Waters™

Using At-Column Dilution in Liquid Chromatography

INTRODUCTION

At-Column Dilution (ACD) is a technique that you could use in all forms of liquid chromatography (LC), preferably for reversed-phase preparative separations. It was developed specifically for injecting relatively large volumes of relatively strong sample diluents. Such injections may distort the chromatography in a conventional system. If injection artifacts are limiting mass capacity or chromatographic resolution, the effects can be ameliorated by applying At-Column Dilution. In addition, At-Column Dilution often increases system ruggedness and column life by preventing bulk precipitation in the sample loop or in the column itself.

CONVENTIONAL HPLC SYSTEM

In a conventional system, the sample is dissolved in a strong diluent, such as DMSO, and carried from the injector to the head of the column as a plug sandwiched in a stream of weak solvent, often 95% aqueous. Precipitation might occur at the edges of this plug where the strong sample solvent is diluted with the weak chromatographic solvent. This precipitation might occlude the fluid path and lead to a high-pressure shutdown. In the absence of such precipitation, the sample enters the column, but there will be no retention until the sample plug is diluted with the initial-strength mobile phase in the pores of the column. With larger injections, the volume required to dilute the sample can only be derived by moving a substantial distance along the column. In such cases, the sample is deposited as a broad band that occupies a large fraction of the column volume. Elution of such samples gives incomplete resolution with the overlapping peaks spread over large volumes of eluent and fractions, as illustrated in Figure 1.

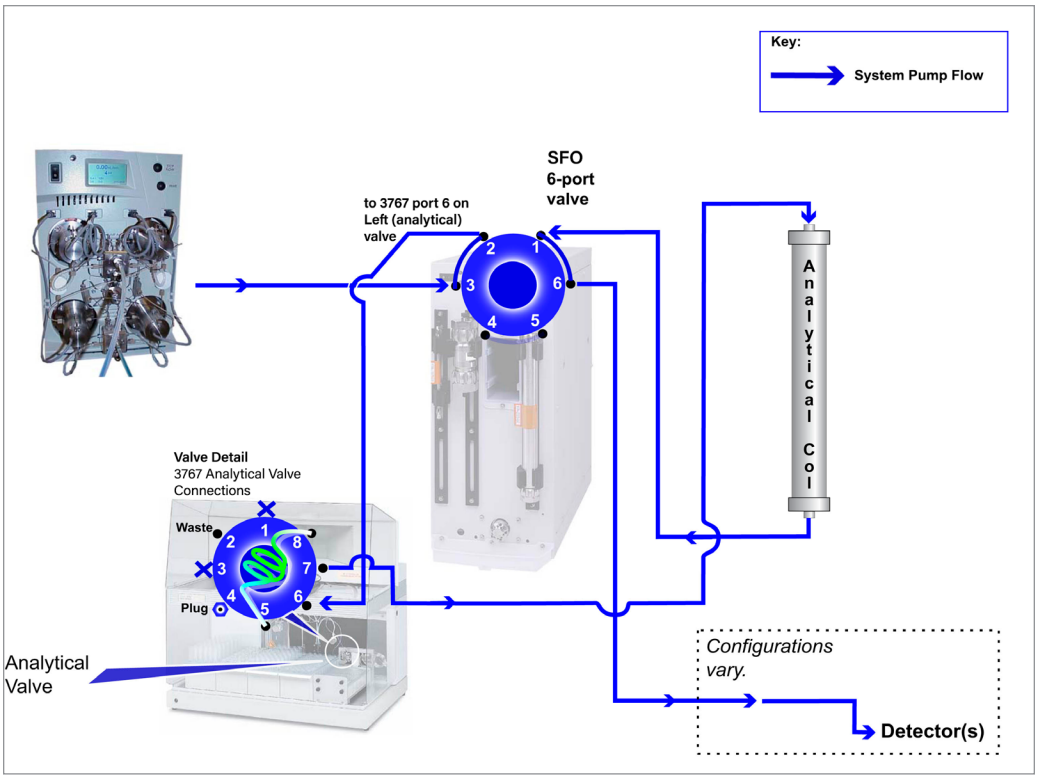

Figure 1. Conventional HPLC separations.

These problems may be reduced by strictly limiting both the volume and mass of sample injected. The alternative is substantial dilution of the sample with water, or, more generally, a weak solvent to ensure adequate retention. Neither approach is completely satisfactory because throughput and recovery are compromised. The required larger injection volume may be incompatible with the injector and fluidics present in the system.

AT-COLUMN DILUTION SYSTEM

In an At-Column Dilution system, the system is reconfigured to allow the sample plug to be carried to the head of the column in a stream of strong solvent. At the entrance of the column, this stream is continuously diluted with a stream of aqueous mobile phase. The rate of transfer into the column is so high that precipitation cannot occur. The sample molecules are then adsorbed to the packing material as very narrow bands that can be eluted as well-resolved, small-volume peaks as illustrated in Figure 2.

