Subcritical Solvent Regeneration of Contaminated HPLC Columns Used for Separating Biomolecules

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Abstract

The regeneration of contaminated chromatography columns using subcritical mobile phases is presented. The method employs minimal amounts of organic solvents (pressurized and heated above their boiling point) to clean strongly retained sample components from hydrophobic stationary phase particles. The process lasts approximately 1 h and is demonstrated to restore the performance of polymeric and bonded-phase columns. The technique is simple to apply and widely accessible, requiring only common laboratory equipment. Results indicate that this could be a beneficial alternative approach for cleaning contaminated columns either directly or in cases in which conventional methods have been unsuccessful.

Introduction

Reversed-phase (RP) high-performance liquid chromatography (HPLC) techniques employing polymeric resin or silica-based columns are widely used for separating biological compounds (1–6). Though proper maintenance normally protects column performance, it can still deteriorate over time because of certain strongly-retained sample materials depositing or accumulating on stationary phase particles. This is especially true for high-molecular-weight or very hydrophobic compounds (e.g., polypeptides, proteins, and lipids) (7). Such column fouling is often indicated through observations of increasing system backpressure, decreasing chromatographic resolution, distorted peak shapes, or shifts in retention time (or a combination of factors). When separations become unacceptable, often the most economical solution is to clean and regenerate the column, hopefully to its original performance level (6,7).

Generally, conventional column regeneration involves washing with a mobile phase of high solvent strength (6,7). This may be performed directly or as part of a cyclic solvent gradient that repeatedly transitions back and forth from polar to non-polar mobile phases. Typically, 5 to 10 cycles are needed, but they may be left to run overnight. Because neat organic solvents (e.g., acetonitrile and methanol) are often ineffective for cleaning proteins or peptides from RP columns, buffers or acids [such as aqueous trifluoroacetic acid (TFA)] are frequently added to help the process (7). When polymeric columns are used, manufacturers often recommend that a plug injection of strong acid (e.g., 1M nitric acid) be left inside of the column for several hours (7). Considering that a combination of the described procedures is also sometimes run, column regeneration can be a time-consuming process lasting several hours to days. Additional care must also be taken to ensure that solvents are miscible and that acids do not damage the chromatographic system. Unfortunately, even after these efforts, column performance is sometimes only partially recovered.

Recently, our laboratory extensively used a polymeric column for separating species of the hydrophobic polypeptide gramicidin by packed-column supercritical fluid chromatography and found that the column would become fouled after numerous runs over approximately six months (8,9). Although various conventional regeneration methods were repeatedly attempted at length, the column performance was never fully restored. In order to continue, a more practical and effective method of regenerating the column was needed. A potentially useful alternative column regeneration method that uses subcritical fluids (i.e., pressurized liquids above their boiling point) as solvents is presented. The method is fast and convenient to apply and requires only a simple lab apparatus and minimal solvent.

Experimental

Materials and reagents

Gramicidin D, benzene, toluene, and *m*-xylene were purchased from Sigma-Aldrich Chemical Company (Oakville, ON, Canada). All solvents were of analytical grade. Methanol and isopropanol were purchased from EMD Chemicals (Gibbstown, NJ). Acetonitrile was obtained from BDH (Toronto, ON, Canada). Both polymeric and silica-based fouled RP-HPLC columns were investigated. The first were two polymeric PRP-

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1 columns [poly(styrene-divinylbenzene) stationary phase], which were originally obtained from Hamilton Company (Reno, NV). The columns had respective sizes of 150 × 2.1-mm i.d. and 50 × 4.1-mm i.d. (both 5-µm particles). The other was a silica-based C18 bonded-phase Supelcosil LC-18 column (250 × 4.6-mm i.d., 5-µm particles) originally manufactured by Supelco (Bellefonte, PA), which was provided by another research laboratory on campus.

