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On-Line High-Throughput Desalting to Prepare Samples for Mass Spectrometry

INTRODUCTION

Mass spectrometry (MS) or liquid chromatography-mass spectrometry (LC-MS) has become a powerful tool for the analysis of complex biochemical samples. These samples often contain high concentrations of salts that interfere with the ionization process and can result in lower peak intensities, compromised peptide/protein detection, and frequent contamination of the ion source. Therefore, it is essential to remove salts from samples before subjecting them to MS detection. Although the most common desalting method is off-line solid-phase extraction (SPE), it has the disadvantage of being time-consuming.¹ To save time, scientists have used on-line desalting based on the valve-switching technique.²⁻⁴

The work shown here describes a simple, fast, and effective on-line method to facilitate high-throughput sample desalting before MS detection. The entire process, including sample desalting and flushing, was completed within 2 min for an oligonucleotide sample and a bovine serum albumin (BSA) sample. This was accomplished with an UltiMate[®] 3000 HPLC system equipped with a dual gradient pump, an autosampler, and a column oven equipped with a 2p–10p valve.

EQUIPMENT

Dionex UltiMate 3000 HPLC system including:

DGP-3600A Pump with SRD-3600 solvent rack with degasser

WPS-3000TSL Autosampler

TCC-3200 Thermostatted Column Compartment

VWD-3400RS UV-vis Detector

DAD-3000RS (for UV spectrum)

Chromeleon[®] Chromatography Data System 6.80 SR7

REAGENTS

Deionized water, Milli-Q[®] gradient A10

Acetonitrile (CH₃CN), HPLC grade, Fisher

Ammonium hydrogen carbonate (NH₄HCO₃),

Formic acid (HCOOH), Potassium bromide (KBr), analytical grade, SCRC, China

SAMPLES

An oligonucleotide sample containing large amounts of salts that remained from the production process was provided by a customer. Purified and desalted BSA was purchased from Shanghai Amin Biotech Co., Ltd., China.

RESULTS AND DISCUSSION

Method Development

In the production process of biochemical products (e.g., oligonucleotides and proteins), some manufacturers apply, or would like to apply, MS as part of their QA/QC analysis. Typically, SPE is used to remove high concentrations of salts from the products. The product is bound to the SPE resin, whereas the salts go to waste. The retained product is then eluted from the SPE resin and analyzed by MS. For best efficiency, desalting and elution must be as fast as possible. On-line SPE using HPLC instrumentation can meet this requirement. In fact, the process can be designed as on-line SPE-MS. Figure 1 shows the schematics of a configuration to perform method development for on-line SPE-UV. In this configuration, an HPLC column is added to create an on-line SPE-LC-MS system. Because most of the salts used in the production process do not have UV absorbance, a salt with UV absorbance (KBr) was added to the sample solution to monitor the success of desalting during method development. The UV detector monitors whether or not the salts are flushed out completely and also is used to decide the valve-switching time for sample elution. After valve switching, this UV detector also is used to confirm if the analytes are eluted. In Figure 1, an HPLC column is added to separate the analytes prior to UV detection. After method development, this system can be configured so that salts go directly to waste, and only the eluted analytes are sent to the column and detector. If desired, the MS detector can replace the UV detector; alternately, it can be added after the UV detector, either with or without the HPLC column. Figure 2 shows the chromatograms of desalting when KBr solutions with concentrations from 100 to 4000 $\mu\text{g/mL}$ were injected onto the SPE column (Acclaim[®] Polar Advantage II [PA2] Guard, 4.3×10 mm, $5 \mu\text{m}$) at a desalting flow rate of 1 mL/min. KBr is eluted completely within 1.5 min, even at 4000 $\mu\text{g/mL}$.

