Purification of ADCs using Continuous Chromatography (MCSGP)

MCSGP (Multi-column Counter-current Solvent Gradient Purification) was used for the purification of Antibody Drug Conjugates (ADCs) with various Drug-Antibody-Ratios (DARs) for a non-specifically conjugated model system, representing the Antibody-Drug-Conjugate Trastuzumab Emtansine (Kadcyla®). The aim of the study was to evaluate the potential benefit of using MCSGP to isolate ADCs with uniform DARs. The purification of product with DAR 2.0, 3.0, 4.0 and 5.0 was performed on a twin-column Contichrom CUBE benchtop system using two cation exchange (CEX) columns. Process performance was compared between traditional single column chromatography and MCSGP. This application note shows that MCSGP is capable of:

1. **Producing product with narrow-DAR profile with higher purity and yield than batch single column chromatography**
2. **Producing product of uneven DAR like 3.0 or 5.0.**

Introduction

Antibody-Drug-Conjugates (ADCs) are a promising class of therapeutics mainly used for treatment of cancer. An ADC molecule consists of an antibody linked to one or more cytotoxic payloads or drug molecules. ADCs can be synthesized by either employing non-specific conjugation or specific conjugation. In case of non-specific conjugation, a common synthesis is to conjugate the payload to Lysine-residues exposed on the surface of the antibody (Figure 1). Therefore, a non-specific conjugation reaction typically results in a broad range of DAR species and positional isomers. A narrow DAR distribution is desirable from a safety and efficacy standpoint. However, in chromatographic purification processes, the different species have very similar adsorptive properties making it difficult to purify target compound with a narrow DAR distribution at acceptable yield.

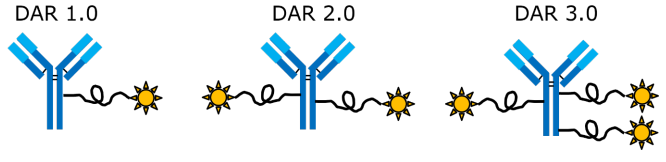

Figure 1 Schematic of ADC molecules with DARs 1.0-3.0

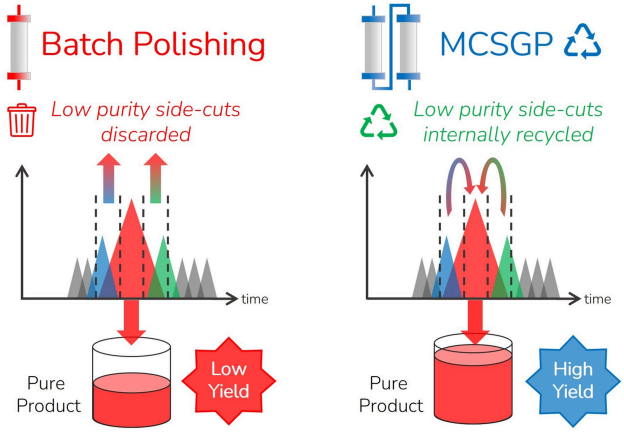

Figure 2 Traditional single column chromatography (left): Side fractions are discarded resulting in low product yield. MCSGP (right): Side fractions are internally recycled, ensuring high product yield.

MCSGP, a high-resolution preparative continuous chromatography technique, can be used to improve the yield when targeting a narrow-DAR product.

MCSGP is a technology that was developed for center cut purifications that require high resolution to deliver the target compound at high yield. The high yield is achieved by automatic internal recycling of impure side-fractions (Figure 2).

The principle of MCSGP relies on a linear gradient elution of the loaded starting material (see Figure 3), containing product P, early eluting impurities W and late eluting impurities S. After directing early eluting impurities to waste (Phase P1), the subsequent eluting overlap of W/P is directed to the second column (Phase P2). Next, the product P is eluted and collected (Phase P3) while new feed is loaded on the other column. Finally, the overlap of P/S is internally recycled to the second column (Phase 4) and the columns

