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REVIEW

# SAMPLE PREPARATION AND COLUMN REGENERATION IN BIOPOLYMER SEPARATIONS

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## 1. INTRODUCTION

Recent advances in the design of high-performance packings and elution conditions for chromatography of biological macromolecules have enabled dramatic increases in speed, resolution and recovery in biopolymer isolation and analysis. However, the complex nature of most biological samples necessitates preliminary sample manipulation to achieve optimal separations and acceptable column lifetime. Moreover, the requirement for recovery of biopolymers with structural and functional integrity often demands that sample pretreatment be rapid and gentle. Because of these constraints and the highly interactive nature of most biopoly-

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mers, many samples will contain components which adversely affect column performance in terms of run-to-run reproducibility or overall longevity. This review describes techniques for preparation of samples prior to chromatography and procedures for regeneration of high-performance liquid chromatography (HPLC) columns whose performance has degenerated to unacceptable levels. The latter topic will focus on the wide-pore silica- and gel-based columns currently used for chromatography of biopolymers.

## 2. SAMPLE PREPARATION

Sample preparation steps in chromatography of biological macromolecules can have several objectives. These include:

(a) removal of unwanted protein or non-protein material which would otherwise be inadequately resolved from the analytes, or which would damage the column;

(b) reduction of the complexity of multicomponent mixtures by prefractionation into analyte subsets, each of which can be sufficiently resolved chromatographically;

(c) removal of low-molecular-mass material (e.g. salts, detergents) which would affect chromatographic resolution or reproducibility;

(d) solubilization of sample components to enable injection under initial chromatographic conditions;

(e) alteration of the physicochemical state of the analyte to achieve desired chromatographic behavior (e.g. addition of ion-pairing, chelating or complexing agents) or to maintain analyte stability;

(f) concentration of sample to provide optimal chromatographic resolution or detection;

(g) dilution of sample to reduce solvent strength or to avoid solvent incompatibility;

(h) removal of particulate material to avoid blockage of HPLC injector, transfer lines, column frit matrix or packing bed.

A variety of techniques and devices are employed to achieve these goals. Most of these are adaptations of procedures developed for traditional purification schemes using low-pressure supports. Others use new concepts and materials developed specifically for HPLC of macromolecules or borrowed from applications of HPLC to analysis of small molecules. HPLC steps are increasingly being incorporated into multistep purification schemes for isolation of macromolecules from complex biological matrices, and here the task of sample preparation is to facilitate introduction of fractions from previous (often non-chromatographic) separations onto the HPLC column. This section will provide an overview of procedures and materials currently available for preparation of samples in the purification or analysis of biological macromolecules.

### *2.1. Prefractionation*

Prefractionation of multicomponent mixtures either to effect class separations of analytes or to remove unwanted protein can be accomplished by solid-phase

extraction or by liquid–liquid extraction. Solid-phase extraction has typically been performed off-line using small cartridges packed with HPLC silica to which an appropriate stationary phase has been bonded. Several manufacturers market products of this type, usually containing hydrophobic, ion-exchange or polar phases coated or covalently bonded to 20–50 $\mu$m porous silica and packed in cartridges which can be attached to a syringe or vacuum manifold [1].

Reversed-phase cartridges containing hydrocarbonaceous phases bonded to silica have found use in fractionation of crude extracts to remove bulk hydrophobic protein in the purification of hydrophilic proteins or peptides. Typical applications involve introduction of the sample in an aqueous buffer followed by washing with an acidic medium (e.g. 0.05–0.1% aqueous trifluoroacetic acid (TFA) to remove salts and low-molecular-mass contaminants. Analytes are then eluted with an aqua–organic mixture of suitable composition (typically a mixture of water and organic solvent such as acetonitrile or propanol containing TFA). Large proteins and hydrophobic peptides remain bound to the stationary phase, eliminating any risk of interference or damage to columns in subsequent HPLC separations. It has been observed [2, 3] that the composition of the solvent in which the sample is introduced to the cartridge can profoundly affect analyte recovery. For example, extraction of proteins from tissues using guanidine hydrochloride as a solubilizing agent can result in poor recovery from octadecyl cartridges, presumably because this chaotropic agent causes at least partial protein denaturation and exposure of hydrophobic surfaces which bind strongly to the cartridge packing.

Bennett [4] and James and Bennett [5] have described the use of Sep-Pak cartridges (Waters Assoc., Milford, MA, U.S.A.) for extraction and class separation in the purification of pituitary peptides from biological tissues. Acid extracts of endocrine tissue were passed through octadecyl reversed-phase cartridges to remove bulk hydrophobic protein, then fractionated into acidic, basic and neutral peptide pools by passage through tandem ion-exchange cartridges. Individual peptide pools could then be resolved by reversed-phase HPLC without the problems of column overload encountered in chromatography of the unfractionated crude acid extract. Fractionation was accomplished by adjusting the acid extract to an appropriate pH, followed by passage through coupled weak cation-exchange (carboxymethyl) and strong cation-exchange (quaternary methyl ammonium) cartridges. Neutral peptides were eluted from the coupled cartridges with low-ionic-strength buffer. The cartridges were then disconnected and eluted separately with high salt to recover the basic and acidic peptides from the cation- and anion-exchange cartridges, respectively. The results of this study illustrate the importance of selecting the proper phase chemistry and eluent conditions for optimal fractionation. For small peptides, retention is governed by their net charge, and a modest change in eluent pH can result in recovery of components in different fractions. These effects can provide powerful tools for handling coelution problems in subsequent chromatography of the peptide fractions. Similarly, the nature of the cartridge phase can affect fractionation selectivity. For example, all acidic peptides were found to bind to the strong anion-exchange cartridge, but

weakly basic peptides failed to bind to the carboxymethyl phase at pH 7 and eluted instead in the neutral fraction.

In addition to advantages in ease of use and good recovery, the use of solid-phase cartridges enables rapid prefractionation of peptides or intermediates in peptide metabolism which might be lost or degraded in prolonged isolation procedures.

Kratzin et al. [6] used octadecyl Sep-Pak cartridges for prefractionation of chymotryptic digests of the $\alpha$ subunit of human class II histocompatibility antigen, an integral membrane glycoprotein. Following reductive alkylation and chymotryptic cleavage, digests were loaded onto cartridges and eluted in a stepwise fashion with increasing concentrations of propanol in 60% formic acid. Hydrophilic peptides eluted at low propanol concentrations, while concentrations up to 80% were required to elute an intramembranous peptide.

Pearson et al. [7] described a novel approach to recovery of proteins resolved by two-dimensional gel electrophoresis using short reversed-phase cartridges. Resolved protein spots were excised and placed directly into a precolumn chamber connected in series with a $2 \times 20$ mm cartridge containing wide-pore $C_3$ reversed-phase packing. Proteins were eluted from the gel matrix with 0.1% TFA and adsorbed onto the reversed-phase column, while gel contaminants, buffer components and stain eluted in the void volume. Proteins were then eluted with a TFA–acetonitrile gradient. This technique has an advantage over electroblotting, in which small proteins are lost by diffusion through the immobilization matrix.