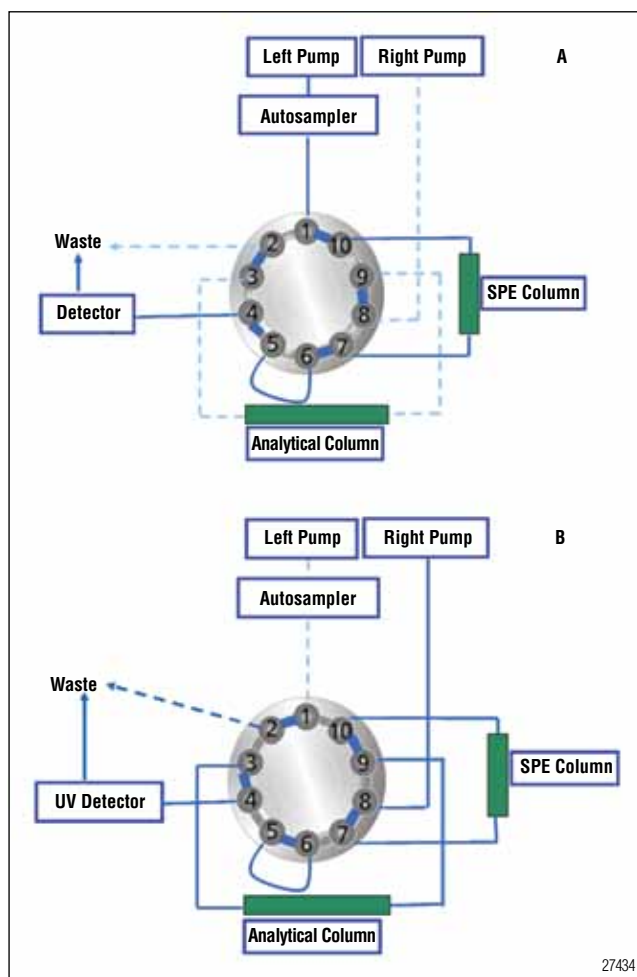


Figure 1. Schematics of valve-switching for sample desalting and elution. A) Loading and desalting (left pump): after injection, the analytes are retained on the SPE column, and the salts in the sample solution are flushed out of the SPE column. If KBr is added to the sample solution prior to injection, a huge peak can be observed at 227 or 215 nm when the SPE column is flushed. B) Sample elution (right pump): after desalting the sample is eluted. The retained analytes are eluted from the SPE column and sent through the analytical column, then to the UV detector.

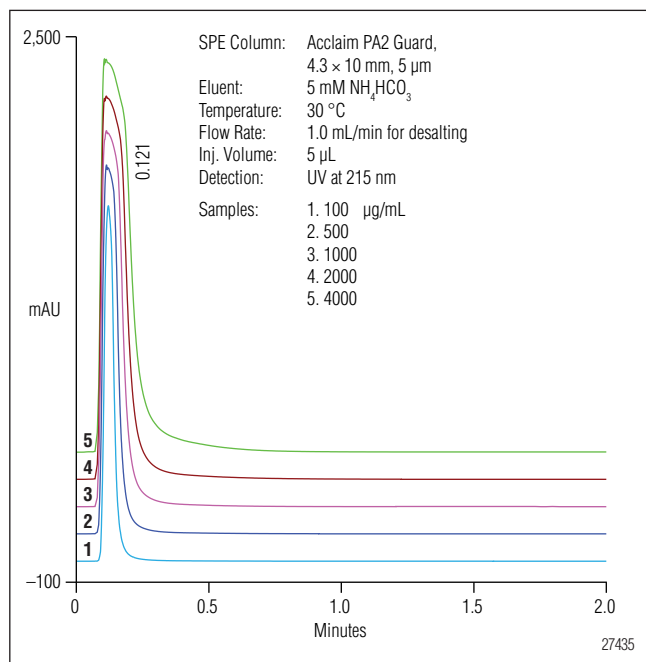


Figure 2. Chromatography of desalting of KBr solutions with different concentrations using an SPE column.

To achieve best peak intensities, the most commonly used mobile phases for LC-MS of oligonucleotides and proteins are basic or acidic solutions in an organic solvent. These mobile phases must be volatile. Solvent volatility is also important if the sample is injected into the MS without an analytical separation. Therefore, NH_4HCO_3 and HCOOH solutions were selected for desalting and SPE. The Acclaim PA2 column was chosen for desalting and SPE was selected because of its wide allowable pH range (pH 1.5–10) and its past success in binding oligonucleotides.⁵

If an analytical column is required for LC/MS, the Acclaim PA2 column is a good choice for the basic conditions of oligonucleotide separations because of its wide allowable pH range. For protein samples, the Acclaim 300 C18 column is a good choice.