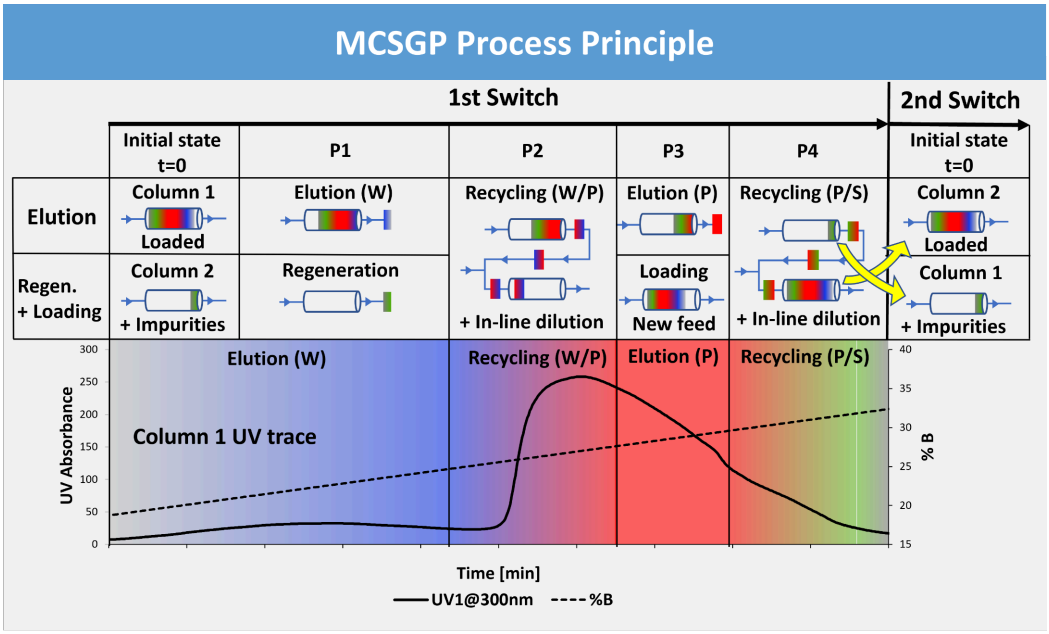

Figure 3 MCSGP process schematic

switch positions and tasks and the process continues in a cyclic manner.

MCSGP requires a **Contichrom CUBE** system to be developed and operated. The Contichrom CUBE is a twin column system enabling the different flow path configurations required for MCSGP to be run. The Contichrom CUBE system is controlled by the ChromIQ® operating software that features the necessary design tools (MCSGP wizard), controls (AutoPeak® dynamic process control), monitoring and evaluation tools.

MCSGP Process Design is done based on a single column linear gradient elution with fraction analysis: The linear gradient chromatogram is loaded into the MCSGP wizard and then divided into sections according to the presence of the impurities. Effectively, in the MCSGP wizard user interface, this is done by dragging & dropping of lines that indicate the section borders (see Figure 3, lower part). The gradient concentrations at the section borders define the gradient segments to be operated by the gradient pump in MCSGP.

Table 1 Preparative method

Parameter	Value
Eluent A: Equilibration/ Wash	25 mM Na-phosphate, pH 6.0
Eluent B: Desorption	25 mM Na phos., 0.1 M NaCl, pH 6.0
Eluent C: Strip	25 mM Na phos., 1 M NaCl, pH 6.0
Eluent D: CIP	0.1 M NaOH
Feed	Trastuzumab, unspecific linkage to Atto-488 using NHS-ester chemistry, Feed conc. = 0.5 g/L mAb
Stationary phase (preparative)	YMC BioPro IEX SmartSep S10 0.5 x 10 cm
Gradient	20-80% in 25 min
Load	4.1 g/L (batch) 3.7 g/L (MCSGP)
System	Contichrom CUBE 30

Study outline

Using the MCSGP wizard several MCSGP runs were designed focusing the product elution window on the regions of the Chromatogram with highest single DAR content. DAR 2.0, 3.0, 4.0 and 5.0 were targeted. For each target DAR, separate MCSGP runs were performed. The eluate from each cycle was collected and analyzed by HPLC and ESI-MS to determine product concentration and the degree of labeling.