There are several guidelines which are critical for the successful use of solid-phase sample preparation cartridges [8, 9]. First, the cartridge must be preconditioned prior to use to solvate the sorbent. This insures that the sorbent will interact reproducibly with the sample, and this step is particularly important with non-polar phases. Preconditioning is accomplished by passage of several bed volumes of a preconditioning solvent (typical bed volume for a 40-$\mu$m particle diameter, 6-nm pore silica sorbent is 120 $\mu$l per 100 mg). The preconditioning solvent should wet both the polar silica matrix and the bonded phase, and should be miscible with the wash solvent used subsequently; organic solvents such as methanol or acetonitrile are typical choices. Following preconditioning, the cartridge is washed with an appropriate solvent to prepare the bed for sample introduction. Sample capacity is a second consideration in the use of solid-phase cartridges. This will vary with the type of sorbent, the elution solvent, the affinity of analyte and contaminants for the sorbent and the relative concentrations of analyte and interferences. It should be noted that the silica most often used in generic sample preparation cartridges contains small (6–10 nm) pores. Proteins will generally be excluded from these materials, and their capacity for most proteins will be low. For fractionation of high-molecular-mass analytes, sorbents with large pore matrices (30 nm or greater) should be used. The third consideration in the application of these products to purification of biopolymers is the choice of elution solvent. It is well known from studies in reversed-phase chromatography [10] that protein retention on hydrophobic phases is extremely sensitive to small variations in the organic solvent composition of the eluent. This

suggests that it should usually be possible to determine elution conditions in which the analyte is recovered with good yield and unwanted protein is retained. Moreover, the potential exists for selective extraction of different proteins or protein classes by discrete changes in the organic modifier content of the eluting solvent. The fourth consideration in the use of sample preparation cartridges is the flow-rate during sample introduction. Flow-rates of less than 10 ml/min are recommended for good retention when using cartridges for low-molecular-mass analytes, and because of the slow diffusion rates of macromolecules, this would certainly constitute an upper limit when using sample preparation cartridges for fractionation of biopolymers. It should be noted that rapid manual passage of solvent through a cartridge/syringe system can achieve flow-rates of 200 ml/min [9].

Recently, several products have been introduced for automated sample preparation which employ bonded-phase sorbent technology. These devices contain sorbents packed in cartridges compatible with high-pressure operation, allowing wash solvents to be introduced by multiport switching valves followed by introduction of the HPLC mobile phase as the eluting solvent. They permit multistep wash and elution schemes to be performed automatically as part of an HPLC analysis, but can accommodate one or at the most a few samples to be processed [11, 12]. An alternative approach employs multiple-sample cartridges combined in a cassette which is processed off-line on a vacuum manifold [13, 14]. Cassettes containing up to 100 samples are then injected into the HPLC by an automatic sample processor which has the capability of delivering a final wash solvent prior to injection. In all of these devices, selection of the bonded-phase sorbent for sample preparation will be conceptually different from the approach used with manual off-line cartridges. In this case, elution is performed using the initial (weak) solvent in the HPLC gradient program. Therefore quantitative elution of the analyte from the in-line cartridge by the HPLC mobile phase requires that the cartridge packing be much less retentive than that of the HPLC column. For example, an HPLC analysis using an octadecyl reversed-phase column might require an octyl or trimethyl phase in the sample preparation cartridge. To date the application of these devices to automated pretreatment in purification or analysis of biopolymers has not been reported. However, they offer promise in settings where automated processing of biological materials will be important, for example automated peptide mapping in the quality control of genetically engineered pharmaceuticals.

The development of high-efficiency HPLC columns capable of effecting rapid high-resolution separations of biological samples has in many cases blurred the distinction between sample preparation steps and segments in a multistep purification scheme. Analytical HPLC columns have thus been used in some cases for rapid prefractionation of samples. For example, commercial cation- and anion-exchange HPLC columns have been used in place of sample pretreatment cartridges in the fractionation of pituitary peptides described above [5]. High-performance gel permeation columns have been used for prefractionation of cyanogen bromide fragments by size prior to HPLC separation by cation-exchange or reversed-phase chromatography [15].

Silica-based sorbents have also been used for batch extraction of polypeptides from crude mixtures. Wolfe et al. [16] have described the use of sulfopropyl silica for isolation of human immune interferon and octyl silica for isolation of human interleukin-2 from conditioned culture medium. The sorbent was added to the medium and proteins were allowed to adsorb with gentle stirring for several hours. Following adsorption, the sorbent was allowed to settle and the supernatant decanted and discarded. In the case of interferon, the sorbent was resuspended in a salt solution which was barely strong enough to elute protein; this step removed unwanted protein but allowed interferon to rebind. Analytes were eluted from the sorbents either by batch extraction with a small volume of eluent or by transfer of the sorbent to a column followed by gradient elution. The latter technique was used when recovery of peptide in a small volume was desired. This extraction procedure was gentle enough to yield biologically active peptides with recoveries ranging from 86 to 100%, with volume reductions greater than 100-fold. The technique is inexpensive and can be used with crude mixtures of high viscosity and high protein content which would be difficult to process by conventional column chromatography. A similar procedure using trimethylsilyl (TMS)-controlled pore glass was employed by Henderson et al. [17] for batch extraction of gibbon interleukin-2 from conditioned medium and applied to extraction of murine B cell stimulatory factor from culture supernatants by Ohara et al. [18]. Under the binding conditions used (culture medium acidified with TFA and incubatd with stirring in the cold), the sorbent preferentially bound proteins of relative molecular mass ($M_r$) below 40 000 and quantitatively bound proteins below 20 000. Unwanted protein was removed by serial batch elution with salt–TFA solutions containing increasing amounts of acetonitrile. The final eluate contained the protein of interest at high yield and could be easily introduced onto a reversed-phase HPLC column following dilution.

In some instances liquid extraction can be used for prefractionation of protein mixtures prior to chromatography. Power et al. [19] have used this approach to fractionate the components of yeast cytochrome C oxidase. This oligomeric membrane protein was isolated as a seven-subunit complex from yeast mitochondria. The holoenzyme consists of three hydrophobic high-molecular-mass (29 500–54 000) proteins and four smaller (4500–14 500) proteins. Reversed-phase chromatography of the dissociated subunits resulted in irreversible adsorption of the three hydrophobic proteins on the stationary phase. This problem was circumvented by a preliminary two-step extraction to selectively remove the four low-molecular-mass proteins from the hydrophobic subunits. In the first extraction with 40% acetonitrile, two of the four were preferentially released. In a second extraction with 50% aqueous acetonitrile–isopropanol (1:1) the remaining two proteins were extracted from the residual hydrophobic species.

### *2.2. Solubilization*

HPLC is frequently applied to purification and analysis of very hydrophobic proteins such as cellular membrane proteins, viral envelope proteins and hydrophobic peptide fragments generated in protein structural analysis. Initial solu-

bilization is the key step to successful chromatography of hydrophobic proteins. In their native state, membrane proteins are imbedded partially or completely in the membrane matrix and exhibit strong protein–protein and protein–lipid interactions. Disruption of the membrane structure and release of proteins into aqueous media result in protein aggregation and precipitation. In order to purify individual membrane proteins, solubilizing agents such as organic solvents, chaotropic agents and detergents are required to displace lipid and maintain the protein in monomeric form. Solubilization techniques for membrane proteins have been reviewed by Hjelmeland and Chrambach [20] and discussed with special reference to HPLC by Goheen [21].

