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Note

Regeneration of protein-contaminated C_4 columns by dimethyl sulfoxide–acetonitrile treatment

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The strong association of proteins with hydrophobic packings is a widely acknowledged phenomenon[1], and the seemingly irreversible accumulation of protein manifests itself as a change in column performance (*i.e.* shifts in k' values and changes in peak shapes are often observed). The current use of shorter brush columns for protein separations is an attempt to deal with the undesirable protein surface interactions by using a bonded phase that is "less hydrophobic" in nature than that of the more traditional octadecyl packing.

In any separation where the brush may be contaminated by solutes accumulated during analysis, it is desirable to regenerate the bonded phase if possible. Methods reported range from general cleaning instructions (with no guarantee of success)[2,3] to the use of tetrahydrofuran or dichloromethane to remove oil-containing solutes[4], and techniques such as flow reversal for regeration of columns used to separate dabsyl amino acids[5].

In this report we describe the successful regeneration of C_4 packing materials using dimethyl sulfoxide (DMSO) and acetonitrile. The surface cleanliness was monitored using a set of well characterized solutes, the alkyl aryl ketones. These solutes separate on hydrophobic surfaces according to carbon number[6,7]. The regeneration of unpacked C_4 stationary phase and an *in situ* regeneration of C_4 stationary phases is described.

EXPERIMENTAL

All separations were performed on a Beckman HPLC system (Model 345/165 Beckman System), in conjunction with a Spectra-Physics Model 4500A recording integrator. All solvents were HPLC grade.

Two 25 × 0.4 cm stainless-steel columns were packed using a Haskel pump with head pressure of 8000 p.s.i. with either Alltech C_4 macroporous stationary phase (P/N 9912), or C_4 stationary phase prepared by the chlorination–Grignard method previously described[7] using 10-μm, 60 Å Partisil (Whatman; P/N 4116-025).

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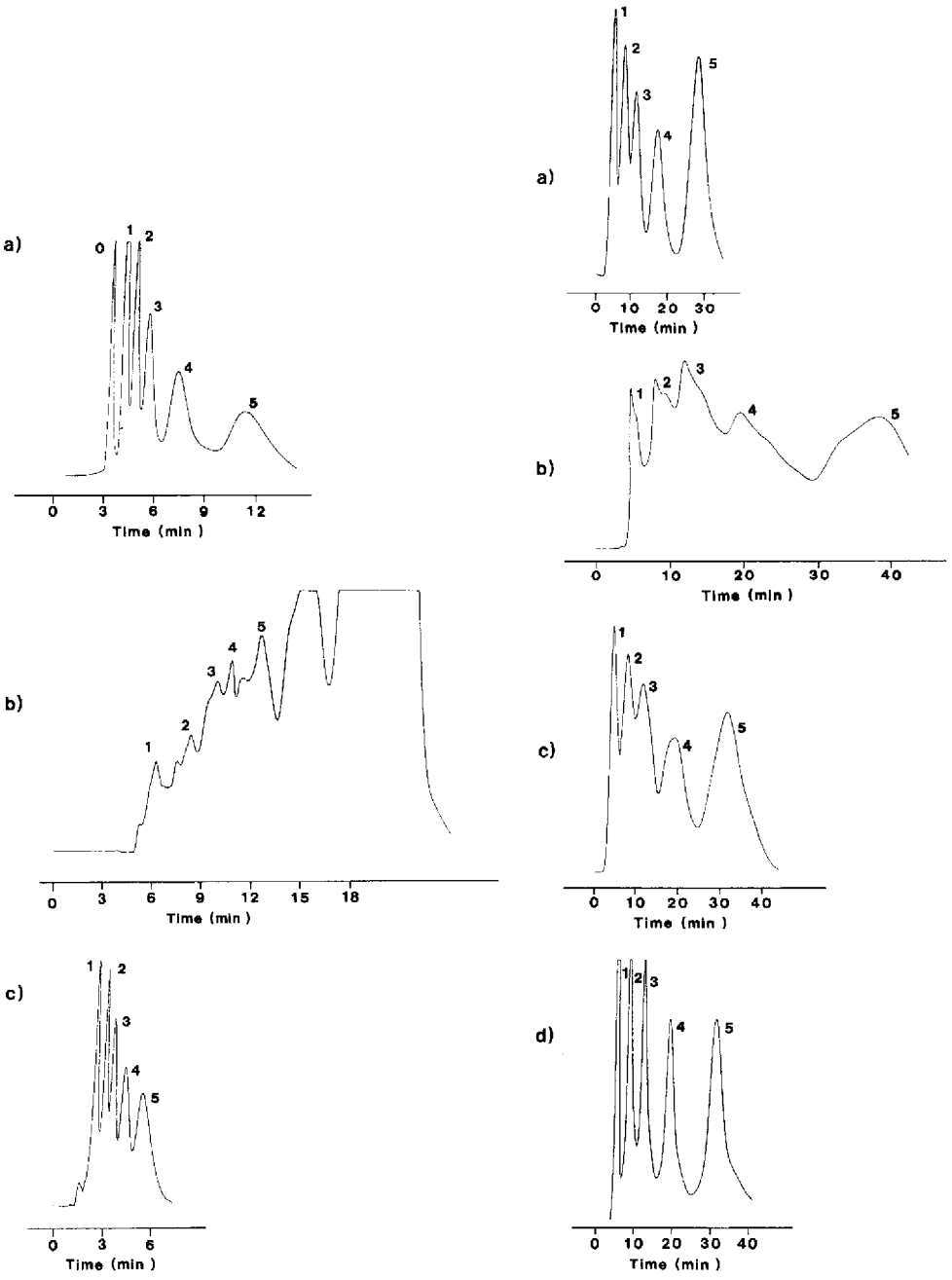

