

## N-Rich® – Automated enrichment and purification of minor compounds from complex matrices

The Contichrom® CUBE Combined and Contichrom® HPLC systems have integrated N-Rich capability enabling the fast isolation of minor compounds amidst complex mixtures. A major application of N-Rich is the acceleration of drug development. Therapeutic antibodies, proteins and peptides have diverse chemical properties and are produced using a wide range of methods, resulting in products with complex impurity profiles. The characterization of these impurities for pre-clinical development is an essential, but often daunting task using standard separation techniques, which are slow to generate sufficient quantities for analysis. N-Rich eliminates this bottleneck by utilizing a highly efficient cyclic/continuous chromatography process for the isolation of impurities in milligram quantities. The technology can save months of tedious work compared to conventional analytical HPLC techniques. In addition, ChromaCon offers customized services and expertise to companies and academics who wish to quickly advance their projects or test the N-Rich capabilities before investing in a system. Here we highlight the capabilities of the N-Rich technology based on two application examples.

### Introduction

N-Rich is an invaluable tool for the isolation and characterization of product- and process-related impurities, as required by regulatory guidelines such as ICH Q6A and Q6B. For biosimilars, the characterization of isoforms is of utmost importance for the substantiation of interchangeability claims. Without N-Rich, these investigations require tedious and repetitive purification procedures using analytical high resolution FPLC/HPLC with low loading capacity. N-Rich solves

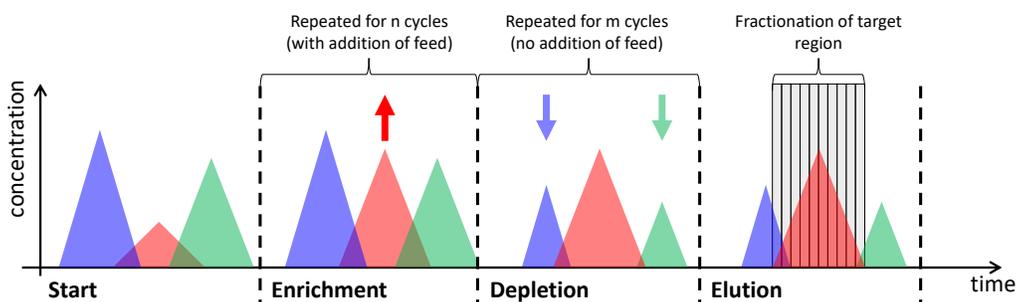
this problem by combining the capabilities of preparative chromatography with the high resolution of cyclic enrichment and polish processes. N-Rich has a proven high efficiency, even for difficult to isolate antibody isoforms, providing milligram amounts in an unparalleled short time. Furthermore, N-Rich finds application in the area of proteome and metabolome screenings. It greatly facilitates the identification of biomarkers by enriching and isolating initially almost undetectable compounds in an automated way.

The Contichrom® CUBE Combined and the Contichrom® HPLC systems have N-Rich capabilities. The N-Rich wizard in the ChromIQ® operating software allows the design of the process in a simple stepwise manner. ChromaCon also offers services for the isolation of product-related impurities of monoclonal antibodies using N-Rich.

### Basic principle of N-Rich

N-Rich is an automated process using two 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 containing several compounds (see application examples below).

The N-Rich process has three main phases (see Fig. 1). The process starts by loading part of the feed material onto the first column and performing a linear gradient elution. The eluting fractions which contain the target substance, even if mixed with other overlapping compounds, are directly loaded onto



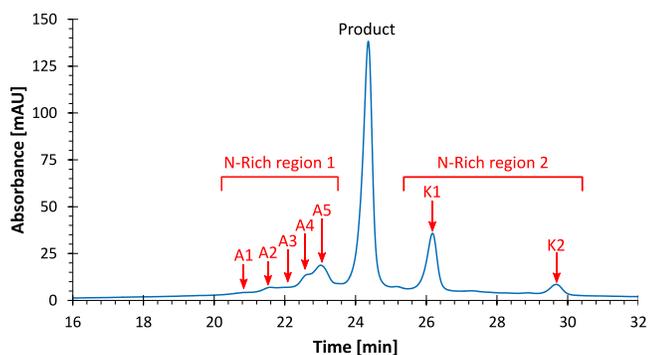
**Fig. 1.** The N-Rich process automatically enriches and isolates compounds in fractions that are initially highly dilute. This is done in three phases, typically overnight: 1) start/enrichment of target compound (red); 2) depletion of interfering compounds (blue, green); 3) elution of target compound with fine fractionation.

