How to Determine Reversed-phase Flash Chromatography Loading Capacity

White Paper

Biotage®

A flash column's loading capacity varies depending on several factors but primarily the selectivity and resolution of the target compound from its closest eluting neighbors. While normal-phase flash chromatography loading capacity is commonly determined from thin-layer chromatography (TLC) separation data, reversed-phase loading capacity determination is typically an empirical process involving several repeated injections with increasing sample mass.

As a general rule, reversed-phase columns have lower loading capacity compared to normal-phase silica columns due to reversed-phase media's lower available surface area (bonding C18 to silica reduces surface area) and different separation mechanism (partitioning vs. silica adsorption/desorption). If you check various flash column vendors you will find silica loading capacities up to 10% and even 20% of media weight. In contrast, typical published reversed-phase capacities are 1% - 2% of media weight. These are suggested maximum loads and not necessarily meant to be average or applicable for every purification. Again, it depends on how good your separation is along with the other criteria mentioned above.

In this document we will show how using a resolution value (Rs), calculated from small-scale empirical scouting runs, can be used to suggest the maximum sample load mass possible while maintaining a specific product purity goal.

Loading Capacity

Column loading capacity is based on a number of factors…

1. Sample complexity (number of compounds)
2. Sample separation method
    a. Isocratic
    b. Linear gradient
    c. Step gradient
3. Sample separation (how well your target is separated from the by-products and impurities)
    a. Retention
    b. Selectivity
    c. Resolution
4. Solubility in…
    a. Mobile phase
    b. Dissolution solvent
5. Compound chemistry – neutral, acidic, basic, polar, lipophilic
6. Load technique
    a. Liquid
        i. Dissolution solvent choice
        ii. Sample concentration
    b. Dry
        i. Sorbent used
        ii. Sample/sorbent ratio
7. Purity and yield goals
    a. Higher purity = lower loading
    b. Higher yield = higher loading

For medicinal chemists, yield usually is more important than purity for *intermediate compounds* with 80+% purity deemed acceptable in many cases. Historically, medicinal chemists have utilized normal-phase flash chromatography for intermediate purification but have started to migrate towards reversed-phase as their synthetic products' chemistry has become more polar and complex. Because of this migration to reversed-phase, understanding reversed-phase column loading capacity has increased in importance.

Method Scouting

As with any chromatography, a proper method must be developed that separates the targeted compound from its nearest eluting impurities. Method scouting is best performed with an analytical HPLC scaling column packed with the same media (particle size and chemistry) as the flash column (Bickler, J. Robert, 2015) or, if a scaling column and HPLC system are not available and you have adequate sample, methods can be developed on small flash columns.

For this whitepaper, the loading capacity of a 12-gram flash C18 column (Biotage® Sfär C18) was evaluated using four reaction mixtures, synthesized in-house using a Biotage® Initiator+.

After synthesis, each reaction mixture was evaporated in a tared scintillation vial providing weights of…

- Reaction 1 – 638 mg
- Reaction 2 – 545 mg
- Reaction 3 – 852 mg
- Reaction 4 – 862 mg

The contents of each vial were then dissolved in DMSO. Reactions 1 and 2 were dissolved in DMSO to a final volume of 5 mL while reactions 3 and 4 were dissolved in 2 mL. DMSO was chosen because of its broad solubility capability and very low retention in reversed-phase chromatography.

Reaction mixtures 1 and 2 were best separated using a 25–60% methanol gradient over 10 column volumes (10 CV) while reactions 3 and 4 needed a 35–85% methanol gradient over 10 CV.

Small aliquots (~10–20 mg) were injected into the 12-gram Sfär C18 column running the appropriate gradient to determine how many compounds were present, where the target product eluted, and the degree of separation achievable, both

selectivity and resolution. The data showed each reaction generated the product and by-products, Figure 1.