Detergents are the most common agents for protein solubilization; their effectiveness depends on the formation of mixed protein–detergent micelles. A wide variety of ionic, non-ionic and zwitterionic detergents has been used successfully. The selection of the proper detergent depends on several criteria including the mode of chromatogaphy to be used, the detection wavelength and the need to recover proteins in a biologically active or undenatured state. In cases where the detergent must be added to the mobile phase to maintain protein solubility during chromatography (e.g. size exclusion or ion exchange), detergent–stationary phase compatibility may dictate the choice of detergent. The most frequently used detergents are sodium dodecylsulfate (SDS), Triton X-100, octyl glucoside and 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS). The anionic detergent SDS binds strongly to proteins and is one of the most effective solubilizing agents. It has several limitations, however. It tends to irreversibly denature protein, and can be adsorbed onto hydrophobic stationary phases or anion-exchange phases, leading to permanent alteration of column selectivity or capacity. Also, the critical micelle concentration (cmc) of SDS varies with ionic strength. Octyl glucoside and Triton X-100 are non-ionic detergents which have been widely used for solubilization. In cases where recovery of biological activity is desired, Triton X-100 is particularly useful since it is a gentle detergent and less likely to cause denaturation. Application of Triton X-100 in HPLC protein purification is limited by its strong absorbance at 280 nm. To circumvent this problem Tiller et al. [22] have used hydrogenated Triton X-100. The reduced form exhibits lower UV absorbance but retains the detergent properties of the aromatic compound. Welling and coworkers [23–26] have used Triton X-100 extensively in the purification of envelope proteins of paramyxoviruses and report the selective extraction of viral proteins under varying salt concentrations. CHAPS is a zwitterionic detergent also known for its gentleness, and is often used when solubilization without denaturation is desired. In comparisons of the common detergents used for solubilization prior to HPLC, Matson and Goheen [27] and Lüdi and Hasselbach [28] observed that SDS was most effective in solubilization of membrane proteins.

Salts and chaotropic agents are also used as solubilizing agents. Neutral salts such as sodium chloride are effective in dissociating peripheral proteins loosely bound with the membrane surface, and salt extraction can be used to isolate these species or to selectively remove them prior to detergent solubilization of integral membrane proteins. Chaotropic reagents such as urea and guanidine hydrochlo-

ride are stronger extractants capable of maintaining integral membrane proteins in monomeric form. They must also be added to the HPLC mobile phase to prevent protein aggregation or irreversible binding to the stationary phase. Care must be taken in the use of salts and chaotropic agents in reversed-phase chromatography to prevent precipitation in the presence of organic modifiers.

Strong acids have long been used as protein solubilizing agents, and because many exhibit minimal absorption at typical HPLC detection wavelengths, they are often used as the weak eluents in gradient reversed-phase HPLC. Solubilization with aqueous TFA or with mixtures of aqueous TFA and organic solvents permits direct injection with little or no additional preparation. High concentrations of formic acid are typically used to solubilize the hydrophobic peptides generated by cyanogen bromide cleavage, and Heukeshoven and Dernick [29] have incorporated 60% formic acid as the initial solvent for reversed-phase chromatography of poliovirus proteins.

### *2.3. Concentration*

Conventional sample concentration techniques such as lyophilization, evaporation, precipitation and dialysis against concentrator resins are widely used to reduce sample volume prior to injection onto HPLC columns. However, these techniques can be cumbersome and time-consuming. Moreover, when using lyophilization or evaporative concentration there is the danger that taking microsamples to dryness will result in chemical modification of the sample and loss of sample due to non-specific adsorption onto the wall of the vessel. Precipitation can cause sample aggregation, leading to poor recovery upon solubilization.

Ultrafiltration is an alternative technique which enables samples to be concentrated rapidly, usually with good recovery and little loss of biological activity. Commercial products are available [30, 31] which permit milliliter samples to be concentrated up to 80-fold. These devices employ centrifugation as the driving force for ultrafiltration. They consist of membrane filters with 10 000–30 000 $M_r$ cut-off held in a centrifuge tube assembly; centrifugation causes solvent and small molecules to pass through the membrane while solutes larger than the cut-off are retained and concentrated above the membrane. Ultrafiltration devices are available for use in fixed-angle rotors which enable unrestricted solvent flow through the membrane and which prevent ultrafiltration to complete dryness. Low non-specific adsorptivity of the membranes generally provides good recovery, although reduced recovery with ultracentrifugation has been reported in at least one case [32].

Nice et al. [33] have reported the use of short microbore HPLC columns for preconcentration of proteins prior to microsequence analysis. Milliliter fractions recovered from chromatography on conventional HPLC columns were diluted and concentrated on 2 or 1 mm I.D. microbore reversed-phase columns. Gradient elution at reduced flow-rates (100–200 μl/min for 2 mm I.D. columns and 20–40 μl/min for 1 mm I.D. columns) enabled elution of samples in eluent volumes as small as 25 μl. The high capacity of the microbore packings permitted sample masses of up to 50–100 μg to be concentrated on-column when introduced in a

weak solvent, while elution at low flow-rates allowed samples to be recovered at high concentrations in the mg/ml range, with concentration factors of up to 80-fold. Although this appears to be a rapid method for concentrating proteins with good recovery, best results require an HPLC system designed or adapted for gradient elution at reduced flow-rates.

### *2.4. Detergent removal*

A variety of techniques have been described for removal of detergents from proteins. Earlier approaches employed dialysis [34] or ion exchange in concentrated urea followed by dialysis [35, 36]; these were time-consuming and did not always provide good recovery. More recent approaches have included adsorption of detergents onto hydrophobic resins, separation on ion-retardation resins, solvent extraction or ion-pair extraction.

Neutral porous polystyrene–divinylbenzene resin beads have been widely used for removal of surfactants from aqueous solutions of proteins. For example, Amberlite XAD-2 resin (Rohm and Haas, Philadelphia, PA, U.S.A.) has been used for removal of Triton X-100 [37] and Holloway [38] has described the use of a similar product (BioBeads SM, Bio-Rad Labs., Richmond, CA, U.S.A. [39]) for removal of this detergent. Triton poses particular problems because of its low cmc. Both batch and column procedures were evaluated. Using the batch method, Triton removal was essentially complete after 2 h of adsorption. The column procedure was a rapid (15 min) alternative for detergent removal from small sample volumes. Results with Triton and sodium deoxycholate suggest that only micellar species are removed by adsorption on polystyrene resins, and that detergent concentration is reduced only to the cmc.