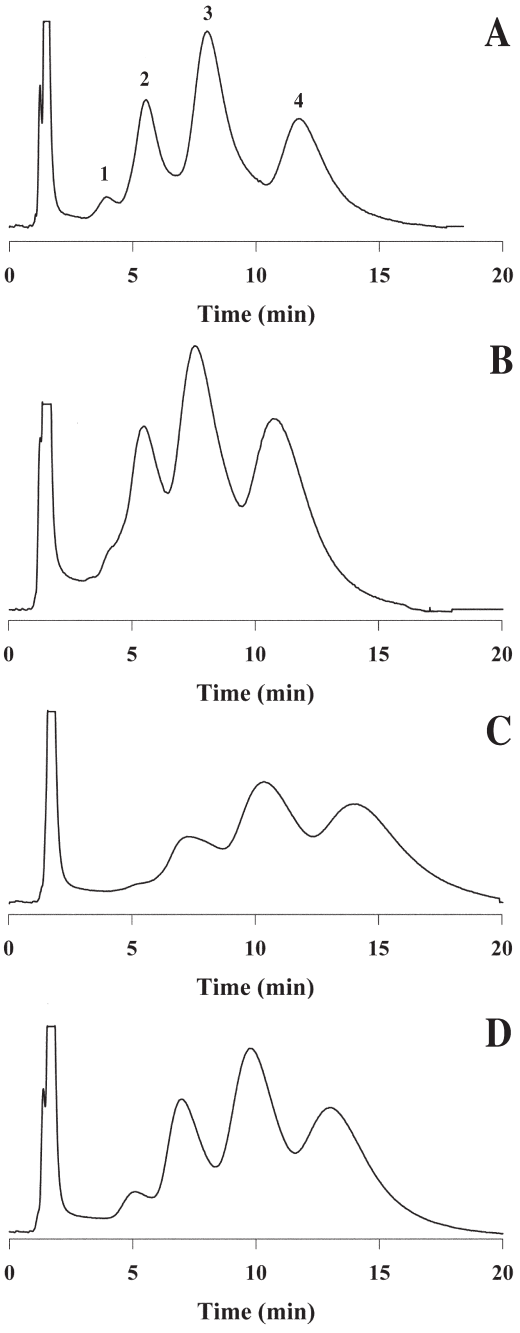

Figure 1. Regeneration of the 150- × 2.1-mm i.d. polymeric column at four different stages: original condition (A), fouled condition (B), After six 8-h treatments with strong acid (C), and after 1 h of flushing with subcritical isopropanol (D). Peaks 1 to 4 are the dimeric species of gramicidin equilibrated in butanol at 5 mg/mL. Analysis conditions: a mobile phase of 35% pentanol in CO_2 at 25 MPa, flow rate of 1.2 mL/min, UV detection at 220 nm, and column temperature of 40°C.

Apparatus

The regeneration system essentially consisted of a common pump, oven, and pressure regulator. Solvents were delivered through the system by an LKB (Bromma, Sweden) model 2150 HPLC pump. Approximately 40 cm of $\frac{1}{16}$ inch o.d. (254-µm i.d.) stainless steel tubing led from the pump and through the wall of a gas chromatography oven (Shimadzu Model GC-8A), which provided temperature control. The fouled column was connected to this tubing and housed inside of the oven. Approximately 20 cm of the same tubing extended from the column outlet and through the oven wall, where it was attached to a stainless steel zero dead volume union (Alltech, Deerfield, IL). A length of deactivated fused-silica capillary tubing (300 × 0.05-mm i.d.,) (Supelco) was connected to the other end of the union and functioned as a fixed restrictor, maintaining system pressure. Solvent exiting the restrictor was directed into an open flask. Note that care was taken to ensure the system was free of leaks and adequately ventilated during operation. This was essential to avoid any potential safety hazards concerning solvent vapor accumulation in the high-temperature oven. Also, system pressure was continuously monitored to ensure that plugging, which could potentially result in a large pressure increase, did not occur.

Procedure

Column regeneration was carried out by systematically increasing the temperature at a constant solvent flow rate through the column. The flow rate is mainly governed by the column size, solvent viscosity, and restrictor. For these experiments the flow rate was normally 0.2–0.3 mL/min for 2.1-mm i.d. columns and 0.3–0.4 mL/min for 4.6-mm i.d. columns. The oven temperature settings used for rinsing the column were: 25°C for 2 min, 50°C for 2 min, 75°C for 5 min, and finally 100°C for 30 min. After the rinse was complete, the oven temperature was decreased to 75°C, then to 50°C, and then to 25°C in 5-min intervals. Alternatively, an oven temperature program could also be used. In this case, the temperature was typically ramped at a rate of 8°C/min from room temperature to a final value of 100°C. After 30 min, the temperature was decreased back to ambient at a rate of 3°C/min. The total process lasted approximately 60 min, and pressure was normally observed between 150 and 250 bar. Column performance was evaluated using a Gilson series SF3 supercritical fluid chromatograph (Gilson, Villiers le Bel, France) equipped with a UV–vis wavelength absorbance detector (8,9). Operation conditions are specified in the text.