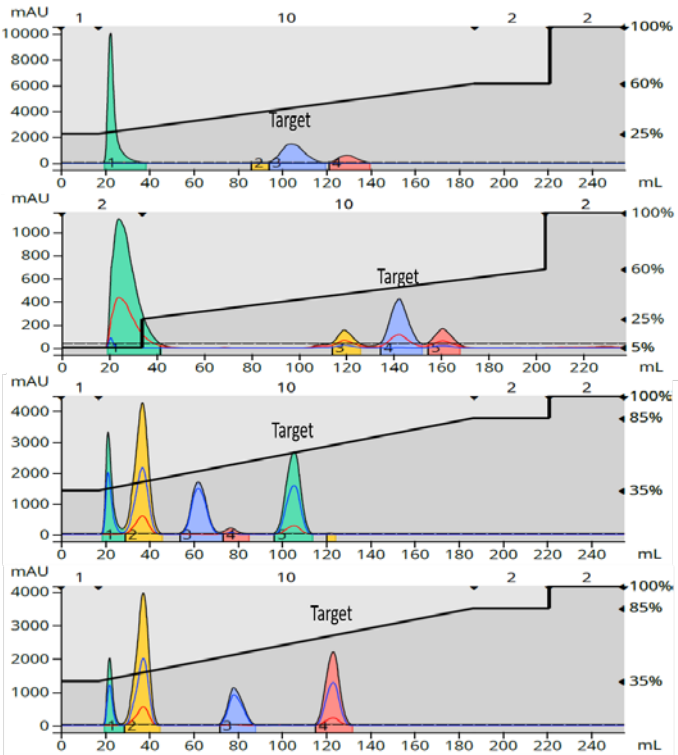

Figure 1. Scouting runs to determine resolution values. From top - reaction 1, reaction 2, reaction 3, reaction 4.

The scouting runs revealed that with reactions 3 and 4 the reaction product was the last to elute and very well separated from the earlier eluting by-products. However, the separations of reactions 1 and 2 showed that the major peak was centered between two other closely eluting peaks making target compound purification more challenging.

To determine each reaction mixture's separation efficiency and maximum load, the target products and nearest eluting by-product peaks retention (in mL) and their peak volume (the difference in volume from the start of peak fractionation to the end of peak fractionation) measured at baseline, were obtained on the flash system (Biotage® Selekt) and tabulated, Figure 2.

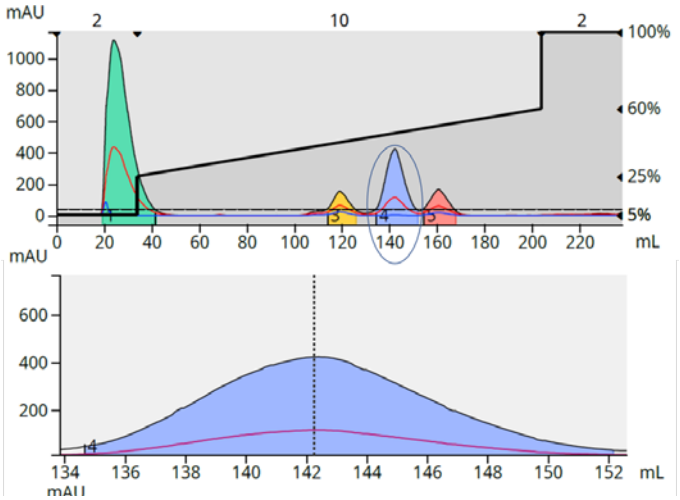

Figure 2. Peak retention volume and elution volume are measured by expanding the chromatogram around the peak of interest. Elution volume is the difference between when fractionation begins and ends, retention volume is the peak apex.

This data was used to calculate the target compound's resolution from each neighbor, which was used to determine loading capacity, Table 1.

Resolution is calculated as follows...

$$Rs = \frac{2(V2-V1)}{(W1+W2)}$$

Where:

- Rs = resolution
- V1 is the elution volume apex for the leading peak
- V2 is the elution volume apex for the product peak
- W1 is the peak volume for the leading peak
- W2 is the peak volume for the product peak

So, what does this provide in terms of determining loading capacity? Well, for starters, the larger the resolution, the higher the load. With a Rs of 2.76, the highest load (or best separation) should be seen with the reaction mixture 4. Since reaction mixtures 3 and 4 have the product eluting last, only the resolution between it and its faster eluting by-product were calculated. Reactions 1 and 2, with the target compound eluting between two by-products, required two resolution values be calculated. In these situations, the lower resolution value is the limiting factor so reaction 1, with a resolution of 0.91, should provide the lowest load. The resolution for reaction 2 is a similar 1.21.