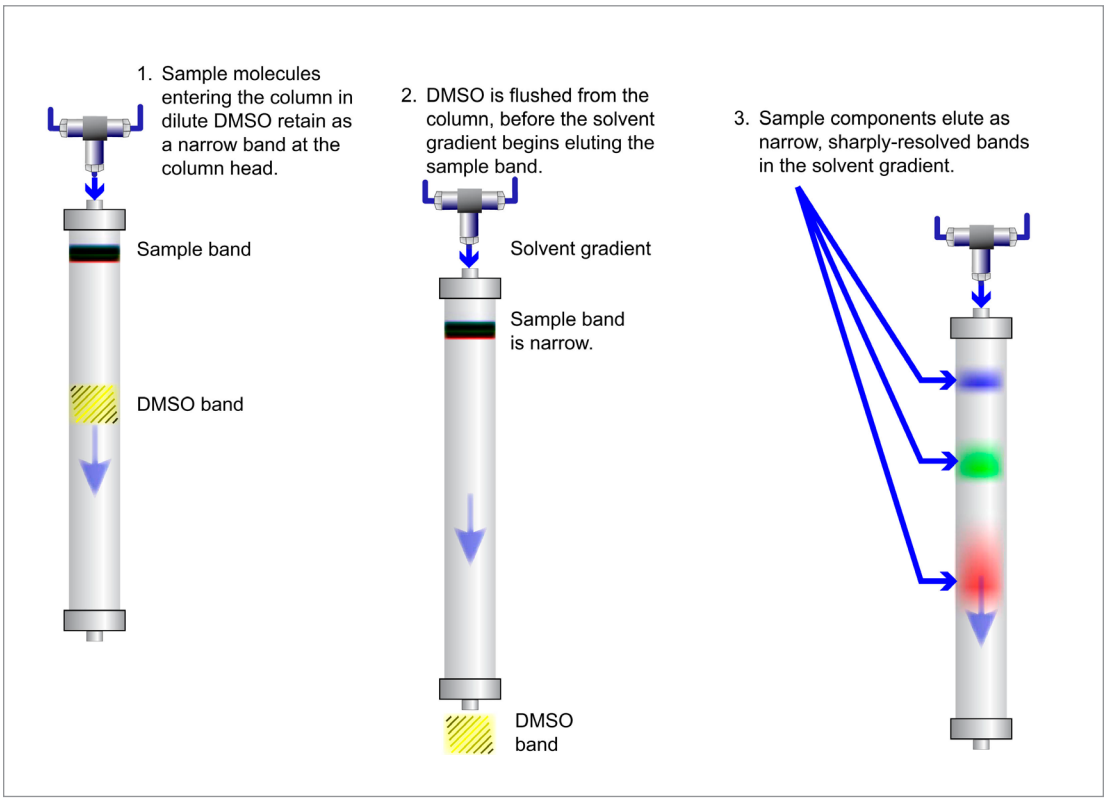

Figure 2. At-Column Dilution separations.

CONFIGURING THE PLUMBING FOR AT-COLUMN DILUTION

A conventional HPLC system flow path is shown in Figure 3. There are two ways to configure the plumbing to establish At-Column Dilution:

- Adding a loading pump to the system as shown in Figure 4 and Figure 5. You can use this approach with a binary gradient (high-pressure mixing) or with a system utilizing Auto•Blend Plus™ Technology (low-pressure mixing). Binary systems include Waters 1525µ, 1525EF, and 2545 BGM solvent delivery systems. Auto•Blend Plus Technology-based systems include the Waters ACQUITY™ H-Class PLUS, ACQUITY Arc™, 25X5 QGM, and 2695 solvent delivery systems. The loading pump must deliver solvent against the full system backpressure with the same accuracy and precision of flow as the rest of the system. The loading pump also contributes to the total flow and solvent composition affecting the separation. The most common loading pump is the Waters 515 pump controlled by MassLynx™ Software through a pump control module.
- Adding a gradient pump, as shown in Figure 6, is known as direct At-Column Dilution. Only binary gradient systems can be used for direct At-Column Dilution.

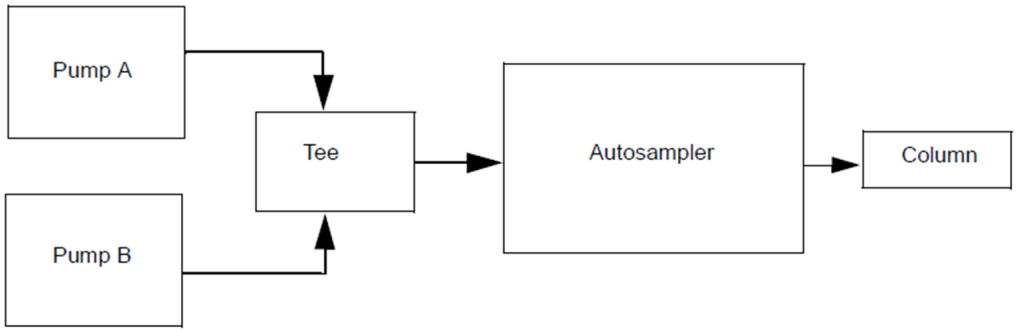

Figure 3. Conventional HPLC system.

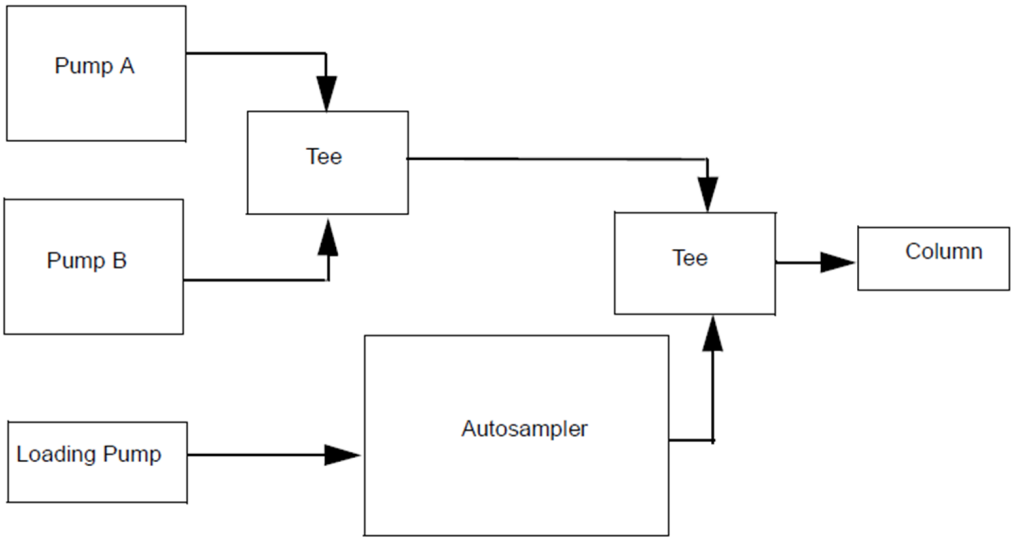

Figure 4. At-Column Dilution with a binary (high-pressure mixing) system and a loading pump.