Table 2 Analytical method

Parameter	Value
Systems	Agilent 1200 (ESI-MS)
Mass spectrometer	Q-TOF Bruker Maxis Impact
Spectra deconvolution	MaxEnt algorithm

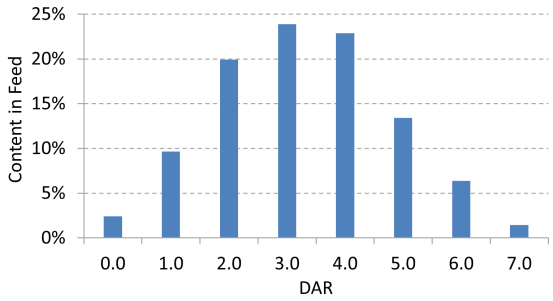

Figure 4 **Analysis of the starting material for MCSGP chromatography**

Materials and Methods

Preparative single column gradient runs and MCSGP runs were carried out using the conditions reported in Table 1.

BioPro IEX SmartSep S10, a high resolving cation exchange stationary phase from YMC, was used to maximize resolution.

Results of mass spectrometry analysis (Table 2) of the starting material are shown in Figure 4 showing the broad DAR distribution resulting from unspecific linkage.

Results and Discussion

MCSGP operation – DAR 2.0

MCSGP operation for DAR 2.0 targeting is shown in Figure 5. The UV signal (only column 1 signal shown) is repetitive, indicating that the run has reached a cyclic steady state where product concentration and purity of the product pools are constant from cycle to cycle. This was confirmed by ESI-MS.

Purifying single DAR 2.0-5.0 products with MCSGP

The MCSGP runs were operated with settings for DAR 2.0, 3.0, 4.0 and 5.0 purification for 4 to 6 cycles. Figure 6 shows the ESI-MS analysis results of each cycle eluate pool of the MCSGP runs. DAR 2.0 product was obtained with 70% purity, DAR 3.0 with 55%-60%

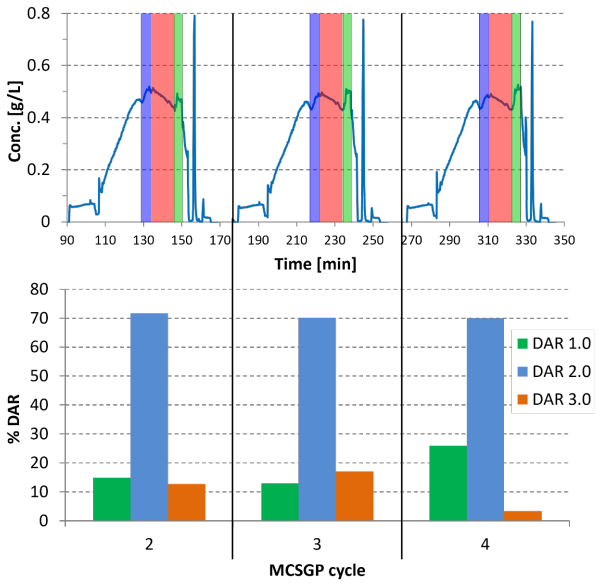

Figure 5 **UV signals of cycles 2-4 of MCSGP targeting DAR 2.0 (top); and corresponding ESI-MS results of product pool analysis of each cycle (bottom).**

purity, DAR 4.0 with 50%-55% purity, and DAR 5.0 with 45-50% purity. The trend to lower purity with increasing DAR can be explained by the increasing number of positional isomers which can lead to a broader elution peak for the selected DAR species. The results show the effective narrowing of the DAR distribution by MCSGP: Apart from the target DAR and the neighboring DAR species, all other DAR species are almost completely eliminated. For example, DAR 3.0 MCSGP product also contains DAR 2.0 and DAR 4.0 but all other DAR species are below 5%. The feed material instead contains 6 DAR species at > 5% (see Figure 4).

Specific DARs obtained at high yield

The performance of single column batch and MCSGP is summarized Table 3 for DAR 2.0-5.0. Thereby the yield was compared for equal DAR purity, if possible.