Table 1. Scouting run separation data.

	Reaction 1				Reaction 2		Reaction 3		Reaction 4	
	Imp. 1	Target	Imp. 2	Imp. 1	Target	Imp. 2	By-product	Target	By-product	Target
Peak volume (mL)	8	25	18.5	12	17.5	13	13	17	16	17
Peak elution volume (mL)	89	104	129.5	119	142	160.5	77	105.5	78	123.5
Resolution Imp. 1/target		0.91			1.56			1.90		2.76
Resolution Imp. 2/target		1.17			1.21					

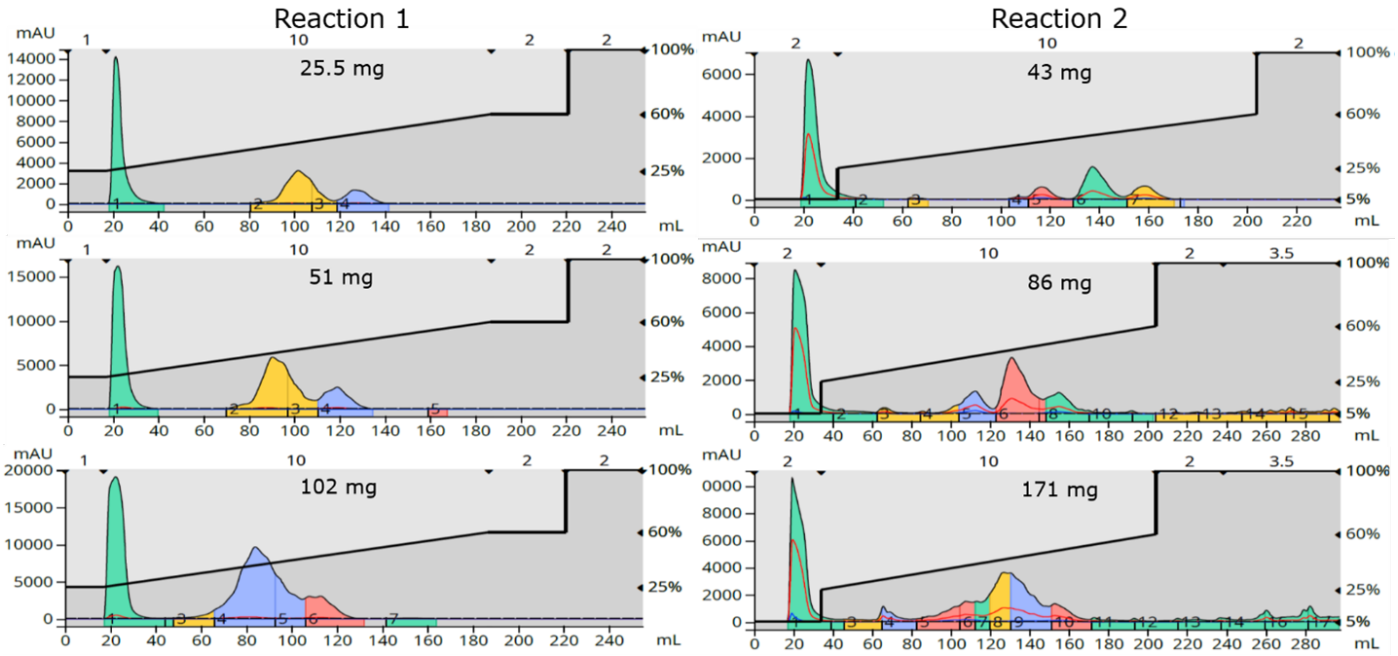

Figure 3. Scale-up chromatograms for Reaction 1 and Reaction 2 show decreasing resolution with increasing load.

Scaled Purification Runs

Since no direct conversion of resolution to load capacity exists, a series of separations with increasing load was performed to empirically determine maximum capacity. This work was done using reactions 1 and 2 injecting volumes 2x larger than the previous load until a complete resolution loss was achieved, Figure 3.