Fig. 1. Time course sequence of C_4 packing (chlorination–Grignard C_4 phase). (a) Initial alkyl aryl ketone profile, (b) profile after *ca.* 12 injections of protein (10 μg/injection), (c) alkyl aryl ketone profile after DMSO treatment, repacked into 15-cm column.

Fig. 2. Time course sequence of C_4 packing (commercial material). (a) Initial alkyl aryl ketone profile, (b) profile after *ca.* 6 injections of protein (10 μg/injection), (c) profile after regeneration of phase *in situ* (Method 2), (d) profile after protein analysis using DMSO in organic phase.

The source and preparation of the alkyl aryl ketones standards also has been previously described[7]. All separations of alkyl aryl ketones were done under isocratic conditions using acetonitrile–water (35:65) with a flow-rate of 1.0 ml/min. Elutants were detected using absorbance at 254 nm.

A standard series of proteins was used consisting of hemoglobin (Sigma P/N H 2625), whale myoglobin (Sigma P/N M 0380), ribonuclease A (Serva P/N 34388), bovine serum albumin (Sigma P/N A 7517), chymotrypsinogen (Sigma P/N C 4879), lysozyme (P-L Biochemicals P/N 27-0267-01), and β-lactoglobulin (Sigma P/N A 7517). Proteins were analyzed with an acetonitrile–water gradient with a flow-rate of 0.5 ml/min. Solvent A was water containing 0.1% trifluoroacetic acid (TFA) and solvent B was acetonitrile also containing 0.1% TFA. A linear ramp from 10% to 90% B in 20 min was used, followed by a 30-min re-equilibration to starting conditions. Typically, 20 μl of 0.5 mg/ml protein solution (10 μg) was injected.

The regeneration of the column packing materials was done by two methods after the elution profile of the alkyl aryl ketones was significantly altered.

Method 1. The C_4 support material was unpacked and washed via suction filtration on a Buchner funnel with 200 ml of DMSO. The material was then washed with 100 ml of methanol and dried at 100°C. Due to the removal of material for other analyses, the treated packing was repacked into a 15 × 0.4 cm column for subsequent chromatographic characterization.

Method 2. The C_4 support material was cycled from 12% to 80% DMSO in a 20-min period then back to 12% DMSO in 20 min. This cycle was repeated three times. Next a cycle from 15% to 75% acetonitrile in 15 min returning to 15% acetonitrile in 15 min was done twice or until no peaks were observed to elute.

RESULTS AND DISCUSSION

The time-course sequence of a column regenerated by Method 1 is depicted in Fig. 1a–c. The packing material was the C_4 phase bonded via a Si–C linkage on 10 μm, 60 Å silica. Restoration (Fig. 1c) of the separation of the alkyl aryl ketones to the original quality (Fig. 1a) is clearly evident after the separation was significantly altered by protein analysis (Fig. 1b). It should be noted that this material also was regenerated successfully by Method 2.

Similarly, the time-course sequence of a column regenerated *in situ* by Method 2 is shown in Fig. 2. The C_4 phase in this case was a commercial packing. Fig. 2a shows a typical alkyl aryl ketone elution profile prior to using the column for protein separations, and is very similar to that of the regenerated column (Fig. 2c). This is in marked contrast to the elution profile after several protein analyses (Fig. 2b). Fig. 2d is the elution profile of alkyl aryl ketone after protein analysis was done as described in the Experimental section, except that solvent B was composed of acetonitrile–DMSO (80:20) containing 0.1% TFA. Thus, column regeneration was not required when DMSO was used in a ternary solvent system for protein separations. A similar effect was not observed when methanol or propanol were used as the primary organic modifier.

In summary, we have found methods for the regeneration of C_4 reversed-phase columns which have been contaminated with residual protein. Furthermore, the addition of DMSO to the acetonitrile phase during the gradient elution of proteins

appears to prevent the bonded phase from becoming contaminated with protein solutes.

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