Removal of SDS from proteins has been accomplished using ion-retardation resin [40, 41]. This material consists of acrylic acid polymerized inside a strongly basic anion-exchange resin composed of quaternary ammonium groups attached to a polystyrene–divinylbenzene matrix [42]. Passage of a protein–SDS complex through the resin results in complete retention of SDS with good (80–90%) recovery of protein. The resin has an SDS capacity of over 2.2 mg of SDS per gram, and passage of SDS–protein complexes in water through an ion-retardation column can reduce the SDS level to less than one molecule of SDS bound per protein molecule. However, adsorption of SDS is reduced in the presence of buffers, resulting in incomplete removal of detergent from the protein. This can be circumvented by prior removal of buffer by gel filtration or, more conveniently, by the addition of a few grams of a size-exclusion gel to the head of the ion-retardation resin bed to retard the buffer. One disadvantage of using ion-retardation resins for detergent removal is the tenacious binding of SDS to the resin. Since it is difficult to remove bound SDS completely, the resin cannot be easily reutilized.

SDS can be removed from proteins by ion-pair extraction as described by Henderson and co-workers [43, 44]. A protein–SDS complex in the dry state is extracted with a solution of ion-pairing agent in an organic solvent, such as acetone–triethylamine–acetic acid–water or heptane–tributylamine–acetic acid–

butanol. Sufficient water must be present in the extractant or protein sample to promote formation of the alkylammonium–SDS ion pair. In the case of the second solvent system, water must be added to about 1%. A single extraction removes up to 95% of total SDS, while protein is recovered as a precipitate which can be separated from extractant by low-speed centrifugation and washed with acetone or heptane to remove residual extractant or SDS. Salts present in the sample may interfere with SDS removal and should be removed prior to extraction. This procedure can be adapted to extraction of small volumes of aqueous protein–SDS solutions by mixing sample and extractant in a 1:20 ratio using extractant prepared without the addition of water. A two-step extraction will quantitatively remove the detergent with good recovery of protein (generally 80% or better). This extraction procedure also removes Coomassie blue, so is a useful technique for preparing proteins recovered from SDS polyacrylamide gels.

A procedure for removing SDS and stain from proteins electroeluted from SDS polyacrylamide gels was described by Wessel and Flugge [45]. Dried fractions from electroelution are dissolved in a small volume of water and mixed with methanol, then extracted with chloroform. Following extraction, protein is washed extensively with methanol, then dried to remove solvent.

### *2.5. Desalting*

Removal of salts from protein solutions traditionally has been accomplished by dialysis or by gel filtration using cross-linked dextran or polyacrylamide gels whose porosity permits proteins to be eluted in the void volume while salts are retarded by permeation into the gel beads. These gels are now commercially available as prepacked disposable columns for rapid desalting [46, 47]. A variation on gel filtration desalting was described by Christopherson [48] in which the gel is packed in a centrifuge tube and centrifugation is used as the driving force to elute protein into an attached collection tube. Size-exclusion HPLC columns can also be used for rapid separation of macromolecules from salts and silica-based size-exclusion chromatography columns have been used for desalting synthetic oligonucleotides via an ion-exclusion mechanism [49]. This approach cannot be used for desalting proteins due to adsorption onto the stationary phase in the presence of low-ionic-strength eluents.

Electrodialysis is an alternative approach for rapid desalting or buffer exchange [50]. In this technique, voltage is applied across a stack of ion-exchange membranes carrying fixed positive or negative charges, resulting in selective permeation of cations and anions into zones of concentration and from zones of depletion. The process is gentle, providing high yields without loss of biological activity. Transfer of water during electrodialysis will result in some sample concentration, depending upon the amount of current applied.

Ion-retardation resins, described above for removal of ionic detergents, are also used for desalting [42, 51]. The resin copolymer consists of adjacent fixed anionic and cationic sites which exist in the self-adsorbed form in the absence of counterions. Upon application of a salt solution to the resin column, ionic species in the sample will adsorb to the resin in an anion-exchange process, disrupting

intramolecular association. Salts of monovalent ions (sodium chloride, potassium chloride, Tris–HCl) will be retained while proteins can be rapidly eluted with water. Other salts such as sodium acetate, sodium citrate, sodium phosphate and ammonium sulfate apparently do not compete as effectively with self-adsorption, and may not be well resolved from the early-eluting protein. In this case, a long column may be required. Unlike SDS, salt and buffer ions are weakly adsorbed, and the resin can be regenerated after desalting by treatment with 1 *M* hydrochloric acid followed by 1 *M* ammonium hydroxide containing 0.5 *M* ammonium chloride, then by extensive washing with water. Use of this regeneration procedure reduces undesirable ion-exchange effects due to unpaired cationic and anionic sites.

The ultrafiltration devices described above for sample concentration can also be used for desalting [30]. Concentrated sample is reconstituted in a ten-fold volume of buffer or water and reconcentrated. Each concentration cycle typically removes 90% of the salt, and two or three washes can reduce salt concentration by 99%. Application of this technique to desalting of supercoiled plasmid DNA, DNA restriction fragments ranging in size from 75 to 1600 base pairs and single-stranded DNA of 200 nucleotides in length has been reported [52]. Essentially quantitative recovery of biologically active DNA was achieved after two desalting cycles following cesium chloride density gradient ultracentrifugation.

Rapid desalting can often be accomplished with reversed-phase sorbents, using either sample preparation cartridges or analytical HPLC columns. Desalting can be performed as a separate step, be included in a sample prefractionation operation or be carried out at the initiation of an analytical HPLC separation. This technique relies upon the strong retention of macromolecules (especially polypeptides) on hydrophobic stationary phases when introduced in aqueous solvents. Under these conditions salts, chaotropic agents such as urea, and other polar compounds are unretained and can be eluted from the column with water, buffer, or the initial solvent in a gradient elution program (e.g. aqueous TFA). Analytes can then be eluted with an aqua–organic solvent either isocratically, or using step or gradient elution. Pan et al. [53] and Lahm and Stein [54] have used this technique in preparation of proteins for tryptic digestion and microsequence analysis. Samples subjected to reductive carboxymethylation in the presence of 6 *M* guanidine hydrochloride were acidified with TFA and loaded onto $20 \times 4.6$ mm cartridges packed with 5-$\mu$m wide-pore octyl reversed-phase material. Cartridges were washed with aqueous 0.1% TFA until the 280-nm absorbance returned to baseline, then protein was eluted in 1–2 ml using 70% acetonitrile in 0.1% TFA. Recovery was generally over 90%, and since the sample was eluted in a volatile solvent it could be easily concentrated prior to digestion or sequencing. The same procedure was used to desalt proteins extracted from polyacrylamide gels with 6 *M* guanidine hydrochloride. In some cases desalting was combined with gradient elution to prefractionate polypeptides bound to the cartridge. Chandrasegaran et al. [55] used reversed-phase sample preparation cartridges to desalt synthetic oligonucleotides during sequence analysis with the Maxam-Gilbert method, and similar techniques could be useful in desalting oligonucleotides prior to HPLC analysis or purification.

### *2.6. Filtration and dilution*

Filtration of the sample prior to injection is always advisable to remove particulate material which would block injector ports, transfer lines, inlet frits or the column bed [56]. For manual operation this is conveniently done using a small-volume syringe and Swinney-type adaptor fitted with a micropore filter. Sample pretreatment columns and cartridges will also act as filtration devices. In-line filters and guard columns should be used as extra precautions but not in lieu of prefiltration as they would rapidly become plugged or saturated. If the sample is dissolved in a solvent of higher eluent strength than the mobile phase, dilution will be necessary to prevent band broadening or irreproducibility. In ion-exchange or reversed-phase applications employing gradient elution, injection of large sample volumes in weak solvents will not adversely affect resolution. However, in gel permeation chromatography, sample volume should be limited to 1% of total column volume.