Purity Evaluation

The middle peak from each purification was evaporated using a Biotage® V-10 Touch, redissolved in DMSO, and analyzed for purity using the same flash method and column. The results showed increasing by-product amounts with increasing loads, a reasonable expectation.

To calculate peak purity, the peak height of each of the retained peaks were measured in mAU. Since the all-wavelength feature was used with a range encompassing the total UV absorption spectrum for each compound (200-300 nm), peak height ratios are valid measurement for comparison. The ratio of the target compound peak height to the cumulative total peak height was calculated and used as the purity measurement, Table 2.

For reaction 1 a load of 102 mg met the ›80% purity goal making it the loading capacity for this crude mixture. However, for reaction mixture 2, the scaling study showed the maximum load is less than 171 mg but more than 86 mg, requiring another purification test at a load between 86 and 171 mg.

Table 2. Target compound purity results.

Reaction 1				Reaction 2			
Load	**Height (mAU)**	**Total by-product height (mAU)**	**% Purity**	**Load**	**Height (mAU)**	**Total by-product height (mAU)**	**% Purity**
25.6	204	20	91%	21.4	902	22	98%
51.2	761	79	91%	42.8	492	11	98%
102.4	1167	250	82%	85.6	1029	51	95%
				171.2	1477	507	74%

Calculating Maximum Load

To help determine the maximum load, a plot of purity vs. load shows a linear relationship useful for determining the load required to achieve the 80% goal, Figure 4.

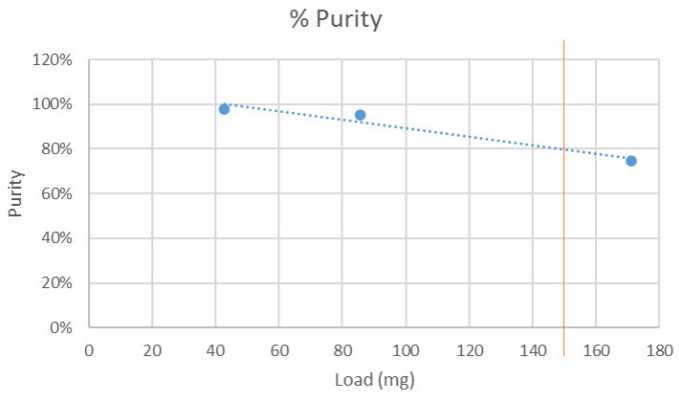

Figure 4. Load vs. purity for reaction 2 shows that a load around 150 mg should achieve the >80% purity target.

The graph indicted a load about 150 mg would meet the purification goal. Unfortunately, only 139 mg of reaction 2 remained. Though less than the calculated load amount, the resulting chromatography revealed a well-separated product from its adjacent by-products, Figure 5. Product purity was determined to be 89%, supporting the viability of a 150 mg maximum load, equating to a 1.3% load (crude weight/column media weight), Table 3.

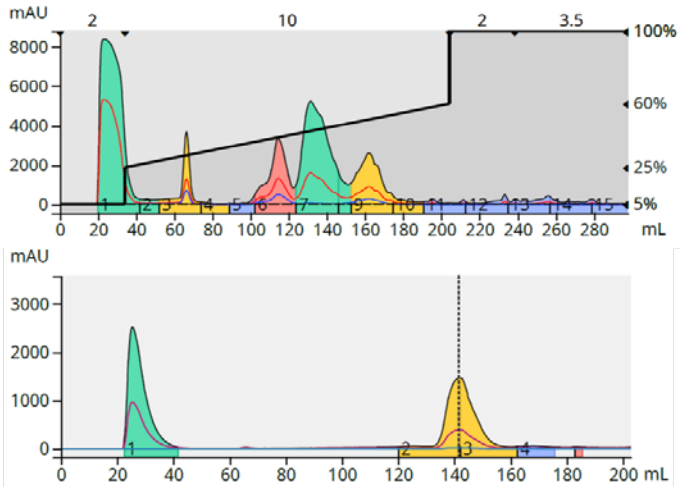

Figure 5. Purification and analysis for reaction 2 with estimated maximum load. Top - 139 mg load purification shows the product, peak 7 (green), well separated from its adjacent by-products. Bottom - Fraction 7 purity analysis indicates high purity.