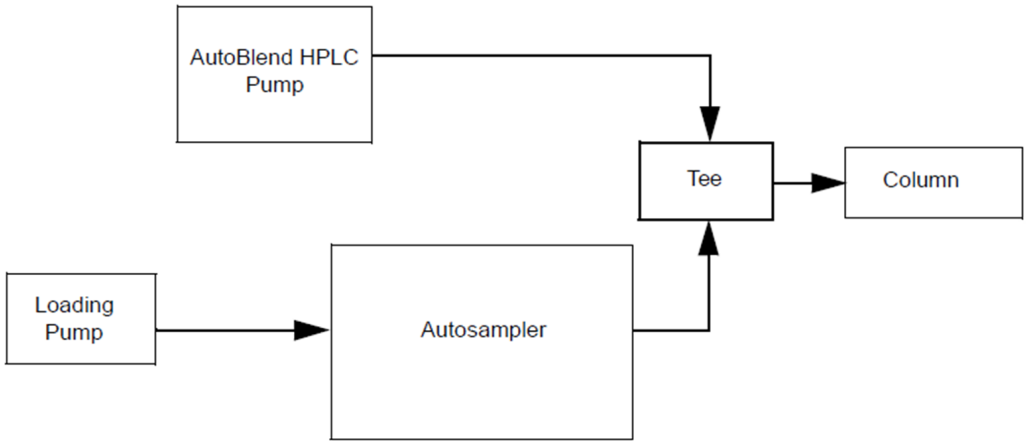

Figure 5. At-Column Dilution with a system using Auto•Blend Plus Technology (low-pressure mixing) and a loading pump.

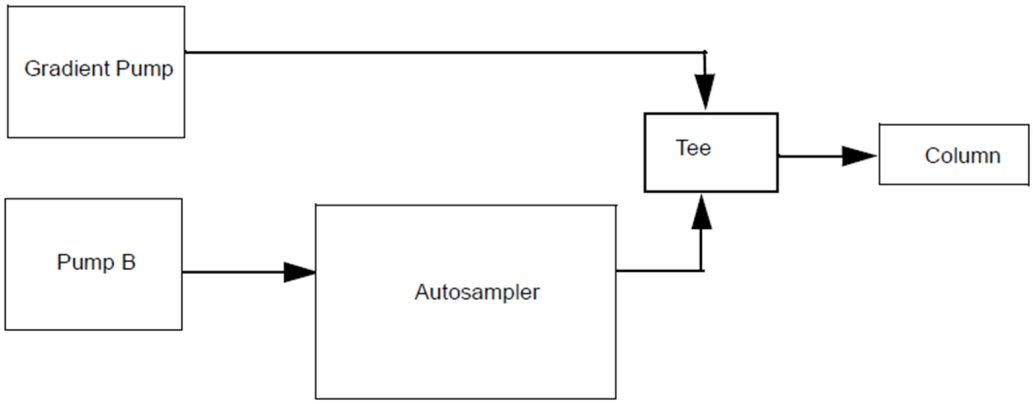

Figure 6. Direct At-Column Dilution with a gradient pump.

CONSIDERATIONS IN SYSTEM CONFIGURATION

Loading pump

You can use any LC pump that delivers flows ranging from 5% to as much as 30-50% of the prep flow. Ensure the pump is free of pulsation at the level generally required for LC. It should also work against the total system backpressure, not just its own low flow.

Sample loop

The sample loop must be large enough to accommodate the range of intended injection volumes. It is usually best to use a loop that is twice the size of the expected injection. If the loop is too large, however, it can result in excessive dilution of sample in the loading solvent with corresponding deterioration of resolution. In addition to the volume being too large, the dimensions can be unsatisfactory. Specifically, 5- and 10-mL injector loops are available as either 1/16- or 1/8-inch tubing, with the larger diameter being a shorter length to provide the same volume. The larger diameter tubing, however, gives excessive band-broadening and dilution, so you should use only 1/16-inch loops.

Mixing tee

Any standard HPLC tee union will work. In the most common configuration, a stainless steel tee has been used (P/N: WAT075215). Avoid tees with very small (<0.010-inch) through-holes.

Connecting tube

The tubing between the tee and the head of the column is the major source of mixing. It must be of sufficiently large diameter to provide mixing while having a short transit time to avoid aggregation after dilution. Typically, this means the shortest possible piece of 0.040-inch tubing, about 1 inch.

System tubing

From the loading pump to the injector and from the injector to the tee, use the tubing diameter that is usual for the flow rate range. Either steel or PEEK is suitable.

Guard columns and inline particle filters

Use either or both devices, if desired. Install the ACD mixing tee at the inlet of the guard column or inline particle filter.

High-throughput systems with rapid parallel re-equilibration

Use a 10-port, 2-position valve to select one of two identical columns for running a given sample while the other column is regenerated and re-equilibrated. These functions are alternated from one injection to the next. Install the At-Column Dilution tee directly on the inlet port of the 10-port valve that is connected to the injector in the standard plumbing.