## 3. COLUMN REGENERATION

Degradation of column performance as evidenced by altered selectivity, decreased efficiency, peak tailing or splitting, or elevated operating pressure can arise from several causes including (a) dissolution of the bed matrix, (b) bed compaction, (c) loss of bonded-phase ligands, (d) contamination of the stationary phase by strongly adsorbed sample or eluent components (e.g. proteins, lipids, metals) or (e) blockage of inlet frits or the column bed by particulate material from the sample or mobile phase. In many cases, complete or partial recovery of column performance can be achieved by regeneration procedures, and this section will review techniques which have been reported in the literature or which are familiar to the author through personal experience or discussions with other practitioners. It should be noted parenthetically that column lifetime can be significantly increased through the use of appropriate sample preparation procedures as described in the previous section, through the use of guard columns and precolumns and through the use of good column installation and maintenance procedures as described in refs. 56–59.

### *3.1. Repair of bed irregularities*

Deterioration of peak shape (such as broadened peaks, asymmetric peaks or split peaks) is often indicative of formation of voids in the column bed and may be accompanied by gradual or sudden increase in operating pressure [60]. Void formation may be due to compaction of less dense regions of the column bed when operating under high-shear conditions, for example with viscous mobile phases or at high flow velocities. In this case void formation is evidenced by a dramatic loss in efficiency (e.g. 80% loss for a 1-mm void at the head of a 30 cm$\times$4 mm, 5-$\mu$m particle diameter column) accompanied by a modest increase in operating pressure (about 20%).

In the case of silica-based columns, void formation often occurs with the use of

alkaline mobile phases which promote dissolution of the silica matrix. Dramatic elevations in column pressure result from the collapse and fracturing of porous silica microparticles during dissolution. Dissolution is exacerbated by operation at elevated temperatures and by the use of cationic mobile phase additives which enhance silica dissolution. The most common example is the use of alkyl amines or alkyl quaternary ammonium salts in reversed-phase chromatography as competing bases to improve peak shape or as ion-pairing agents to modify retention or selectivity. Under neutral-to-alkaline conditions (pH > 6) these species accelerate silica dissolution, decreasing column lifetime.

Void formation due to silica dissolution can be reduced by incorporating a silica-packed saturator precolumn before the analytical column [61], although use of saturator precolumns can require additional precautions to insure good chromatographic performance [56]. Increases in the saturator void volume during operation can cause poor chromatographic reproducibility in gradient elution, and gelation of dissolved silicate in the saturated mobile phase can result in formation of microparticles which can plug the inlet frit or bed in the analytical column. Column packings based on polymeric materials such as polystyrene, polymethacrylates or polyethers may be operated under alkaline conditions where silica materials would fail. However, certain solvent conditions (e.g. high concentrations of organic modifiers) may cause swelling or shrinkage of polymeric gel materials, resulting in channeling or void formation when these solvents are introduced into packed columns. The column manufacturer generally stipulates these solvent restrictions in the column installation and operating instructions.

Void formation can be verified as the cause of deteriorated column performance by removal of the inlet terminator and inspection of the column bed. If the void is small (on the order of a few millimeters in depth), a simple repair procedure can be used which usually provides substantial recovery of column performance [62]. This entails removal of a small amount of packing to form a level surface, followed by addition of an appropriate packing material as a slurry to bring the bed surface flush to the face of the column tube. Either porous or non-porous microparticulate materials may be used for repacking, as long as the selectivity of the stationary phase is reasonably well matched to that of the column. Settling often occurs when the column is returned to operation, and it is usually necessary to repeat the repacking procedure two to three times to recover adequate performance.

If the depth of the void is greater than a few millimeters, topping-off procedures will probably not be successful in recovering adequate column performance. Also, internal voids and channels cannot be repaired by these procedures. In both cases, the only alternative is repacking the column. Because of the high cost of HPLC columns (particularly gel permeation and large-pore ion-exchange columns) repacking of damaged columns may be an economic necessity for many laboratories. Considerations in reuse of HPLC column hardware and stationary phases have been reviewed by Dewaele et al. [63].

The condition of the inner surface of the column tube is commonly assumed to affect the regularity of the bed structure at the wall, and hence the efficiency of the column. This subject is controversial, however, with conflicting reports on

the requirement of highly polished walls for best column performance [64, 65]. In practice, other factors such as the packing material and packing procedure may be the major contributors to column quality. Dewaele et al. [63] demonstrated that column hardware could be successfully repacked with fresh material but wall defects limit the number of times a column can be repacked. This limit depends upon the wall thickness and tube dimensions; 8 mm I.D. semipreparative and 22 mm I.D. preparative columns may have a lower success rate for repacking. In the opinion of these authors, silica-based stationary phases can be reused only if several precautions are taken. First, the upper centimeter at the column head should be discarded as it is most likely to be contaminated. Second, elimination of fines by flotation may be required and the packing may need to be end-capped or rederivatized to eliminate silica surfaces exposed by particle fracturing during packing and unpacking. Third, conditioning the packing by sonication in the packing solvent may be necessary to disrupt particle aggregates. This last precaution is even more important in reuse of polymeric gel packings which, because of their semirigid nature, are more prone to aggregate formation during packing. Avery and Light [66] demonstrated that at least 95% of the original efficiency of a 5-$\mu$m octadecyl reversed-phase column could be recovered by repacking the column with the same packing material using slurry packing at 300 bar. The 2-propanol used as packing solvent also served to strip adsorbed contaminants from the column. The same column could be repacked up to four times with recovery of acceptable performance.

Although conventional small-pore silicas yield high-efficiency columns when packed at high pressure, the macroporous materials with pore diameters of 30 nm and larger which are used for biopolymer separations are more fragile. These can fracture upon impact during high-pressure packing, plugging the outlet frit; packing these materials at pressure below 300 bar is recommended.

### *3.2. Frit repair*

The majority of commercial HPLC columns incorporate stainless-steel frits in their inlet and outlet terminators to contain and protect the column packing. These frits, which are usually composed of 316-grade stainless steel and possess pores of 0.5–2 $\mu$m diameter, can themselves contribute to degradation of chromatographic performance.

Blockage of the inlet frit by microparticulate material may occur if the sample or mobile phase has not been adequately filtered. This is evidenced by a gradual rise in operating pressure without an accompanying loss in column efficiency. This problem can be minimized by the use of guard columns packed with microparticulate material or the addition of a low-volume in-line filter with disposable frit. Devices of both types are commercially available which, if properly installed, should not introduce excessive extra column band broadening with high-efficiency analytical columns. If the geometry of the inlet terminator is such that solvent flow is not distributed evenly across the frit surface, the central part of the frit may become preferentially plugged. In this case, impeded flow in the frit

center relative to the annulus can result in multiple flow paths, giving rise to asymmetric or split peaks.

