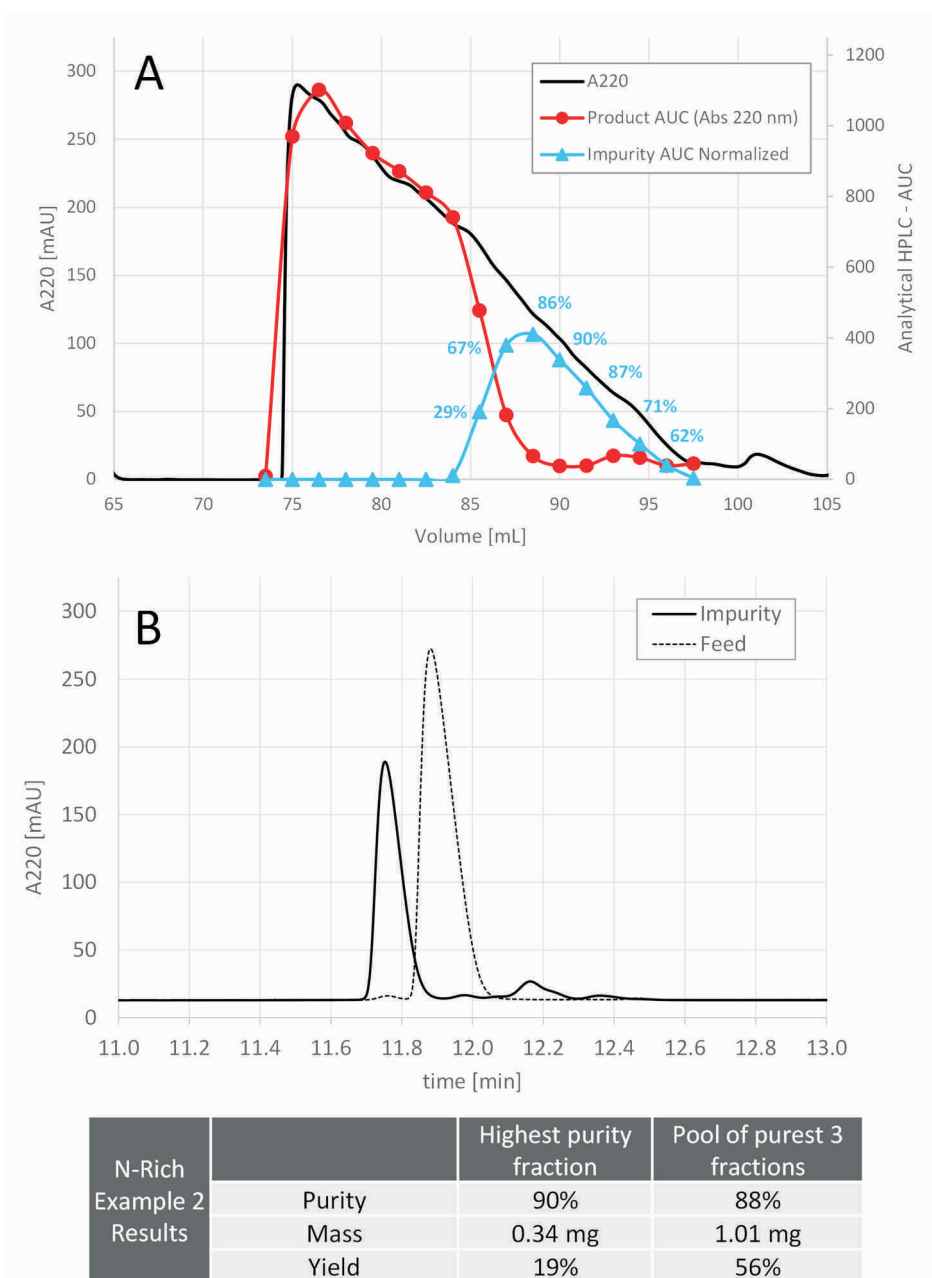
divided into 4 phases, as described in Fig. 1., using five adjustable section borders (Fig. 3A and 3B, t1-t5). Once section borders for impurity recycling and non-target compound removal were configured, the wizard automatically determined the necessary load per switch and in-line dilution factors (Table 3). In addition, N-Rich example 1 required a UV-threshold-based control set point (AutoPeak) to trigger main compound removal to waste. This was necessary to stabilize performance by compensating for small deviations in the retention time that occur during a long, multi-cycle enrichment phase. Upon finalization of the method parameters, the N-Rich wizard automatically created four methods as described in Fig. 1 and outlined in Table 3. (Startup, Enrichment, Depletion & Final Elution).