In this document, an oligonucleotide sample and a protein (BSA) sample were used to develop the desalting method. The oligonucleotide sample was spiked with 2000 $\mu\text{g/mL}$ KBr and then desalted with the Acclaim PA2 column, followed by elution from and subsequent separation on the PA2 column. Figure 3A shows the results of this experiment at a desalting flow rate of 1.0 mL/min.

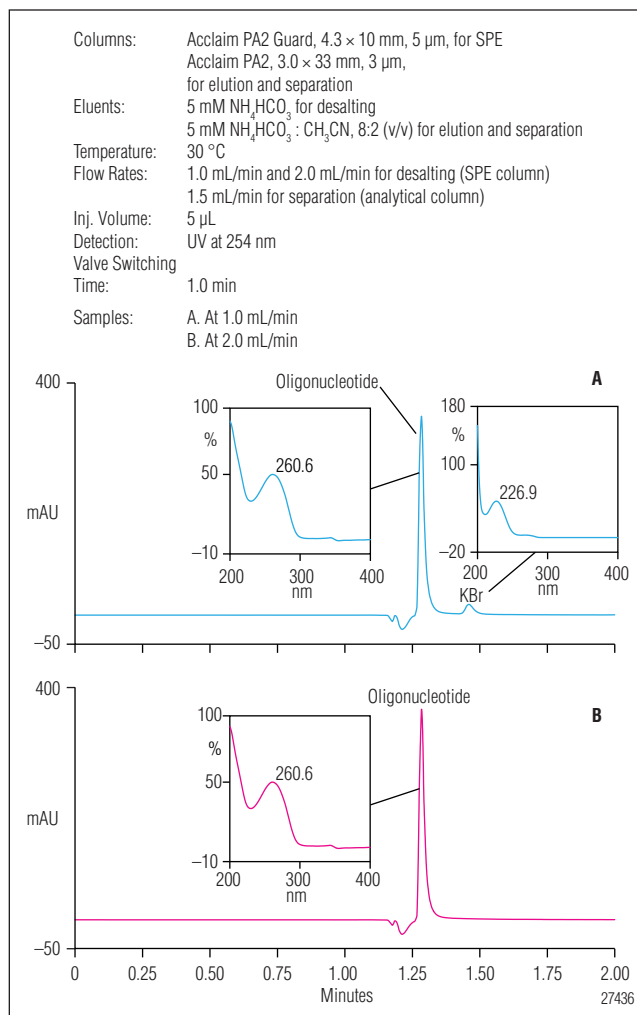


Figure 3. Chromatograms of an oligonucleotide sample spiked with 2000 $\mu\text{g/mL}$ KBr and desalted at different flow rates. A) 1.0 mL/min and B) 2.0 mL/min.

A small peak was found following oligonucleotide (characteristic absorbance at 260 nm for oligonucleotide). Upon comparing the UV spectrum of the small peak to that of bromide, the authors concluded that the small peak was bromide and desalting was incomplete. There are two possible solutions for this problem. One possibility is to lengthen the desalting time. For example, KBr can be removed completely when the valve-switching time increases from 1.0 to 1.5 min. Another possible solution is to increase the flow rate from 1.0 to 2.0 mL/min, as shown in Figure 3B. The increased flow rate completely removes KBr. For the BSA sample, the same results are obtained. The higher flow rate also allows higher throughput desalting.

On-Line High-Throughput Desalting Using a Tandem Configuration

The desalting and elution can be accelerated using the tandem configuration shown in Figure 4 in conjunction with the program shown in Figure 5. The reproducibility of this tandem configuration was investigated using 10 consecutive injections of BSA spiked with 4000 $\mu\text{g/mL}$ KBr (5 injections each on the two SPE columns). An overlay of the chromatograms from this experiment shows that this method is reproducible (Figure 6). The retention time RSD was 0.10%.