The results show that MCSGP provides the selected DAR product species consistently with higher yield than single column preparative chromatography. Apart from the runs targeted at DAR 5.0, the yield of MCSGP is always larger than 60%, while single column batch

Table 3 **Performance summary of batch and MCSGP**

Process	DAR 2.0		DAR 3.0		DAR 4.0		DAR 5.0	
	Purity [%]	Yield [%]	Purity [%]	Yield [%]	Purity [%]	Yield [%]	Purity [%]	Yield [%]
Batch	59.0	34	30.9	22	50.7	56	47.4	33
MCSGP	70.0	61	57.1	100	53.9	92	48.1	42
Improvement	n/a	**+ 80%**		**4x**		**+ 50%**		**+ 25%**

Table 4 Performance summary of batch and MCSGP for DAR 2.0 conjugate purification

Process	Purity [%]	Yield [%]	Product conc. [g/L]	Load [g/L]	Productivity [g/L/h]	Buffer cons. [L/g]
Batch	59.0	34	0.5	4.1	0.11	142
MCSGP	70.0	61	0.5	3.7	0.20	64
Improvement	+ 11%	+ 80%	/	- 10%	+ 80%	**- 55%**

yields are between 20% and 60%, depending on the species. There seems to be a trend that the yield gap between batch and MCSGP narrows with increasing DAR. It is possible that by adjusting the linear gradient, the yields for higher DARs can be improved. The results show that MCSGP enables the production of uneven DARs (DAR 3.0) with high yield, a task that is infeasible with batch chromatography. It is worth mentioning that this task is also not possible for starting materials originating from site-specific conjugation of ADCs which only allows manufacturing of even-DAR products (DAR 2.0, 4.0, or 6.0).

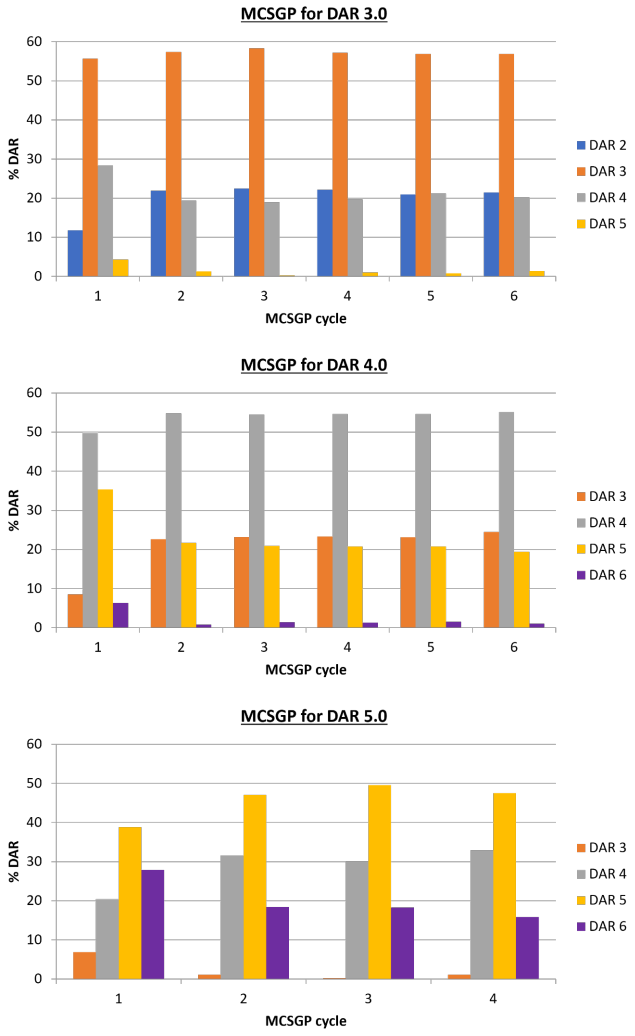

Figure 6 **ESI-MS results of eluates of MCSGP runs targeting DAR 3.0, 4.0 and 5.0 conjugates, showing relative DAR species content.**

Process performance benefits of MCSGP

The yield improvement enabled by MCSGP entails several advantages which are seen when comparing process performance for DAR 2.0 purification (Table 4). The yield increase from 34% to 61% leads to an increase of productivity from 0.11 to 0.20 g/L/h at comparable load and avoids 50% of synthesis runs for the same output. Alternatively, the same amount of product can be purified in about half the time or with half the column volume, compared to batch chromatography. Also, the buffer consumption of MCSGP is 50% lower.