### 3.3. Example 1: N-Rich for broad isolation of Angiotensin II impurities

N-Rich example 1 used a small load of 0.4 g/L resin per switch to maximize resolution with the aim of accumulating a broad spec-

trum of impurities in one procedure (Fig. 3A). A total of 18 enrichment cycles were run to process 38.2 mg of feed, after which the concentration of the impurities on column was sufficiently high to carry out the depletion and final elution steps Fig. 4A shows the results of the enrichment phase where cyclical overlays were used to visualize the accumulation of the impurities. The load applied per switch was in equilibrium with the main compound removed (red shaded area) so as impurities accumulate, the main peak is always in steady state. The UV-based process control successfully compensated for changes in the retention time Fig. 4.B shows the final switch without feeding (depletion) before the final elution step was carried out (Fig. 4C). Total processing time was ≈27 h.

A clear increase in resolution was seen between the depletion step and the final elution step due to the use of a high-resolution method. Also evident in the final elution step is the depletion of the main compound peak relative to the target impurities and this results in very little of the Angiotensin II main compound present



**Fig. 7.** N-Rich Example 2 – evaluation of enriched fractions by analytical HPLC. **A** – Quantification of product vs. critical impurity (\*) using analytical HPLC, overlaid with final N-Rich elution chromatogram. The % purity of the target is reported next to the data labels for each fraction. **B** – Analytical HPLC overlay of input feed vs. fraction with highest enrichment of critical impurity (\*) (90% pure). The table shows the % purity, mass recovered, and % yield of the highest purity fraction vs. a pool of 3 high purity fractions.

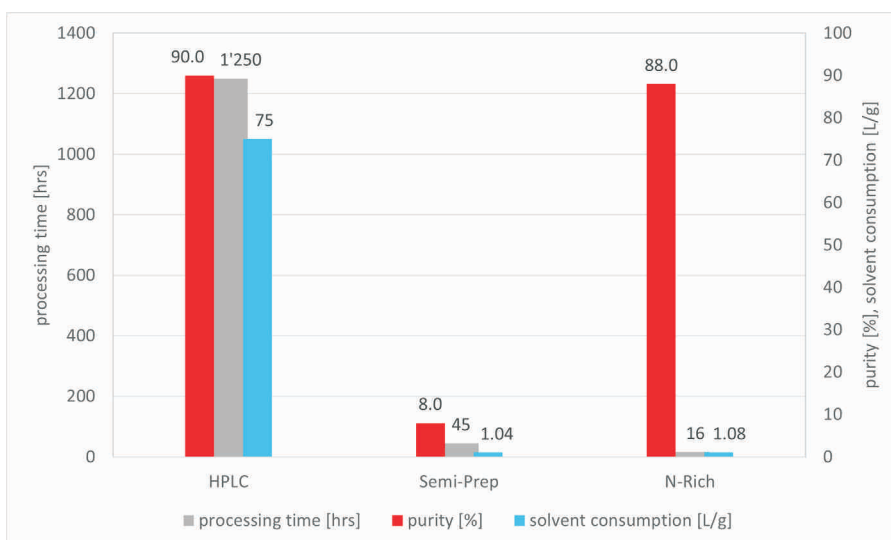
in any of the impurity fractions. The fractions were collected and analyzed for purity using analytical HPLC.