the second column where they re-adsorb. The other fractions are discarded. Additional feed material is loaded onto the second column, which already contains the previously isolated fractions. This step leads to an enrichment of the target compound, absolute and relative to other compounds in the mixture. The process step is repeated in a cyclic fashion between the two columns, each time increasing the concentration of the target compound. In a second phase, closely eluting and thus interfering compounds are depleted further, increasing the purity of the target compound and leading to a relative enrichment. This is achieved by a cyclic process similarly to the one in the first phase. However, impure side fractions are now discarded and no new feed material is added anymore. In a third phase, the enriched target material is eluted with a shallow gradient and the target material is collected with a fine fractionation. Thus, the maximum amount of pure target material can be collected by pooling sufficiently pure fractions.

### First application example: antibody isoforms

An important application for N-Rich is the isolation of antibody isoforms for subsequent characterization in secondary assays. In this industrial application example, N-Rich was used to enrich and isolate seven isoforms of a biosimilar antibody in multi-milligram quantities at high purity. This allowed characterization of the isoforms in structural and functional assays, as required by regulatory agencies as part of the CMC (chemical, manufacturing & control) characterization data package. The data supported interchangeability claims for a biosimilar monoclonal antibody with its originator drug.

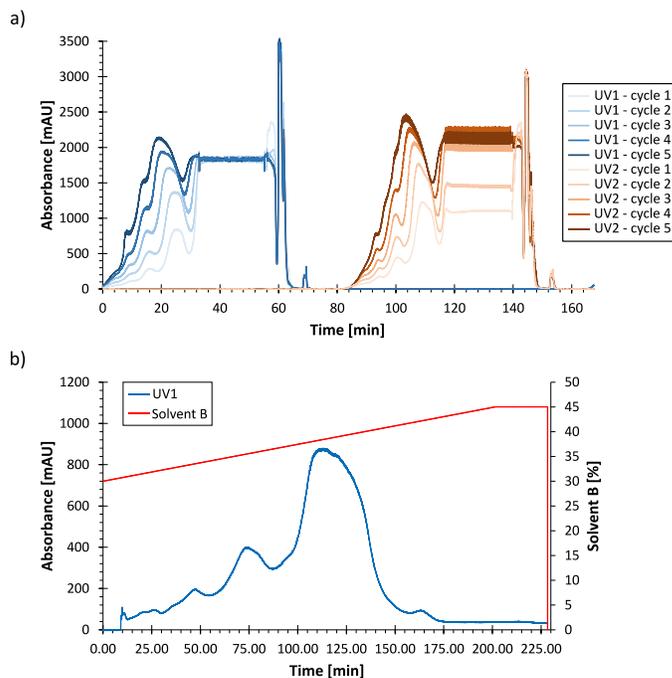
The analytical IEX profile of the purified biosimilar showed seven minor isoforms, besides the main product species (see Fig. 2). Each of the minor forms made up between 1.5% and



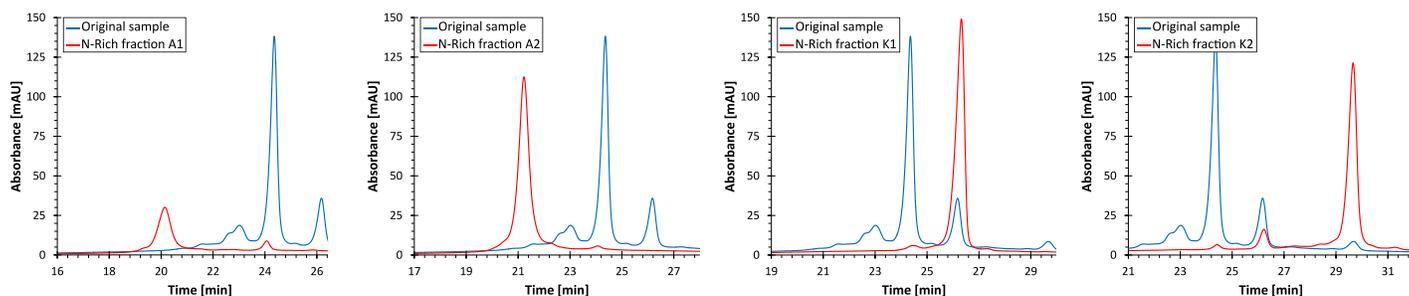
**Fig. 2.** Analytical IEX chromatogram of an antibody biosimilar. The profile shows the product peak and seven isoforms (A1-A5, K1, and K2), all with potentially varying biological effects. Two regions for N-Rich runs were defined for isolating all seven isoforms.