METHODS FOR AT-COLUMN DILUTION

General principles

At-Column Dilution methods mimic the separation methods used for standard separations. In general, the flow rate and solvent composition generated with the gradient pump in a standard system are distributed between the gradient pump and the loading pump in an At-Column Dilution system. The solvent entering the column is the same in both systems, as illustrated in Figure 7. With total flow and percentage composition thus modified, alter the gradient table to include an isocratic composition period (initial hold) sufficient to completely transfer the sample through the At-Column Dilution tee to the head of the column. Add the separation and other segments of the gradient to this initial hold. There is seldom any reason to further modify the gradient to account for the contribution of the loading pump to the percentage of strong solvent.

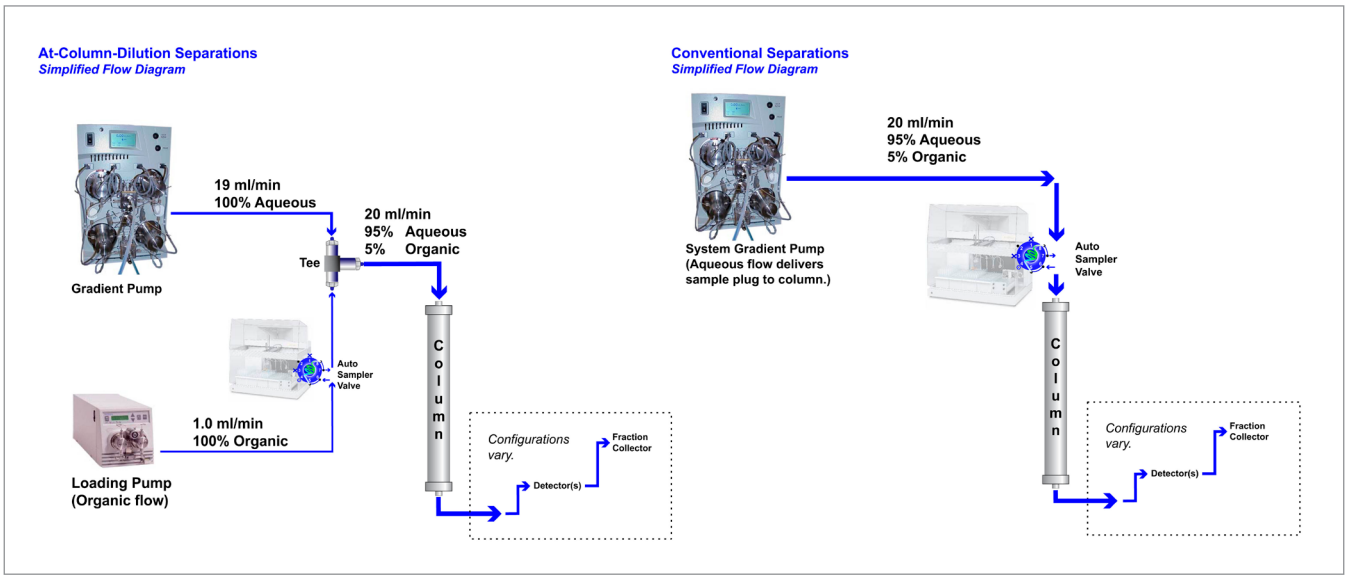

Figure 7. At-Column Dilution and conventional separation methods.

Selecting the Loading Solvent

Selection of loading solvent is critical. It should be as similar as possible to the sample diluent and also to the strong solvent of the separation method. For samples in DMSO, you can use either acetonitrile or methanol as loading solvent. In this case, the strong solvent of the separation method is favored. For samples that are dissolved in water at a pH where they are 100% ionized, the loading solvent can also be water at that pH. Such samples are usually separated in a reversed-phase column with a mobile phase buffered to a pH that suppresses ionization. In this case, At-Column Dilution serves to continuously titrate the unretained ionic form to the strongly retained nonionized form. On occasion, some samples that dissolve in aqueous-organic mixtures have precipitated in 100% organic loading solvents.

Gradient table examples

Table 1. Standard system.

Time	Flow	%A	%B	Curve
0.00	25.00	95	5	*
1.00	25.00	95	5	6
6.00	25.00	5	95	6
7.00	25.00	5	95	6
7.10	25.00	95	5	6
10.00	25.00	95	5	6

Table 2. At-Column Dilution System with a 5-mL Sample Loop. This table illustrates the loading pump running at 1.25 mL/min (5% of total flow).

Time	Flow	%A	%B	Curve
0.00	23.75	100	0	*
5.00	23.75	100	0	6
10.00	23.75	5	95	6
11.00	23.75	5	95	6
11.10	23.75	100	0	6
14.00	23.75	100	0	6

Table 3. Direct At-Column Dilution System with a 5-mL sample loop. This table illustrates no loading pump, with only B solvent passing through the injector loop.

Time	Flow	%A	%B	Curve
0.00	25.00	95	5	*
5.00	25.00	95	5	6
10.00	25.00	5	95	6
11.00	25.00	5	95	6
11.10	25.00	95	5	6
14.00	25.00	95	5	6

TROUBLESHOOTING AND CONSIDERATIONS

Insufficient hold

The most common cause of failure in At-Column Dilution experiments originates with an insufficient hold at initial conditions. If the hold is too short to completely empty the sample loop, the sample plug mixes with the increasing solvent strength gradient during loading. Resolution is severely degraded, and peak shapes are very distorted.