Results and Discussion

Figure 1 depicts the changes in chromatographic behavior observed for the four dimeric conformations of gramicidin with the 150- × 2.1-mm i.d. polymeric column. The original separation of the dimers is shown in Figure 1A. After more than six months of intensively separating gramicidin samples, the

column was fouled and the separation was degraded (Figure 1B). As seen, overall resolution, selectivity, and retention has decreased, and two dimers (peaks 1 and 2) now coelute, thereby impeding the analysis of each individual dimer. To correct the problem, initial attempts employed pure methanol, isopropanol, and acetonitrile as solvents under ambient conditions at 0.3 mL/min for 30 min each. However, this was not successful, and no change was subsequently observed in the chromatogram. Next, following manufacturer recommendations, the column was filled and sealed with a 1:1 mixture of 12N HCl and 0.1% TFA in acetonitrile for a minimum period of 8 h. Afterward, a 20 min gradient from 0% to 60% acetonitrile in water (each containing 0.1% TFA) was run through the column. Furthermore, this entire procedure was repeated six times, and the resulting chromatogram is displayed in Figure 1C. As shown, even after at least 48 h of total exposure to concentrated HCl, the dimer separation is only partially restored. For instance, resolution and retention are increased, but the peaks have broadened to the extent that peak 1 is barely detectable; thus, the column is still rendered impractical for use. In addition, no further improvements were realized by subsequent repetitions of this approach. As such, one trial was finally carried out employing subcritical isopropanol as the solvent using the proposed method. As seen in Figure 1D, this resulted in an improved separation of the four peaks and a chromatographic profile that more closely reflects that of the original column, to the extent that it can now be used again for the analysis of the individual dimers. The same results were also obtained for the 50- × 4.1-mm i.d. fouled polymeric column.

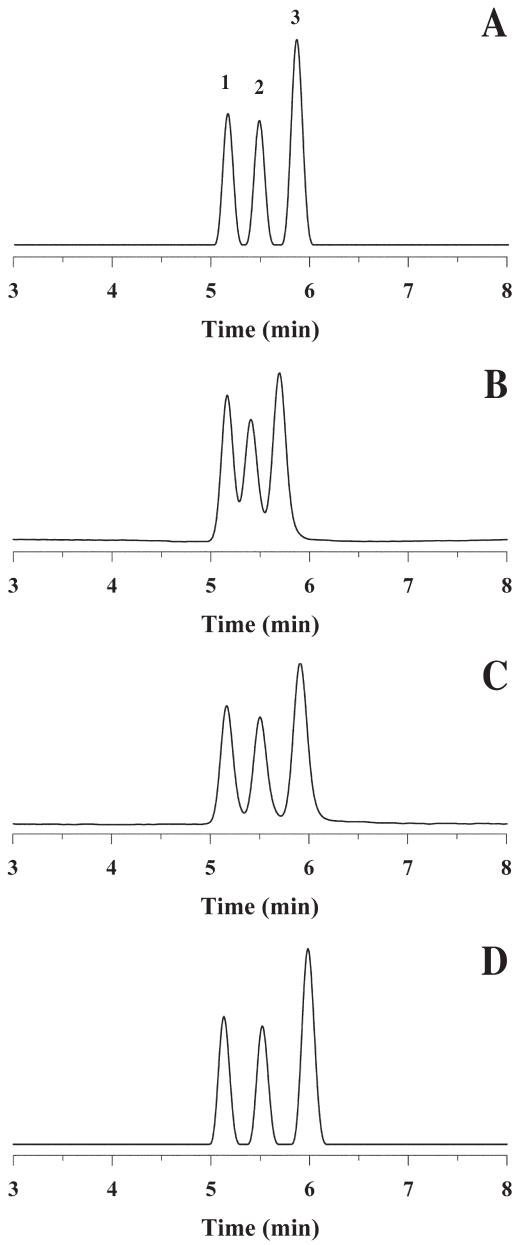

Figure 2. Regeneration of the bonded-phase C18 column at four different treated stages: a clean, unfouled column (A), fouled column (B), after treatment with subcritical isopropanol (C), and after treatment with subcritical isopropanol–acetonitrile (1:1) (D). The test sample order of elution is benzene, toluene, and *m*-xylene (100 ppm each in methanol). Analysis conditions: a CO_2 mobile phase at 8 MPa, a flow rate of 0.6 mL/min, UV detection at 200 nm, and a column temperature of 40°C.