14.5% of the mixture. The goal of the project was the enrichment and individual isolation of all isoforms. A conventional preparative batch FPLC separation using a small-particle cation exchange resin was optimized towards separation of the isoforms by screening different buffers and gradient elution conditions. The optimized batch process was then used to design two N-Rich separations, one each for the early eluting isoforms A1-A5 (region 1 in Fig. 2) and for the late eluting K1 and K2 (region 2).

The N-Rich processes were designed with the wizard tool integrated in the ChromIQ® operating software of the Contichrom® CUBE Combined. After loading a batch chromatogram into the wizard, the user sets the region that should be enriched, i.e. the region containing isoforms A1-A5. The user then defines the column size, washing and cleaning protocols, the desired number of enrichment cycles, and the fractionation conditions. The software automatically turns this information into a continuous N-Rich process requiring no further user interaction for the individual phases (Fig. 3).



**Fig. 3.** a) Twin-column chromatograms during the enrichment phase. The signals of column 1 are shown in blue, column 2 in brown. A gradual increase of the concentration of the target compounds from cycle to cycle is demonstrated, with the maximum after five cycles (UV2 – cycle 5). b) After enrichment and depletion phase, only the N-Rich region is left and is eluted using a shallow gradient and a fine fractionation (2 min) to ensure that the eluted species can be isolated at a high purity.



**Fig. 4.** Comparison of original feed material (blue) and N-Rich fractions (red) for isoforms A1, A2, K1 and K2, respectively. The isolated amounts and purities (see Table 1) were sufficient for further isoforms analysis.

**Table 1.** Overview over results of N-Rich processing.

Isoform	Percentage in feed	Purity N-Rich fraction	Amount N-Rich fraction	Enrichment
A1	1.5%	80%	>1.5 mg	53x
A2	2.5%	85%	>4.0 mg	32x
A3	1.5%	90%	>1.5 mg	69x
A4	5.0%	>90%	>7.0 mg	18x
A5	8.5%	>90%	>3.0 mg	10x
K1	14.5%	90%	>8.0 mg	6x
K2	3.5%	75%	>8.0 mg	21x

All seven targeted isoforms were successfully isolated and enriched using two N-Rich runs (see Fig. 4, Table 1). Between 1.5 mg and 8 mg were isolated, depending on the amount of material in the original solution, with enrichments of up to 70-fold. Importantly, all seven isoforms were collectively produced in a total of two N-Rich runs, greatly reducing the subsequent analytical burden compared to the classical approach based on pooling of repeated analytical runs. The isolation time was thus cut from 32 days, as required with conventional approaches, down to only 3 days, including analytical verification runs. The isoform samples were fit for further evaluations regarding their structure and therapeutic activity.

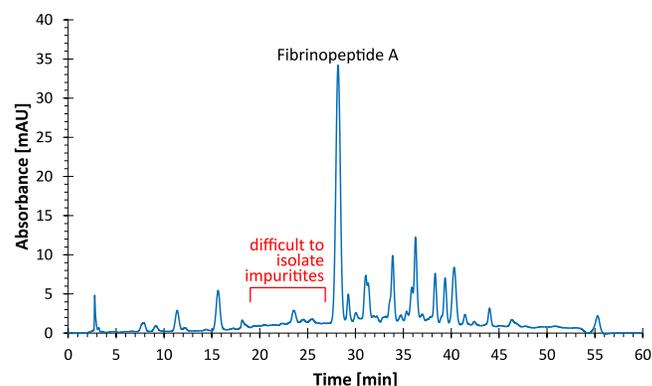
### Second application example: product-related impurities of a synthetic peptide

Fibrinopeptide A is a 16-amino acid sequence at the N-terminal end of fibrinogen. Within the blood coagulation cascade, fibrinopeptide A is cleaved from fibrinogen. Thus, fibrinopeptide A is a useful biomarker for coagulation events.