Fig. 5 shows an analytical overlay of 11 fractions containing enriched impurities compared to the original feed. Most regions of the chromatogram were enriched except for two impurities (4 & 5<sup>(\*)</sup>) which were co-eluting with the main compound and sent to the waste. Impurity 5<sup>(\*)</sup> was targeted separately in N-Rich example 2 below. Depending on the starting abundance of a particular impurity in the feed, between 13  $\mu\text{g}$  and 58  $\mu\text{g}$  was obtained after N-Rich fractionation. The purity of the 3 weakly adsorbing impurities was between 80% - 94%, whereas strongly adsorbing impurities were overlapping with their neighbors resulting in lower purities between 48% and 75%. In theory, purity of these strongly adsorbing impurities could be further im-

proved by using a shallower gradient in this region of the chromatogram and collecting smaller fractions in the final elution step.

By comparison, a well optimized analytical HPLC method used to accomplish the same task (assuming 50- $\mu\text{g}$  load per run, a 30 min run time, a high purity of 85–95%), would require >500 chromatography runs and 10 days of continuous running time (>9x longer than N-Rich). Finally, a high-resolution semi-preparative batch method would give similar purity to N-Rich, but with a load of 0.5 g/L per run, a total of 15 runs taking 4.6 days would be required to complete the task (4x longer than N-Rich), and the pool concentration would be 15x lower, meaning up-concentration (with potential sample loss) would be an essential step before subsequent analysis.





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**Fig. 8.** Process performance comparison of optimized analytical HPLC, semi-preparative batch chromatography, and N-Rich chromatography for the purification of 1 mg of critical impurity (\*), in terms of purity, processing time and solvent consumption.

In summary, N-Rich example 1 shows that a broad enrichment strategy facilitates fast purification of  $\mu\text{g}$  quantities of multiple peptide impurities simultaneously and produced at higher concentration than analytical or semi-preparative batch chromatography. This is ideal for generating samples for tentative characterization using, for example, MS analysis. For generating larger quantities with high purity, the following N-Rich example demonstrates a second approach.

### 3.4. Example 2: N-Rich for targeted isolation of a specific Angiotensin II impurity

In the second example, N-Rich was designed with one enrichment window (Fig. 3B) targeting a single critical impurity (\*) that was not recoverable in N-Rich example 1. This impurity was resolved in analytical HPLC, but co-elutes with the main compound when using semi-preparative batch chromatography, giving a purity of only 8% (Fig. 2E). N-Rich example 2 used a far higher load per cycle compared to N-Rich example 1, with a total of 231 mg of feed processed, of which 1.8 mg was the target impurity (\*). The high load greatly facilitated the productivity of the method, minimizing the number of cycles needed to enrich the target sufficiently. 8 cycles of targeted enrichment were carried out and it was notable that almost no perceptible change in the UV profile was visible (Fig. 6A). After enrichment, a switch without feeding (depletion step) was carried out, and a final elution step was done using a high-resolution method with fractionation (Fig. 6B). Total processing time was  $\approx 16$  h.

Following fraction analysis, the relative mass of main compound and impurity (\*) was plotted against the chromatographic profile (A220 nm) of the final elution step (Fig. 7A). The % purity of the target (\*) is shown next to the data labels. We can clearly see a large accumulation of the target (\*) relative to the main compound and the main compound was almost completely displaced from this region of the chromatogram. As can be seen in Fig. 7B, the purity of the target is 90% in the purest fraction compared to 0.8% in the feed. Moreover, this is a 11-fold improvement in purity compared to a semi-preparative batch method which gave a purity of 8% (Fig. 2E).

The table in Fig. 7 shows that the yield of the target impurity is 19% at 90% purity, with a total mass of 0.3 mg obtained. By pooling 3 fractions, purity is slightly less at 88% but with 1 mg obtainable

(56% recovery). Possibly, additional cycles would further improve purity, but that remains to be tested.

