The use of a Reverse Phase (C18AQ) Flash Chromatography Cartridge in Sample Desalting

Cong Zhang[1], Bo Xu[1], Andre Couture[2]*
1: Santai Technologies Inc., 78 Qingyang Road, Xinbei District, Changzhou, Jiangsu Province, 213125, China
2: Santai Science Inc., 214 Brunswick, Pointe-Claire, Montréal, QC H9R 1A6, Canada
*Corresponding Author: andrecouture@santaitech.com

Reversed phase liquid chromatography (RPLC) is a widely used separation mechanism in liquid chromatography. RPLC has been used to separate numerous compounds, from organic acids with a low molecular weight (MW) to proteins with a MW of up to 150 kDa. However, analytes with good water solubility (i.e., hydrophilic or polar) exhibit poor retention on conventional C18 RP columns. In this case, the aqueous ratio in the mobile phase needs to be increased in order to enhance the interaction between the polar analyte and the hydrophobic stationary phase, thereby increasing the retention of polar analytes. The greater the polarity of the analyte, the higher the proportion of water required in the mobile phase. When regular alkyl bonded reversed phase columns e.g. octadecyldimethylsilane (ODS, C18) are used in pure aqueous or highly aqueous mobile phases for a long time, problems can occur including loss in retention time for the analyte and irreproducible separation results. This phenomenon is referred to phase collapse [1]. The classic explanation for this phenomenon is that the C18 stationary phase bonded to the surface of the silica gel changes the spatial arrangement of the carbon chain in highly aqueous mobile phase, that is, changes from perpendicular to the surface of the silica gel to lying flat on the same plane as the silica surface (as shown in *Figure 1*). The change in the spatial arrangement of the stationary phase reduces its interaction with the analyte, thus reducing the retention time of the analyte and often causing it to be unretained.

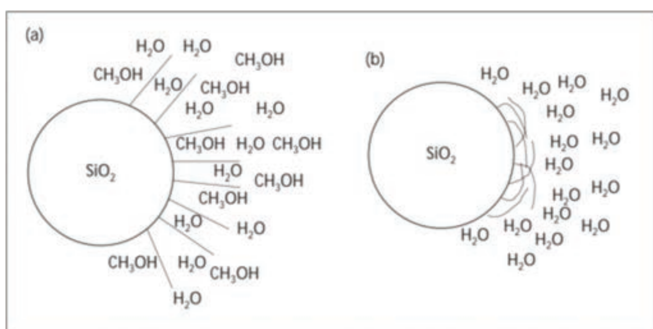

Figure 1. Illustration of the classic explanation of phase collapse in reversed phase chromatography (reproduced from Ref.1). Shown are the configurations of long-chain bonded alkyl phases (a) in water-methanol mixtures and (b) in 100% water.

Researchers have conducted in-depth studies of the phase collapse phenomenon. The continuous accumulation of experimental evidence has led many researchers to accept another new theoretical explanation, that is, the loss in retention time is actually due to the 'dewetting' which occurs in the fine pores of the stationary phase particles [2]. In the new theoretical explanation (*Figure 2*), a high surface tension is generated between the aqueous mobile phase and the surface of the hydrophobic stationary phase. Therefore, the mobile phase is easily expelled from the porous space of hydrophobic stationary phase. In this case, the mobile phase no longer remains in the porous space of the stationary phase particles, so the chance of the stationary phase and the analyte coming into contact with each other is also reduced, which in turn causes the reduced retention time of the analyte. An experiment confirmed that when the mobile phase in the C18 column was suddenly switched from a high proportion of organic solvent to 100% aqueous solvent, the volume of the mobile phase in the column was significantly reduced, and the magnitude of the decrease in such volume was closely related to the loss of retention time for the analyte [3, 4].

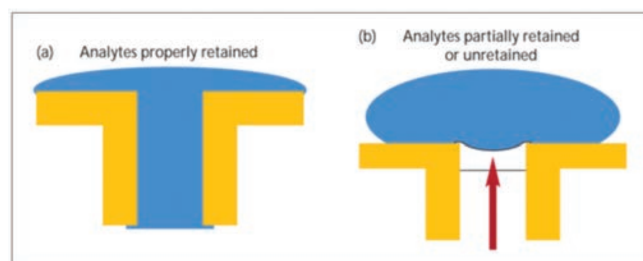

Figure 2. Illustration of a possible mechanism of pore dewetting for reversed phase chromatography (reproduced from Ref. 1). The analytes are properly retained when the alkyl chains on the stationary phase are properly solvated with pressure using a 100% aqueous mobile phase (a). When the flow has been stopped to allow expulsion of water from the pores, with flow resumed the pores are still dewetted and analytes cannot enter pores and have little or no retention (b).