Polar compounds

For compounds that are extremely polar, that is, those that elute with low k' (low retention), At-Column Dilution may degrade resolution rather than improving it. The initial hold for loading the sample may be on the order of 10 column volumes, so compounds with intrinsic k' of that magnitude will spread down the column much more extensively than they would in a conventional system. At-Column Dilution was developed for samples that must be dissolved in relatively large volumes of strong solvents where the large injection of strong solvent results in poor retention. It does not increase retention properties inherent to the sample-column-mobile phase combination.

Mass capacity and resolution

At-Column Dilution can only improve mass capacity and resolution in separations where those characteristics are limited by the sample diluent and injection volume. Where this is true, there should be no deterioration of the separation, and the technique may still be valuable for increasing both system ruggedness and column life.

Hydrophobic samples

Some very hydrophobic samples may still precipitate during the At-Column Dilution process. For samples of this class, the initial strength mobile phase can include a concentration of strong chromatographic solvent. The usual guideline is an initial strength mobile phase that is about 20% lower in organic solvent than the solvent expected to elute the compounds.

Software control

When MassLynx Software and FractionLynx™ Software control the preparative HPLC system, the fraction collection method controls how much liquid is deposited in each collector tube, that is the percentage fill, before moving to the next empty tube. This feature requires, however, that the software relate the time duration of collection to the flow rate. The software only considers the flow from the gradient pump for this purpose. Since the flow from the loading pump is not included, you must reduce the percentage tube fill to allow the extra flow.

Automated configurations for At-Column Dilution

Figures 8-13 show various plumbing configurations for automating the selection of At-Column Dilution or conventional chromatography in a system.

CONVENTIONAL ANALYTICAL SCALE AND AT-COLUMN DILUTION PREPARATIVE SCALE

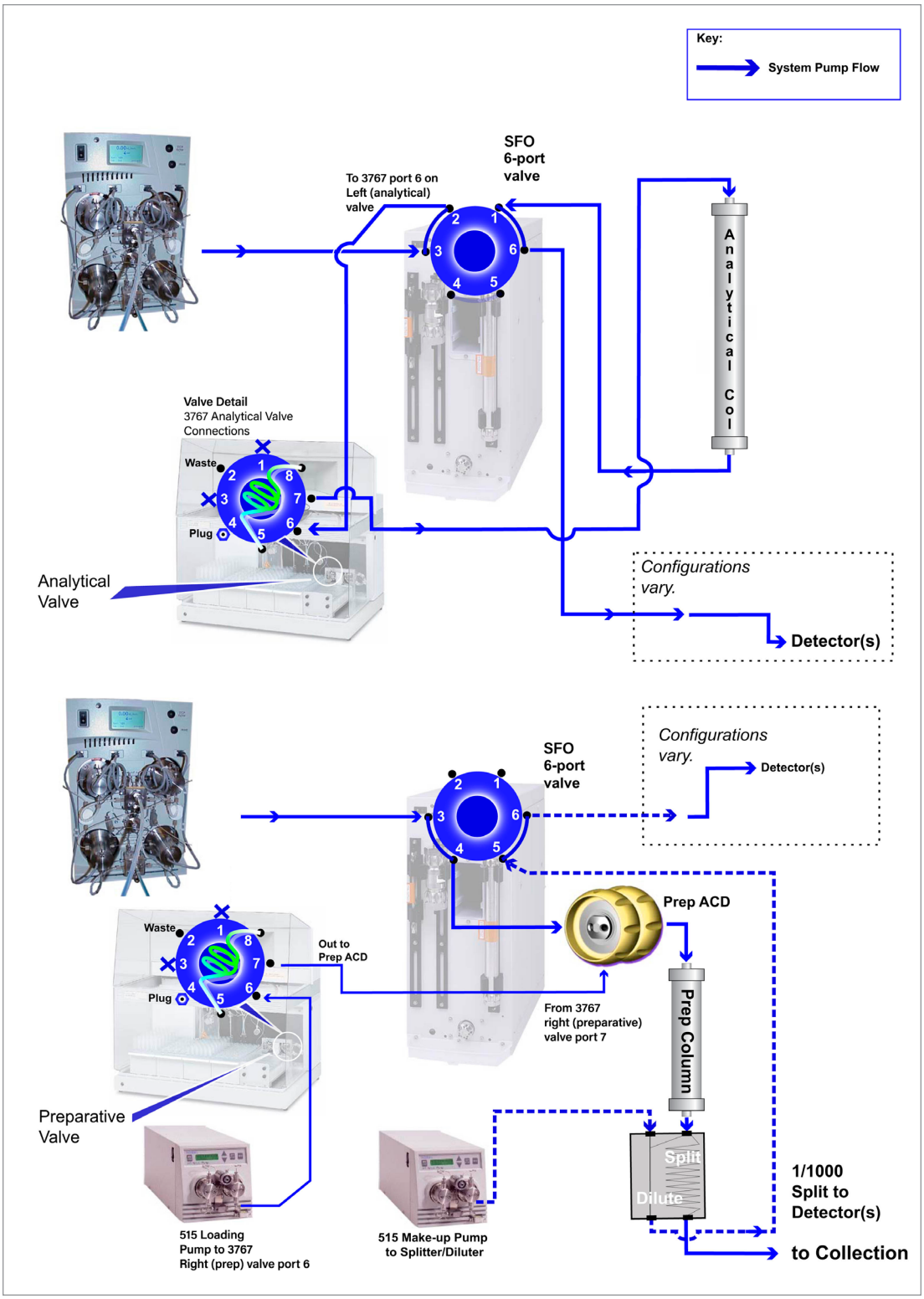

Figure 8. Full-time conventional analytical separation for analytical and preparative separation.