HPLC, semi-preparative batch, and N-Rich chromatography were compared in terms of purity, processing time and solvent consumption (Fig. 8) to obtain 1 mg impurity (\*) that is present at 0.8% content in the starting material. The analytical HPLC method used in the comparison represents a hypothetical, optimized analytical HPLC allowing a loading of 50  $\mu\text{g}$  per HPLC run. The actual HPLC method used in the experimental part of this study was not well adapted for the task of impurity production because a load of  $< 1$   $\mu\text{g}$  per HPLC run (very low) had been used. For the hypothetical HPLC method, achievement of 90% purity, 100% yield and 30 min run time were assumed. Nevertheless, even when using this “best-case” HPLC method, 2500 runs including fractionation would be required to complete the task, taking 52 days of straight operation and using 75 Liters of solvent. As demonstrated experimentally, N-Rich completes the task within 16 h (79-fold faster) using about 1.1 Liters of solvent (69-fold less), achieving 88% purity. For the sake of completeness, the performance of semi-preparative batch chromatography is also shown in Fig. 8, although this process delivers the target compound at far lower purity (8%).

In summary, N-Rich example 2 shows excellent results, isolating the target impurity in one step at semi-preparative scale and with a high purity. In addition, whereas scaling analytical HPLC requires parallelization of the task using multiple HPLC systems or packing of larger columns with very expensive analytical stationary phase, N-Rich is scalable to larger column dimensions using a single system if necessary and works with cost-effective semi-preparative stationary phases. For example, a 20-fold increase in scale can be obtained simply by increasing the column inner diameter from 0.46 cm to 2 cm, and with no need for extra equipment besides columns. The two examples show that by a simple change of the process design (i.e. by selecting a different region of the chromatogram for enrichment, see Fig. 3A, Fig. 3B), the N-Rich process can be focused to purify on a specific impurity that was inaccessible with a previous design, exploiting displacement effects.

## 4. Conclusion

The advantages of N-Rich continuous chromatography over traditional methods for Angiotensin II peptide impurity isolation were

shown in two examples and can be summarized as follows. Firstly, N-Rich enables the use of semi-preparative scale resins with a high loading capacity to complete a task that is usually carried out using analytical resins. Thus, a high productivity and high resolution are achievable in one procedure, eliminating the tradeoffs of traditional methods. Secondly, the buffer savings are also proportional to the productivity increase, leading to huge reduction in solvent consumption compared to analytical HPLC. Thirdly, target impurities generated from N-Rich elute at a far higher concentration compared to analytical HPLC or batch chromatography, reducing the time required for subsequent up-concentration steps. Fourthly, the single step elution protocol of N-Rich eliminates the need to carefully monitor fraction quality from “n” number of HPLC runs thus reducing potential fraction pooling errors. Finally, the problem of longer term storage, handling and stabilization of multiple fractions generated during a multi-run analytical HPLC project is also solved.

The barrier to use N-Rich includes the need to procure specialized lab-scale equipment and training. In addition, the setup of an N-Rich procedure, while greatly facilitated with software, can require extra effort upfront to characterize the impurity profile of a preparative batch “design” method needed to design an N-Rich procedure.

Overall, this study demonstrates that N-Rich is a highly productive process that enables the isolation of large quantities of peptide impurities with the high purity required for detailed structural analysis, safety and efficacy studies, or for generation analytical standards. Provided that sufficient starting material is available, the process represents an attractive alternative to targeted synthesis and purification of impurities.

#### Author contributions

Richard Weldon: Conception or design of the work, Data collection, Data analysis and interpretation, Drafting the article

Thomas Müller-Späh: Data analysis and interpretation, Critical revision of the article.

#### Authorship statement

All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript

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#### 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.

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#### Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:[10.1016/j.chroma.2022.462894](https://doi.org/10.1016/j.chroma.2022.462894).

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