Table 3. Reaction 2, 139 mg load purity analysis.

Load (mg)	Target compound height (mAU)	Total by-product peak height (mAU)	% Purity
139	1473	189	89%

Pushing the Limits

The preceding research provides a pathway to determining maximum loading capacity for low resolution purifications.

Reactions 3 and 4, however, had far better resolution values and only one major by-product as a potential contaminant, a situation that enables increased loading and throughput.

Plotting the log of the load vs. the log of the resolution using the previous data's maximum load and resolution (Rs 0.91=102 mg, Rs 1.21=150 mg) along with the resolution values and crude synthetic masses of reactions 3 and 4 generated a relationship with enough linearity (R^2=0.9004) to test purification of each reaction's total crude mix, Figure 6.

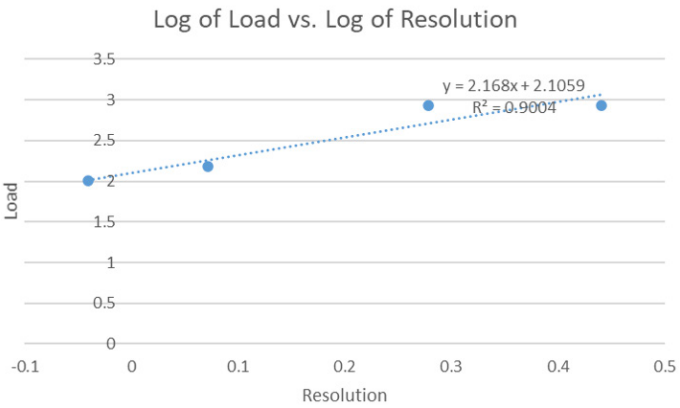

Figure 6. Log load vs. log resolution plot shows enough linearity to predict loading capacities for reactions 3 and 4.

Each crude mixture was loaded in its entirety (RxN 3 = 852 mg and RxN 4 = 862 mg) using the same 12-gram Sfär C18 column, Figures 7 and 8. The resulting purification yielded product purities of 86% for reaction 3 and 95% for reaction 4 indicating a loading capacity for both crude mixtures in excess of 7%, Table 4.

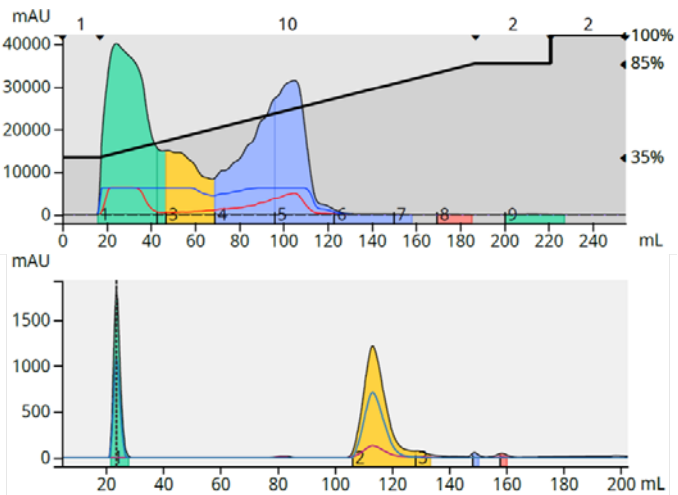

Figure 7. Reaction 3 purification (852 mg) and purity analysis. Top – crude reaction mix purification with product eluting in fractions 4 and 5 (blue peak). Bottom – analysis of fractions 4 + 5 indicates a high purity level.