Next, in order to test this method directly and accurately quantitate column performance changes at each stage, separations on the bonded-phase C18 column were investigated. This column had been used to separate various membrane proteins and other polypeptides in a routine biochemical laboratory over several years until its performance significantly deteriorated and was discarded. Because much of the contamination source was unknown, first methanol, isopropanol, and acetonitrile were each run through the column in the forward and reverse directions at 0.4 mL/min for 30 min with the detector disconnected. This cautionary measure revealed any potential flow impediments and helped expel any particulate matter to prevent system plugging. In this case, no major problems were observed. A methanol solution of three test probes, benzene, toluene, and *m*-xylene (100 ppm each), was then used to evaluate column performance (6). To facilitate this process, a clean column of the exact same type was also evaluated for comparison. Figure 2 illustrates the changes observed, and Table I quantitates column performance parameters measured at each stage. Figure 2A presents the test separation performed on the unfouled column, which displays the three analytes eluting with baseline resolution. In contrast to this, the same separation on the fouled column (Figure 2B) illustrated that retention, selectivity, and plate numbers were reduced, and resolution was eroded. No effect on peak asymmetry was observed. After the fouled column was flushed using one trial of subcritical isopropanol (Figure 2C), the column performance was nearly re-established. Finally, after a second trial using a 1:1 mixture of subcritical isopropanol–acetonitrile (Figure 2D), it could be seen that the separation had recovered and that the selectivity, resolution, and plate number for each analyte had been regenerated. Therefore, 1 to 2 direct subcritical solvent treatments adequately restored column performance.

As might be anticipated (10), in our experience, subcritical isopropanol is often a useful choice for removing more hydrophobic contaminants, whereas the addition of acetonitrile

Table I. Column Performance Parameters for Each Peak in Figure 2 (A–D)

Figure	Peak	Asymmetry factor	Selectivity	Plate number	Resolution
2A	1	1		10609	
			1.06		1.6
	2	1		11968	
			1.07		1.9
	3	1		13689	
2B	1	1		4715	
			1.05		0.83
	2	1		5184	
			1.06		1.0
	3	1		5776	
2C	1	1		6790	
			1.07		1.4
	2	1		7744	
			1.07		1.6
	3	1		8911	
2D	1	1		10568	
			1.07		1.9
	2	1		12188	
			1.08		2.3
	3	1		14304	

can facilitate the removal of more polar residues. Though these and other solvents are normally ineffective at removing peptides and proteins from RP columns under ambient conditions (7), it is likely that interactions between the stationary phase, mobile phase, and contaminants differ considerably as the higher temperature critical region is approached (11). For example, under high temperature conditions, protein denaturation could break or weaken any binding forces experienced with the stationary phase and lead to increased protein solubilization in the mobile phase. Furthermore, such large biomolecules may also decompose into smaller, more soluble fragments that are more readily stripped from the column by the subcritical solvent, similar to approaches involving strong acid or base injections.

In this regard, further clarification of the term "subcritical" is warranted. Formally, a subcritical fluid can be considered one that is held at an elevated pressure and temperature but still below its critical temperature (12). Isopropanol has a critical pressure of 47 bar and a critical temperature of 235°C, though a 1:1 isopropanol–acetonitrile solvent has respective values of 56 bar and 252°C. As such, for the conditions employed currently (i.e., 150–250 bar, 100°C), these fluids are described as "subcritical". Still, such designations are merely formal because the high temperature nature of the fluid is the important experimental aspect (e.g., "supercritical" isopropanol would likely also hold similar properties).

Finally, it should be mentioned that manufacturers often recommend column operation at temperatures lower than 100°C. This warrants consideration when adapting the current method for use in different applications. Admittedly, though, this is less of a concern if other regeneration methods have failed and the column has already been slated for disposal. However, many columns have also been safely operated above the recommended limit in higher temperature sub- and supercritical fluid applications (13–15). Still, an important exception to note in this regard is that high temperature water should never be used for silica-based columns, as it will erode the stationary phase (15).

Conclusion

The regeneration of fouled chromatographic columns can be achieved by using subcritical solvents as mobile phases. The approach appears to be an efficient alternative to conventional methods using normal liquids for removing proteins, peptides, and other large biomolecules from stationary phases. The technique employs a simple apparatus found in many labs. It is easy and practical to apply, thus making it widely accessible. The process is quick to optimize (for known and unknown contaminants) and requires only approximately 60 min to complete rather than several hours to days for more complicated conventional procedures. This, in turn, may also reduce the amount of hazardous organic solvent and strong acid that is typically consumed for this purpose. The regenerated columns perform normally, often yielding several months of regular usage before they begin to foul again. Thus, subcritical solvent regeneration might be beneficial to employ as a first (or last) resort for restoring chromatographic column performance.

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