According to the Young-Laplace equation, the surface of the dried hydrophobic stationary phase requires very high pressure to drive the aqueous phase solvent into the pores contained on the surface. This equation relates the intrusion pressure to the surface tension of the water and to the contact angle of the water and air in the sorbent surface:

$$\Delta P = \frac{4\gamma\cos\theta}{d}$$

where ΔP is the intrusion pressure required to drive liquid into the pores, γ is the surface tension, d is the effective pore diameter, and θ is the contact angle made between water and air on the adsorbent surface. Since the contact angle made between 100% aqueous mobile phase and hydrophobic alkyl-bonded silica-gel pore is greater than 90°, therefore, θ from the above mentioned equation is smaller than 90° and ΔP should be a positive number, which means positive pressure is required to drive the aqueous mobile phase into the silica-gel pore. In regular liquid chromatography separation, the pressure driving aqueous mobile phase into the silica-gel pore is larger than the pressure required to drive the mobile phase flowing through the whole chromatography column. So, when the separation is suddenly stopped, the pressure inside the silica-gel pore is greater than the pressure outside the silica-gel pore, as a result the aqueous mobile phase will be squeezed out from hydrophobic alkyl-bonded silica-gel pore. When this column is reused without special treatment, due to the dewetting status of the adsorbent pore, the interaction between the adsorbent and the analyte will be greatly reduced, resulting in a significant loss of retention time for the analyte. However, when the stationary phase surface becomes more hydrophilic, the contact angle made between 100% aqueous mobile phase and hydrophobic alkyl-bonded silica-gel pore will be reduced. When this contact angle is smaller than 90°, which means θ is greater than 90°, ΔP should be a negative number. Under this condition, the pressure outside the silica-gel pore is greater than the pressure inside the silica-gel pore and the mobile phase will spontaneously enter the silica-gel pore. This is when the adsorbent pore is wetted.

The above theoretical analysis shows that for a conventional C18 column, hydrophilic modifications can be performed to the silica gel surface to improve the wettability in highly aqueous mobile phase [5], including

- non-endcapped, short-chain alkyl phases;
- hydrophilic, polar-endcapped, and polar-enhanced stationary phases;
- polar-embedded alkyl phases;
- long-chain alkyl phases; and
- wide-pore-diameter phases.

Flash chromatography cartridges prepacked with a hydrophilic C18-bonded silica gel, in which hydrophilic cyano groups are introduced on the silica gel surface (as shown in *Figure 3*) have been prepared. The alkyl chains on the silica surface can be fully extended under highly aqueous conditions and the phase collapse/wettability phenomenon can be avoided. These modified C18 columns are called aqueous C18 columns, namely C18AQ columns (SepaFlash, Santai Technologies), are designed for highly aqueous elution conditions and can tolerate a 100% aqueous system. C18AQ columns have been widely applied in the separation and purification of highly polar compounds, including organic acids, peptides, nucleosides and water-soluble vitamins.

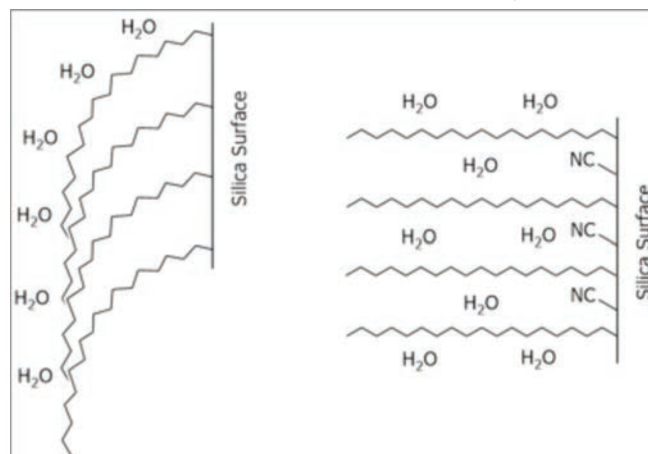

Figure 3. The schematic diagram of the bonded phases on the surface of silica gel in regular C18 column (left) and C18AQ column (right).

Desalting is a typical application of C18AQ columns in the Flash chromatography purification of samples, where the salt or buffer components in the sample solvent are removed to facilitate the use of the desalted sample in subsequent studies. In this application, a highly polar compound was used as the sample and purified on a C18AQ cartridge. The aqueous salty components contained in the raw sample were removed and the sample solvent replaced by organic solvent, thus facilitating the subsequent rotary evaporation saving both solvents and operating time.