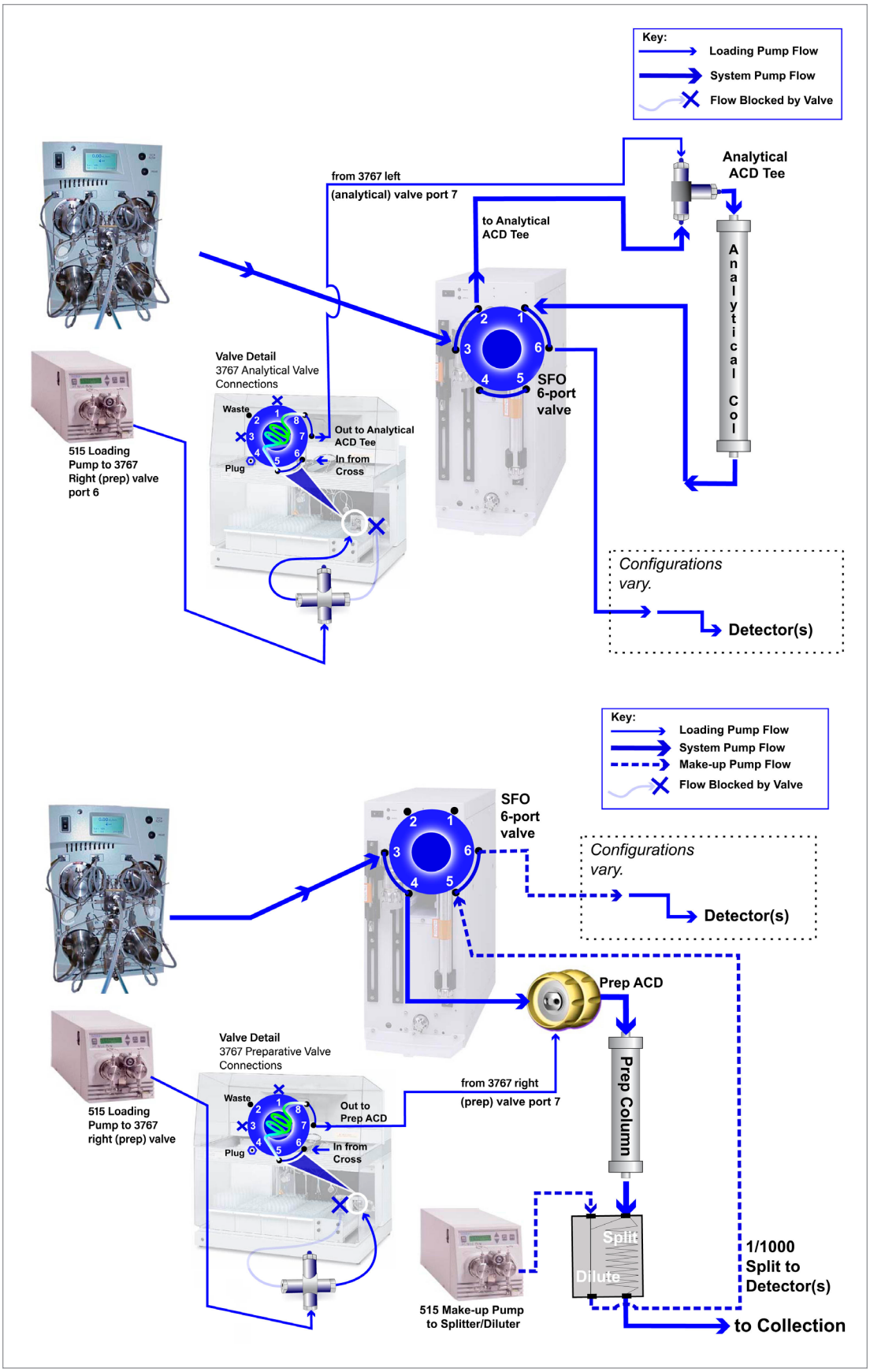

Figure 9. Full-time At-Column Dilution for analytical and preparative separation.