Requirements for recovery of native biological macromolecules have led to increasing concern about the effect of metal ions leached from the hydraulic components of stainless-steel HPLC systems upon biological activity. These species may indeed bind to proteins and nucleic acids, leading in some cases to structural changes or loss of function. Of perhaps greater importance, however, is the gradual deposition of metals onto the surface of the stationary phase materials, leading to changes in column capacity or selectivity. This may be of particular concern considering the trend to small columns and low-surface-area packings for analytical and micropreparative HPLC. The high surface area of stainlesss-steel frits (approximately 200 $cm^2$) makes them the major source of metal ions leached from the HPLC hardware.

Proteins have been shown to adsorb to stainless steel [67, 68] and Sadek et al. [69] have shown that stainless-steel frits are a significant contributor to irreversible protein loss. Their work demonstrated that the percentage losses on frits was greatest for small protein samples (below 1 $\mu g$) and that the weak solvents typically used during injection in a gradient elution scheme enhanced protein adsorption on frits.

If frit blockage is suspected as the cause for elevated operating pressure, backflushing the column is a simple technique for freeing a partially blocked inlet frit [56]. Several precautions should be noted: first, column operating instructions should be consulted to determine whether the manufacturer recommends backflushing. Second, elevated operating pressure should not be accompanied by efficiency loss. This would indicate void formation, in which case backflushing might irreversibly disturb the column bed. Third, elevated operating pressure when using silica-basd columns with alkaline mobile phases, alkyl amine mobile phase additives or elevated column temperatures can indicate blockage of the outlet frit by degraded packing material. In this case, backflushing may produce only a transient reduction in pressure. Fourth, the column should be disconnected from the detector during backflushing to prevent dislodged contaminants from blocking detector transfer lines or flow cell. If backflushing the column is unsuccessful, it indicates that contaminants have penetrated to the interior of the frit matrix or to the column bed, and other steps must be taken. In the case of replaceable frits, replacement is generally straightforward. In the case of non-replaceable (pressed-in) frits, replacement of the entire terminator assembly is necessary; this may be difficult if the terminator ferrule has been deformed during manufacture. An alternative approach is to remove the terminator and attach it to the outlet end of an empty column or guard column. It can be then be backflushed under vigorous conditions with aggressive solvents without risk to the analytical column. The column should be capped with a spare terminator during this process to prevent damage to the column head by drying.

Poor recovery due to adsorption onto the frit surface or leaching of metal ions may be minimized by thorough passivation of the frit with nitric acid or by silanization [69]. However, one report suggests that passivation with nitric acid may not be effective in reducing protein loss on stainless-steel frits [70].

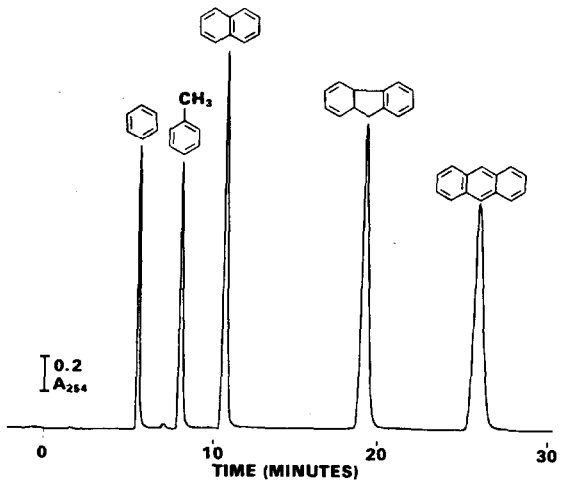


Fig. 1. Separation of an aromatic hydrocarbon test mixture. Sample: 1 μl containing 4 μg benzene, 4 μg toluene, 0.5 μg naphthalene, 0.2 μg fluorene, 0.03 μg anthracene. Column: MicroPak SP-C18-5, 150×4.6 mm. Elution conditions: acetonitrile–water (55:45) at a flow-rate of 1.0 ml/min. Detection: absorbance at 254 nm.

Replacement with low-surface-area screen frits, or with non-ferrous or non-metallic frits, appears to be the best solution.

### *3.3. Stationary phase regeneration*

Changes in retention and selectivity can both point to changes in the chemical characteristics of the stationary phase. Alterations in stationary phase properties can arise from loss of bonded phase by hydrolytic attack on the siloxane bond, chemical reaction of bonded phase functions with sample or mobile phase constituents, and irreversible adsorption of sample or mobile phase components onto the stationary phase. Recognition of the cause of column failure and proper selection of regeneration procedures will be greatly aided by routine monitoring of column performance with suitable test compounds and by failure diagnosis using test probes. Aromatic hydrocarbons such as those shown in Fig. 1 are useful for monitoring column efficiency and capacity, and will detect loss of stationary phase (reduced capacity factor) or void formation [reduced number of theoretical plates (N), peak asymmetry, appearance of doublets]★. Agents which complex or chelate metals, such as acetylacetone [63], tetracycline or benzoin, will detect metal contamination of the stationary phase (Fig. 2). Basic compounds such as N,N-diethylaniline [71] or thiamine which interact strongly with silanols can be used to detect loss of stationary phase or incomplete end-capping in silica-based columns (Fig. 3). In the chromatography of proteins and peptides, a set of carefully selected polypeptide standards can be useful in monitoring column performance. Proteins interact with chromatographic supports through mixed hydrophobic,

★The figures serve to demonstrate column testing or some deteriorative effects occurring with the column packing; use of protein test mixtures may reveal such processes with difficulty or not at all.