Operational benefits of MCSGP

With dynamic UV-based control (AutoPeak) of both the automatic internal recycling and product collection, MCSGP has the following advantages:

1. No re-chromatography of side cuts
2. No product peak fractionation and detailed fraction analysis required for determining the product pool
3. Faster batch release times
4. Fewer operators required

MCSGP improves ADC safety

By avoiding yield losses, MCSGP reduces the number of synthesis operations by 50-80% (DAR 2.0 – 4.0), limiting expensive operations, isolator time, and (toxic) starting material consumption and exposure. ADC product from MCSGP has a much narrower DAR distribution than the starting material, improving drug safety and efficacy.

About the Contichrom® CUBE 30 system

The Contichrom CUBE is a versatile two-column benchtop system for continuous chromatography process development. It can run MCSGP, CaptureSMB (a continuous capture process to improve resin utilization and lower eluent consumption in affinity capture applications), N-Rich (a process to automatically purify impurity standards), and

automated two-dimensional purifications with inline dilution. A UV detector with 4 freely selectable wavelengths in the range of 200-600 nm is mounted behind each column, allowing a view inside the process when columns are operated in interconnected mode as well as monitoring the final chromatogram. Likewise, conductivity is monitored at the outlet of each column while pH is monitored at the outlet of either column 1 or column 2. The fraction collector can be equipped with various rack types, such as 50 mL, 15 mL tube racks or a rack for 96-well microtiter or deep-well plates. Processes developed on the Contichrom CUBE can be scaled up to the Contichrom TWIN systems for manufacturing under GMP.

The **ChromIQ® operating software** includes software tools "wizards" for the design of single- and twin-column chromatography processes. Moreover, it features a buffer management system, cycle overlay, and evaluation tools that are useful to plan, set-up, control and evaluate continuous chromatography runs. ChromIQ includes the MCSGP wizard, a process design tool to transfer single column chromatography processes recorded on the Contichrom CUBE into MCSGP chromatography processes. The UV-based dynamic process control option AutoPeak is integrated in the MCSGP wizard.

Table 6 Contichrom® CUBE 30/100 Ordering Information

Product	Order #
Contichrom® CUBE 30	220060
Contichrom® CUBE 100	220062
Fraction collector R1 (holds 1 rack)	300001
Fraction collector R2 (holds 2 racks)	300002
50 mL Tube rack (36 tubes)	300049
15 mL Tube rack (72 tubes)	300052
96-well plate rack (2 plates)	300048

Table 5 Contichrom® CUBE 30/100 System Specifications

Parameter	Value
Flow rate range	0.1 – 36 / 0.5 – 100 mL/min
Pressure rating	100 bar
Number of columns	1-2
Number of buffers	Up to 18
Fractionation	Fraction collector with multiple rack options
UV Detectors	2x 4-channel external detectors. 200-600nm behind each column
Conductivity detectors	2 detectors behind each column
pH	1 pH probe at product outlet

Acknowledgments

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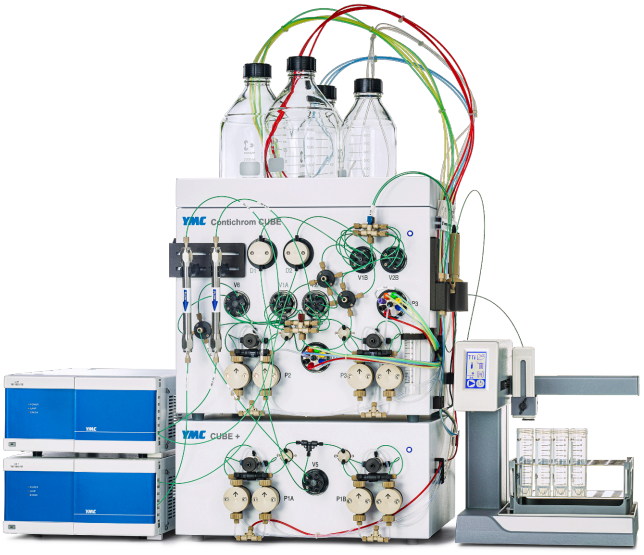

Figure 7 Contichrom CUBE benchtop chromatography system

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