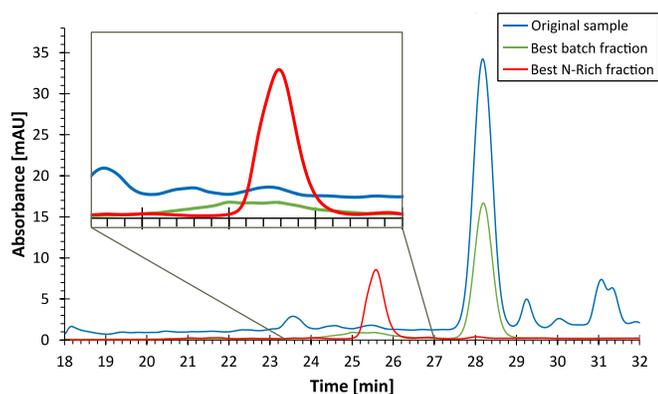
Since the peptide is relatively short, it can be produced synthetically by solid phase peptide synthesis (SPPS). SPPS is a highly efficient process, but with 16 coupling reactions and just

as many deprotection reactions, it is common to produce a number of side products which differ from the target peptide only in a minor way, for example by missing a single intermediary amino acid. Fig. 5 shows an analytical HPLC chromatogram of crude fibrinopeptide A on a reversed phase C18 column. It is of great interest to identify the product-related impurities marked in red, because structural identification would allow a target-oriented synthesis optimization. Furthermore, toxicological studies could determine if complete removal is necessary. With conventional methods it is tedious, sometimes even impossible, to identify such low concentration variants eluting close to the actual product.

A single N-Rich run was designed for all targeted impurities, as described in the previous section. After a total of eight enrichment cycles, the concentration of the impurities was sufficiently high. A single depletion cycle and finally elution with a shallow gradient followed. The impurities targeted by N-Rich were more than tenfold more concentrated than in the starting mixture. Even more so, the concentration relative to



**Fig. 5.** Analytical reversed phase (C18) HPLC chromatogram of the crude product from a fibrinopeptide A synthesis. The impurities marked in red are especially difficult to isolate from the main product peak. These impurities are majorly responsible for yield loss during preparative purification, where target purities have to be met.



**Fig. 6.** Overlay of analytical chromatograms prior to purification (blue) and upon isolation by batch (green) and enrichment by N-Rich (red). The zoomed window shows the elution region of a targeted impurity.

the main product, Fibrinopeptide A, was more than 600x higher than in the crude product. Importantly, purities of more than 80% could be achieved, even for targets eluting very close to the main product. In comparison, these impurities could not be isolated by normal batch chromatography (see Fig. 6).

### N-Rich using Contichrom® CUBE system

N-Rich is available on the Contichrom® HPLC and the corresponding FPLC instrument, Contichrom® CUBE Combined. Both systems are premium chromatographs with full-featured cyclic/continuous chromatography capabilities.

ChromIQ®, the operating software, comes with a wizard for designing N-Rich processes in four simple steps:

1. Load chromatogram of batch run and select region to enrich by drag-and-drop.
2. Set the column size.
3. Set desired washing and cleaning protocols.
4. Finalize the method by setting the number of enrichment cycles and fractionation parameters.

For enquiries regarding the Contichrom® systems, please contact [sales@chromacon.com](mailto:sales@chromacon.com).

### N-Rich as a Service

ChromaCon also offers a contract service for the isolation of product related impurities. Typically, an N-Rich fee-for-service project consists of two work packages (WPs):

**WP1: Method development (2-4 weeks).** The analytics for the target compound has to be pre-established by the customer and will be confirmed by ChromaCon. The method for the preparative isolation is then developed by ChromaCon. Initial test samples from preparative N-Rich runs will be isolated, and can be formulated and shipped to the customer. At the end of WP1, the customer and ChromaCon jointly review the data obtained thus far, before moving to WP2.

**WP2: Isolation of product-related compounds of the protein/peptide (2-6 weeks).** The target compounds will be isolated in suitable quantities and purity. The result is captured in a final report and a certificate of analysis will be issued for all shipped samples.

Typical projects take 4-10 weeks, depending on the amount and number of compounds to be isolated. A typical separation scale is in the milligram range, as this allows for further testing by the customer with his own assays (e.g. biochemical assays or even animal models).

The service offering can be handled directly between customers and ChromaCon. Enquiries can be sent to [sales@chromacon.com](mailto:sales@chromacon.com). Alternatively, if desired by the customer, the service offering can also be arranged through the Scientist.com platform, where it is listed in the [Innovation hub](#).