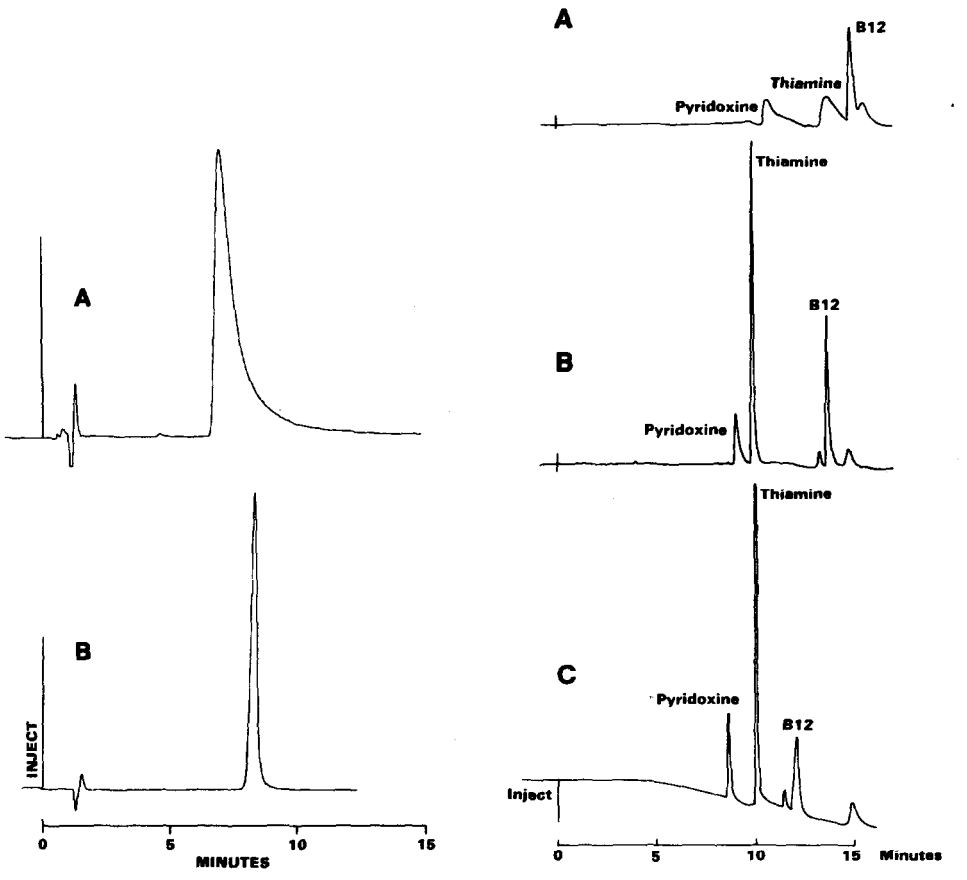


Fig. 2. Chromatography of benzoin on reversed-phase columns packed with octadecyl phases bonded to spherical silica containing (A) high and (B) low amounts of metals. Solvent: water–acetonitrile (60:40).

Fig. 3. Effect of end-capping and addition of a competing base to reduce silanol interactions in the chromatography of a water-soluble vitamin test mixture. (A) Separation using non-end-capped column without addition of tetramethylammonium chloride (TMA); (B) separation using non-end-capped column with addition of 10 m*M* TMA; (C) separation using end-capped column without addition of TMA. Column: octadecyl phase bonded to 3-$\mu$m spherical silica, 150×4.6 mm.

hydrophilic, electrostatic and exclusion mechanisms; Table 1 presents a group of proteins which exhibit a range of $M_r$, hydrophobicity and charge characteristics. These are commercially available in high purity and have been used regularly to characterize chromatographic media. Mant and co-workers [72, 73] have developed a series of synthetic peptides for use in monitoring reversed-phase column performance and in evaluating commercial HPLC columns. These are closely related sequence variants whose chromatographic behavior could aid in the diagnosis of column failure (Fig. 4).

Loss of stationary phase is evidenced by gradual reduction in retention of all sample components. With silica-based columns, cleavage of the bonded phase is favored by operation at high pH, high ionic strength and low concentrations of

TABLE 1

STANDARD PROTEINS USED IN THE CHARACTERIZATION OF HPLC COLUMNS

| Protein | Molecular mass | Isoelectric point |
|---|---|---|
| Insulin | 5700 | 5.3 |
| Cytochrome C | 12 400 | 10.6 |
| Ribonuclease | 13 700 | 7.8 |
| Lysozyme | 14 400 | 11.0 |
| Myoglobin | 17 000 | 7.0 |
| Chymotrypsinogen | 24 700 | 9.1 |
| Ovalbumin | 45 000 | 4.6 |
| Serum albumin | 65 000 | 4.8 |

organic modifiers [74]. This is a common problem in the ion-exchange purification of oligonucleotides which is typically carried out at pH 7 with mobile phases containing 20% organic modifier and salt concentrations of up to 0.5 *M*. An extreme case is exemplified by the use of quaternary ammonium anion-exchange columns for this application; the strong base of the stationary phase ligand evidently promotes cleavage of the siloxane bond, and column failure may occur within a few hours of normal operation.

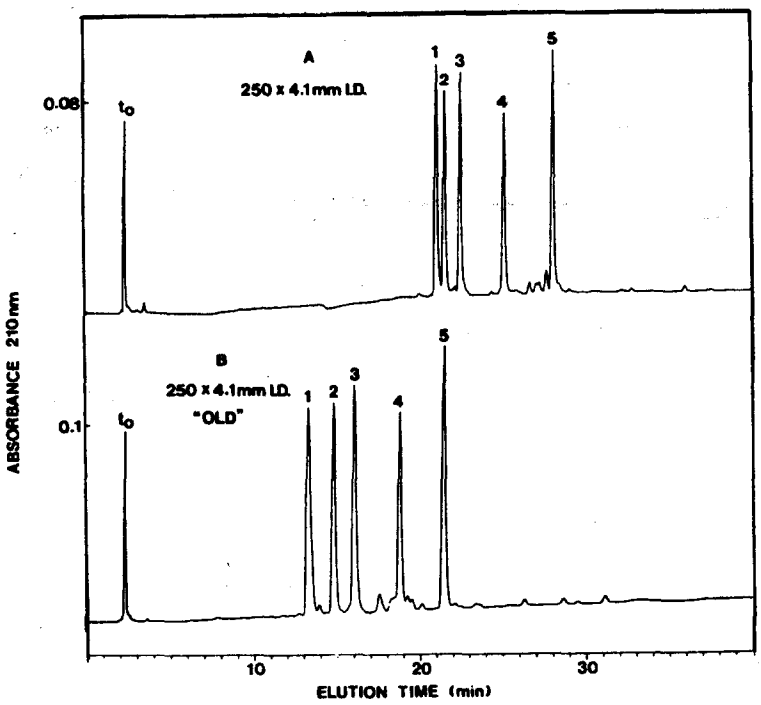


Fig. 4. Separation of synthetic peptide standards on a new reversed-phase column (A) and on the same column after four months of extensive use (B). Column: SynChroPak RP $C_{18}$. Elution conditions: gradient from 0.1% TFA in water to 0.1% TFA in acetonitrile at 1%/min, 1 ml/min. Reprinted from ref. 73 with permission.

In reversed-phase chromatography, loss of stationary phase is minimized by the use of hydrophobic *n*-alkyl ligands, which shield the siloxane bond, and by the use of high average organic modifier concentrations, which minimize hydrolytic attack. For the octyl and octadecyl phases typically used for reversed-phase chromatography of polypeptides and polynucleotides, column lifetime is good. However, short-chain alkyl phases are being used with increasing frequency for chromatography of large and hydrophobic proteins. Glajch et al. [71] have shown that operation of silica-based reversed-phase columns bonded with short-chain alkyl ligands ($C_1$–$C_4$) under typical gradient elution conditions with 0.1% TFA in combinations of water and organic modifier can promote bonded-phase cleavage. Chromatographic studies and element analysis of the eluents suggested that cleavage occurred very rapidly, with losses of 20–80% of the bonded phase over a four-day period of normal operation, corresponding to an average ligand loss of 1–25 nmol of ligand per milliliter of eluent.

Comparison of different commercial columns indicated that proper preparation of the silica prior to bonding was the major determinant of column longevity in operation under acidic conditions. Interestingly, these authors [71] observed that acceptable peak shape and resolution were retained despite the loss of over half the bonded phase, suggesting that complete coverage of the silica surface is not always necessary for usable chromatographic performance. If column performance has deteriorated due to loss of stationary phase, the column may be regenerated by passage of an appropriate silane reagent through the column bed [74]. Wilson [75] described a procedure for in situ rebonding in which octadecyltriethoxysilane and trimethoxysilane reagents were pumped through 5- and 10-$\mu$m particle diameter reversed-phase columns. Results were variable, depending on the source of the column. At best only partial regeneration of chromatographic efficiency and resolution was achieved, and excessive pressure was observed following rebonding of 5-$\mu$m columns. Given the decreasing costs of generic reversed-phase columns and the trend towards small, high-efficiency cartridge columns, one must weigh the advantages of in situ rebonding procedures against the costs in time (4–8 h) and reagents.