Experimental Section

Sample information

The sample was the final product of a synthesis reaction which was shown in *Figure 4*.

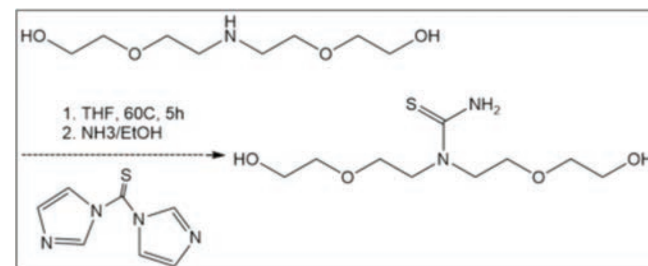

Figure 4. The synthesis reaction formula of the sample.

As shown in *Figure 4*, in addition to the target product in the raw sample, there is also excess ammonium salt (tetrabutylammonium fluoride, MW 261) used in the synthesis reaction. The sample should be desalted in order to obtain the target product meeting the purity requirement. In chromatographic separation technology, gel filtration based on the principle of molecular sieving is generally used to desalt the macromolecular sample, including proteins, peptides, nuclei acids, etc. However, for the target product with MW of 252 in this post, it is a nearly impossible task for gel filtration to distinguish the target molecule from salt impurities with very similar MW. Other separation modes must be considered.

Sample purification by Flash chromatography

The sample was purified by a Flash chromatography system (SepaBean machine 2) according to the parameters as shown in *Table 1*.

Table 1. The experimental setup for Flash purification.

Instrument	**SepaBean machine 2**	
Flash cartridge	12 g SepaFlash C18AQ cartridge (spherical silica, 20 - 45 µm, 100 Å, Order number: SW-5222-012-SP(AQ))	
Wavelength	254 nm; 220 nm	
Mobile phase	Solvent A: water Solvent B: methanol	
Flow rate	15 mL/min	
Sample load	0.5 mL (100 mg)	
Gradient	Time (CV)	Solvent B (%)
	0	0
	20	0
	21	100
	30	100

Results and Discussion

The sample used in this post was highly polar and soluble in water. Due to its high polarity and strong retention on normal phase silica cartridges normal phase separation was impractical and excluded. In a reversed phase separation mode, if a conventional C18 RP Flash cartridge was used, the sample will be eluted from the stationary phase quickly since the organic phase ratio was more than 10% in the initial mobile phase. Furthermore, there is still certain amount of salt impurities in the collected fractions, leading to a bad desalting result. Therefore, an C18AQ cartridge was utilised to purify the sample.

A step gradient was set for the Flash chromatography. To ensure that the salt impurities with a MW very close to the target product are fully removed, pure water was used as the mobile phase to flush the cartridge for about 20 column volumes (CV). As shown in *Figure 5*, the sample was fully retained on the C18AQ cartridge when pure water was used as the mobile phase. Next, methanol in the mobile phase was directly increased to 100% and the gradient was maintained for 10 CVs. The target product was eluted out from 22.5 to 24 CVs. In the collected fractions, the aqueous sample solution was replaced with methanol. The methanol was easily removed by rotary evaporation in the subsequent step, which facilitates following research on the purified sample.

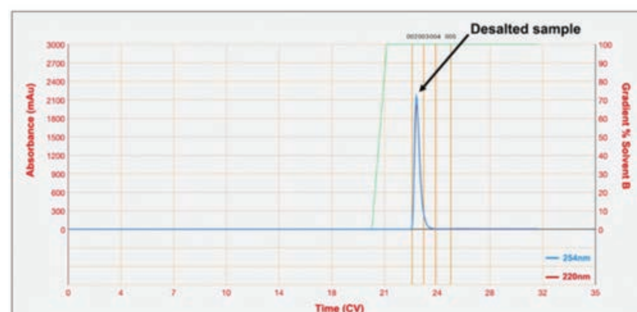

Figure 5. Flash chromatogram of the sample on a C18AQ cartridge.

Compared to a linear gradient, the use of step gradient has the following advantages:

- Solvent usage and run time for sample purification is reduced.
- The target product elutes in a sharp peak, which reduces the volume of collected fractions and thus facilitates the following rotary evaporation as well as saving time.
- The collected product is in methanol which is easily evaporated, thus drying time is reduced.

In conclusion, for the purification of a sample which is strongly polar or highly hydrophilic, a SepaFlash C18AQ cartridge combiined with a Flash chromatography system (SepaBean machine) is undoubtedly a fast and efficient solution.

References

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