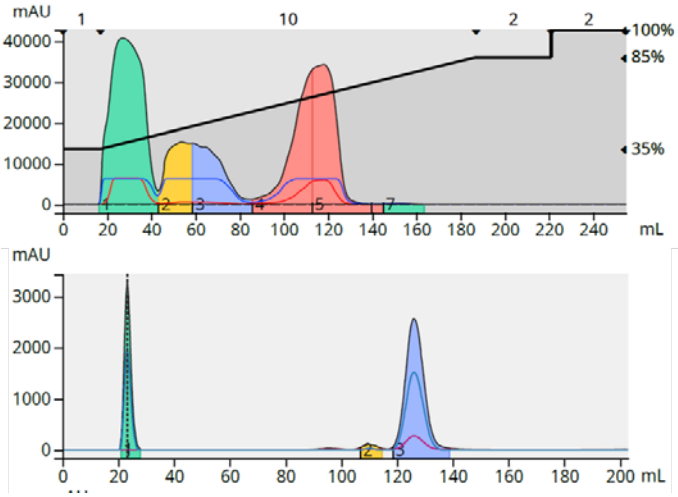

Figure 8. Reaction 4 purification (862 mg) and purity analysis. Top – crude reaction purification with product in fractions 4 and 5 (pink peak). Bottom – Fraction 4 and 5 analysis showing very high product purity.

Table 4. Post-purification purity assessment for reactions 3 and 4.

	Reaction 3	Reaction 4
Load (mg)	852	862
Product peak height (mAU)	1218	2558
Total by-product peak height (mAU)	195	140
Product purity	86%	95%
Loading capacity	7.1%	7.2%

As predicted, the highest load (862 mg) was achieved with the best resolution (2.76) and provided product purity of 95%.

During purification of reactions 3 and 4 an interesting phenomena was occurred - the product immediately began crystalizing in the collection vessels, an indication of high fraction purity, Figure 9.

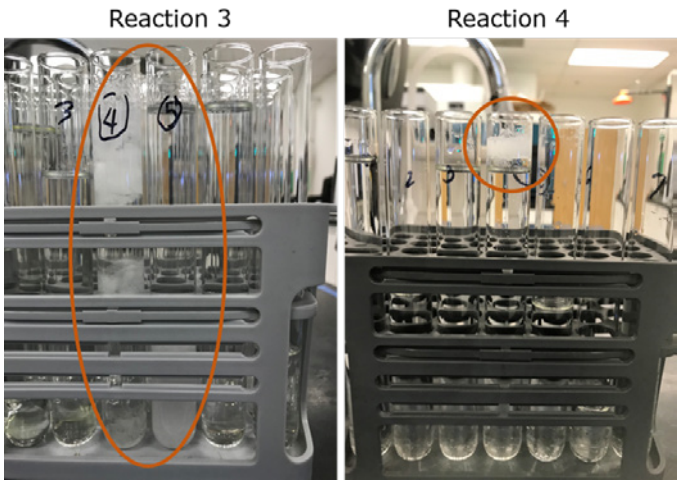

Figure 9. Reactions 3 and 4 crystalizing during fractionation.

Conclusion

Reversed-phase flash chromatography loading capacity can be determined for crude mixtures from just a few small-scale purification runs by calculating the resolution of the target compound from its closest eluting by-products. High loading capacity is achievable with reversed-phase flash chromatography when resolution is maximized.

In the examples shown above, loads of ~7% were possible with two crude reaction mixtures when product resolution was ›1.90 and DMSO was used as the reaction mixture dissolution solvent.

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EUROPE
Main Office: +46 18 565900
Fax: +46 18 591922
Order Tel: +46 18 565710
Order Fax: +46 18 565705
order@biotage.com
Support Tel: +46 18 56 59 11
Support Fax: + 46 18 56 57 11
eu-1-pointsupport@biotage.com

NORTH & LATIN AMERICA
Main Office: +1 704 654 4900
Toll Free: +1 800 446 4752
Fax: +1 704 654 4917
Order Tel: +1 800 446 4752
Order Fax: +1 704 654 4917
ordermailbox@biotage.com
Support Tel: +1 800 446 4752
us-1-pointsupport@biotage.com

JAPAN
Tel: +81 3 5627 3123
Fax: +81 3 5627 3121
jp_order@biotage.com
jp-1-pointsupport@biotage.com

CHINA
Tel: +86 21 68162810
Fax: +86 21 68162829
cn_order@biotage.com
cn-1-pointsupport@biotage.com

KOREA
Tel: +82 31 706 8500
Fax: +82 31 706 8510
korea_info@biotage.com
kr-1-pointsupport@biotage.com

INDIA
Tel: +91 11 45653772
india@biotage.com

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