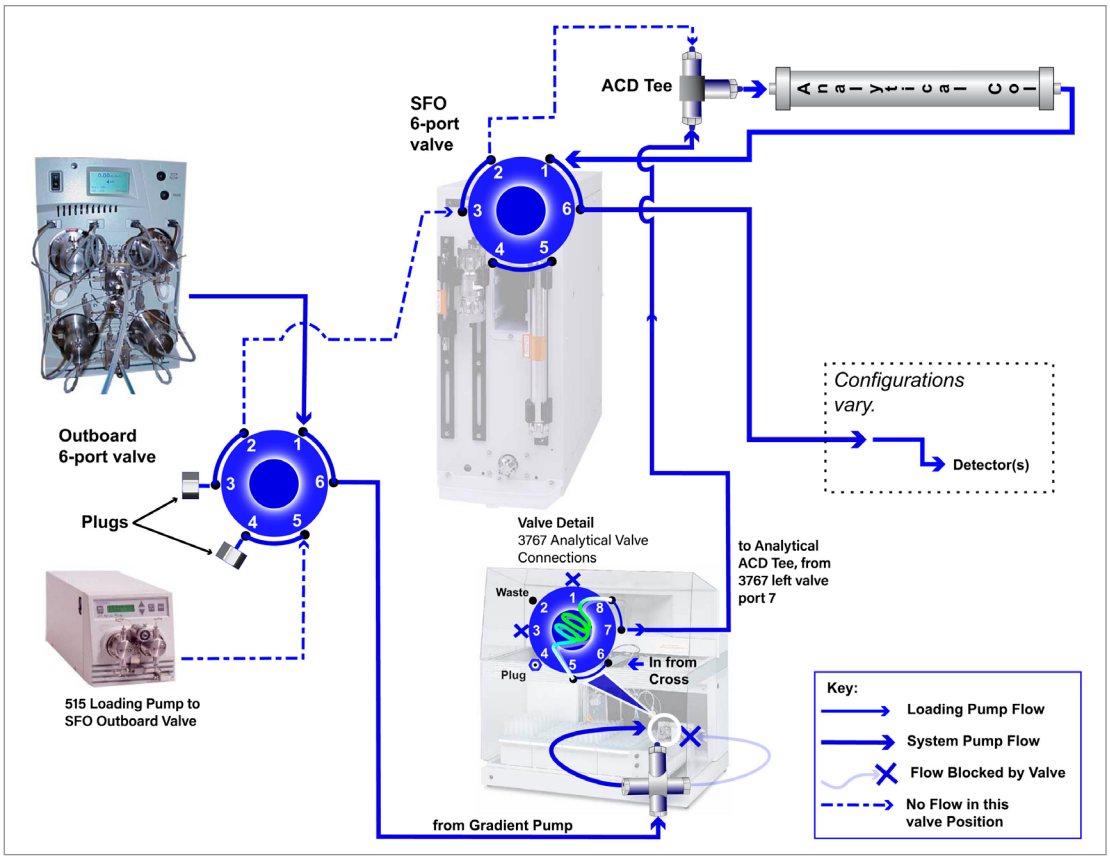

Figure 10. Analytical scale, conventional valve position.

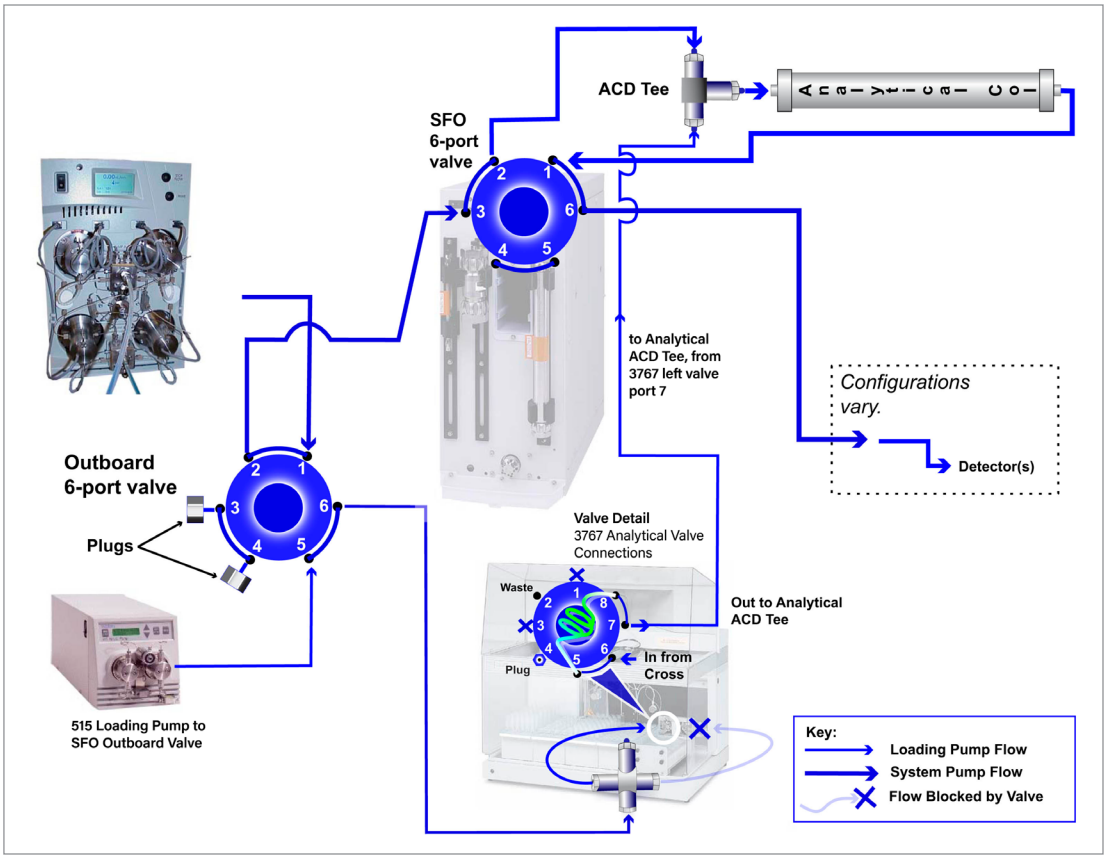

Figure 11. Analytical scale, At-Column Dilution valve position.