Chemical reaction of stationary phases with sample or eluent is known to occur in two instances in chromatography of biopolymers. Alkyl amine columns are often used in both the ion-exchange and normal-phase modes for chromatography of charged or neutral oligosaccharides [76, 77]. The primary amine functions on these columns react with aldehydes and reactive ketones through Schiff base condensation [74]. Low-molecular-weight sugars such as ribose are reactive and repeated injection of samples containing high concentrations of these species will lead to a gradual conversion of the stationary phase; hexoses and higher-molecular-weight saccharides are unreactive. Cyano columns are frequently used in the reversed-phase mode for chromatography of peptides and proteins. It has been reported [74] that cyano bonded phases prepared from trimethoxyalkylsilanes exhibit a gradual reduction in retention time when operated with aqueous eluents, probably due to silanol-catalyzed hydrolysis of the nitrile to hydroxylamine. The use of a dimethyl-methoxyalkylsilane reagent prevented this reaction from taking place, and the use of cyano columns prepared in this manner is recommended

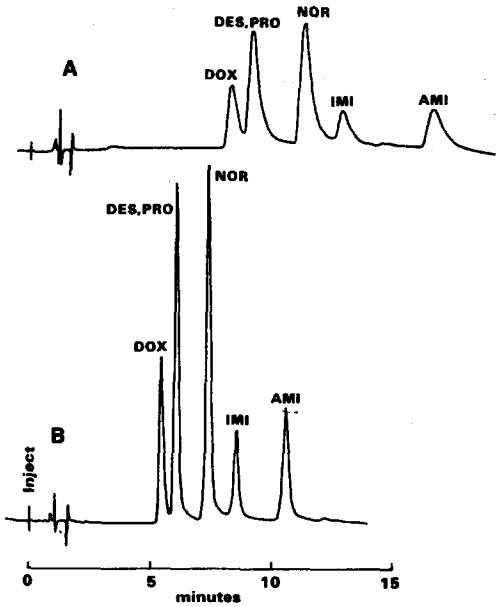


Fig. 5. Effect of acid washing to reduce surface metal on the separation of tricyclic antidepressants. (A) Reversed-phase separation of tricyclic antidepressants on a column packed with untreated 3-$\mu$m $C_{18}$-bonded silica; (B) separation of same compounds using acid-washed material.

for reversed-phase applications. In both of these cases of chemical reaction of the stationary phase, no convenient column regeneration procedure is available.

Accumulation of strongly retained contaminants on the column can in some cases cause a reduction in retention due to saturation of active sites on the stationary phase. In other cases the contaminants also serve as an interactive surface, resulting in changes in selectivity and peak shape. Accumulated material may block inlet frit pores or interstitial spaces in the column bed, resulting in increased operating pressure and formation of multiple flow paths. Removal of strongly adsorbed material can be accomplished by washing the column with a solvent of suitable eluent strength. The key is determining which solvent will displace contaminants from stationary phase binding sites. In the case of hydrophobic contaminants such as lipids, a non-polar organic solvent (e.g. acetonitrile, tetrahydrofuran, hexane) would be an appropriate choice. In the case of adsorbed metals, washing with strong acid or chelating and complexing agents such as phosphoric acid, citric acid and EDTA have been used [63], as shown in Fig. 5. However, as noted above, passage of aqueous solutions of strong acids through bonded-phase silica columns may strip stationary phase ligands from the silica.

Removal of adsorbed proteins poses a more difficult problem since they interact at multiple sites with the stationary phase ligands and support matrix through a variety of mechanisms. For example, a wash solvent for reversed-phase columns must disrupt both the hydrophobic and silanophilic interactions involved in protein binding. Therefore the neat organic solvents commonly used to strip lipids and other hydrophobic contaminants are rarely effective in regenerating protein-

TABLE 2

WASH SOLVENTS FOR HPLC COLUMNS

Consult manufacturer for column compatibility.

| Solvent | Composition |
|---|---|
| Acetic acid | 1% in water |
| Formic acid | 60% in water |
| Trifluoroacetic acid | 1% in water |
| 0.1% Aqueous trifluoracetic acid-propanol★ | 40:60 |
| Triethylamine phosphate★★-propanol★ | 40:60 |
| Aqueous urea or guanidine | 5–8 *M* |
| Aqueous sodium chloride, sodium phosphate or sodium sulfate | 1–2 *M* |
| Dimethylsulfoxide | 100% |

★High viscosity solvent: pump at reduced flow-rate to prevent overpressure.
★★0.75 *M* phosphoric acid adjusted to pH 2.5 with triethylamine.

contaminated columns. Instead, protein-solubilizing agents, such as concentrated solutions of salts or chaotropic agents, or surfactants have been used. Table 2 lists several wash solvents which have proven effective in regeneration of high-performance reversed-phase, ion-exchange and gel-permeation columns; two or more of these solvents may have to be used in series for complete regeneration. Regnier [78] has observed that the TFA–propanol solvent systems used for reversed-phase chromatography are able to solubilize many ionic and hydrophobic proteins, and five to ten repetitive gradient–retrogradient cycles with TFA–propanol are effective in regeneration of ion-exchange and gel-permeation as well as reversed-phase columns.

When using multiple wash solvents, care must be taken to insure that sequential wash solvents are compatible. For example, a high salt or urea solvent should not directly follow or precede a wash containing an organic solvent, but should include an intervening water wash. Column washing should be carried out with the column in the backflush position, with the column disconnected from the detector (off-line washing with a low-pressure pump may be more convenient). Washing with 20–30 column volumes of solvent per step should be effective in most situations. If the contamination level is low or the wash solvent is strong enough to strip contaminants rapidly, small volumes can be introduced via an injector valve fitted with a 1–2 ml loop. This approach has been described by Power et al. [19] for removal of strongly adsorbed protein with a 1-ml injection of dimethylsulfoxide.

## 4. SUMMARY

Successful use of high-performance liquid chromatography (HPLC) for biopolymer separations requires adequate sample preparation procedures for prefractionation of complex mixtures, solubilization of the compounds of interest, or removal of contaminants which would interfere with chromatography or dam-

age the HPLC column. Rapid techniques have been described for solid-phase and liquid–liquid extraction, desalting, detergent removal, concentration, and filtration. A number of commercial products are available for off-line batch sample preparation and for on-line automated sample processing. These include ultracentrifugation cartridges and a wide variety of sorbents sold in bulk form or packed in disposable cartridges or minicolumns.

In cases where HPLC column performance has degenerated to unacceptable levels, procedures have been described to achieve partial or complete recovery of performance. These include addition of material to fill voids caused by compaction or dissolution of the packing, replacement or cleaning of contaminated frits, regeneration of lost stationary phase material, and stripping of strongly retained contaminants from the stationary phase with suitable strong solvents.

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