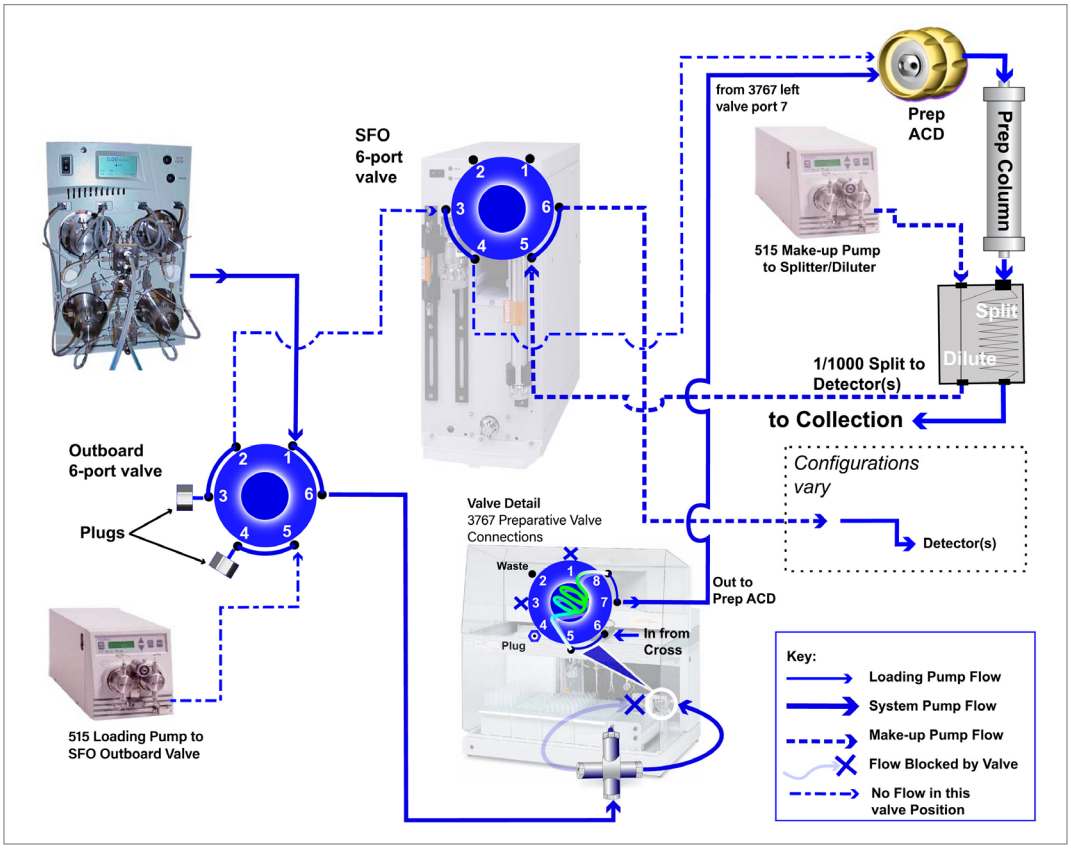

Figure 12. Preparative scale, conventional valve position.

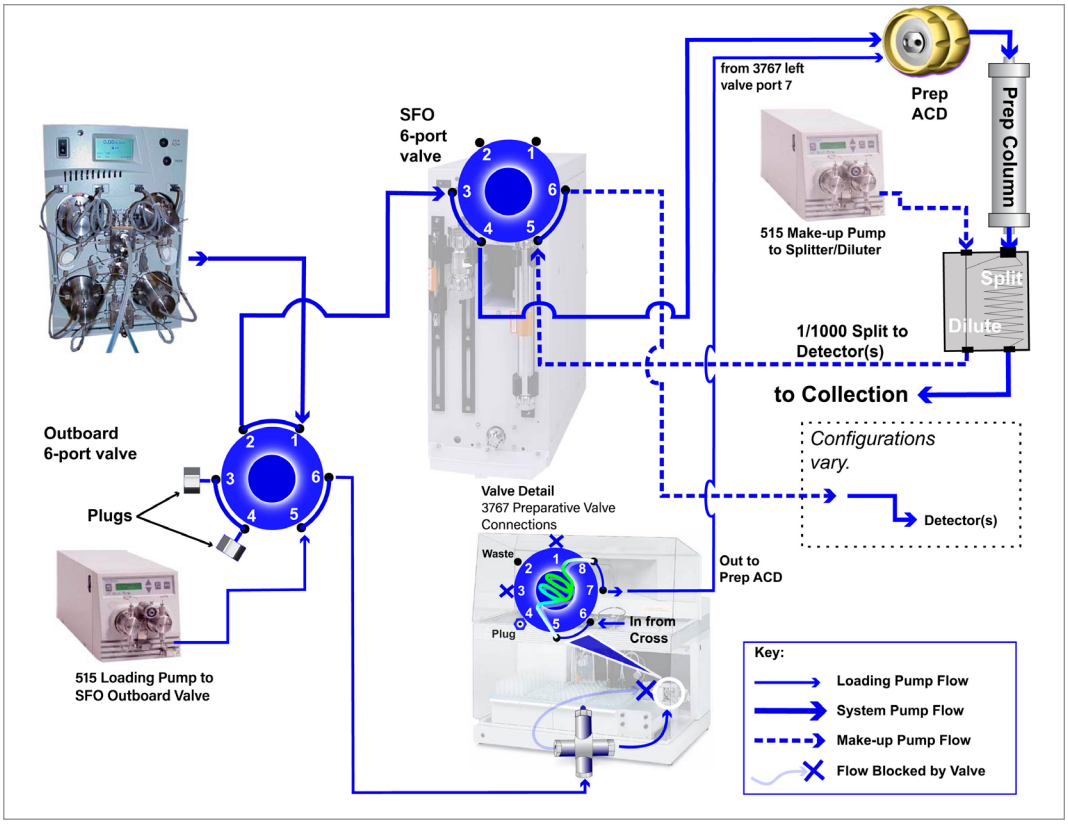

Figure 13. Preparative scale, At-Column Dilution valve position.

CONCLUSION

Sample loading onto a column can be highly compromised due to the solubility of the compounds or their compatibility with the initial mobile phase conditions, resulting in low yields and productivity. The white paper comprehensively illustrates at-column dilution, which can help improve mass capacity and chromatographic resolution by mitigating injection artifacts. It is particularly beneficial for preparative separation involving large sample volumes and strong solvents, making it an indispensable tool in the field.

The paper also shows ACD addresses precipitation issues in conventional liquid chromatography systems and increases system robustness and column life by preventing precipitation in the sample loop or column. It provides tips for selecting loading solvents to ensure sufficient retention of diverse sample types, such as polar and hydrophilic compounds.

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Waters™

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
waters.com