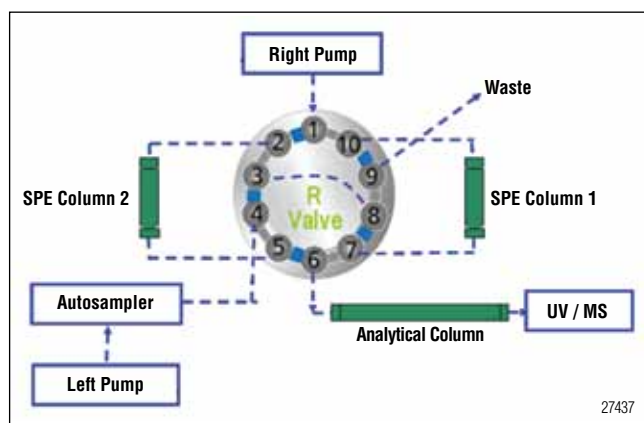


Figure 4. Schematics of valve-switching for fast on-line desalting using a tandem configuration. Cycle 1: 1) Valve switching to 1-2. Loading and desalting (left pump): after injection, the analytes are retained on SPE Column 1, and the salts in the sample solution are flushed out of SPE Column 1 to waste. 2) When the desalting completes, the valve switches to 1-10. Sample elution and analysis (right pump). Meanwhile, SPE Column 2 is now in line with the left pump. Cycle 2: same as Cycle 1, but the position of valve switching is reversed for injection to SPE Column 2. Two programs are required for each cycle and run in turn.

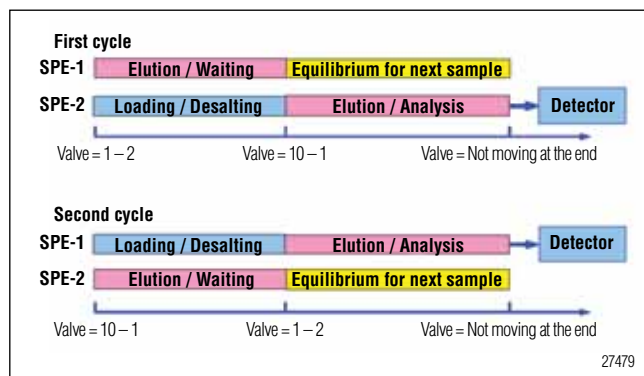


Figure 5. Program of the on-line tandem configuration for fast desalting.

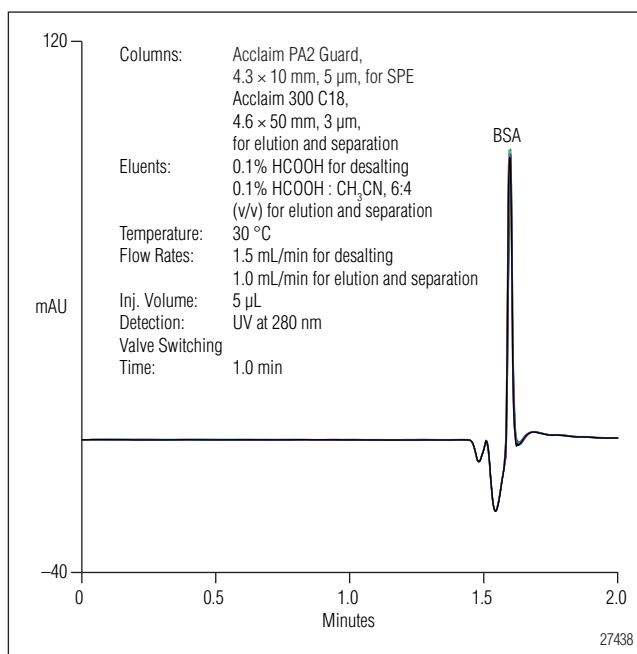


Figure 6. Overlay of ten chromatograms of a BSA sample spiked with 4000 $\mu\text{g/mL}$ KBr.

When the BSA sample was desalted and separated using this tandem configuration, a small peak also was found with absorbance at 214, 254, and 280 nm, even when the desalting flow rate was 2.0 mL/min. Figure 7 compares chromatograms of BSA, BSA spiked with KBr, and UV spectra of KBr. Detection at three wavelengths revealed that the peak is an impurity from the BSA sample, and that sample desalting was complete.

When the sample salt concentration is not high, faster on-line high-throughput desalting and separation can be achieved by shortening the valve-switching time in the tandem configuration. Figure 8 overlays chromatograms of four consecutive injections of an oligonucleotide that was injected onto two SPE columns (two injections on each SPE column). The desalting, elution, and separation of the oligonucleotide sample (salt concentrations less than 1000 $\mu\text{g/mL}$) may be completed within 1.1 min when the valve-switching time is 0.4 min. The 0.0% RSD for retention time and 1.5% for peak area show that the faster on-line high-throughput desalting and separation tandem configuration is reproducible.

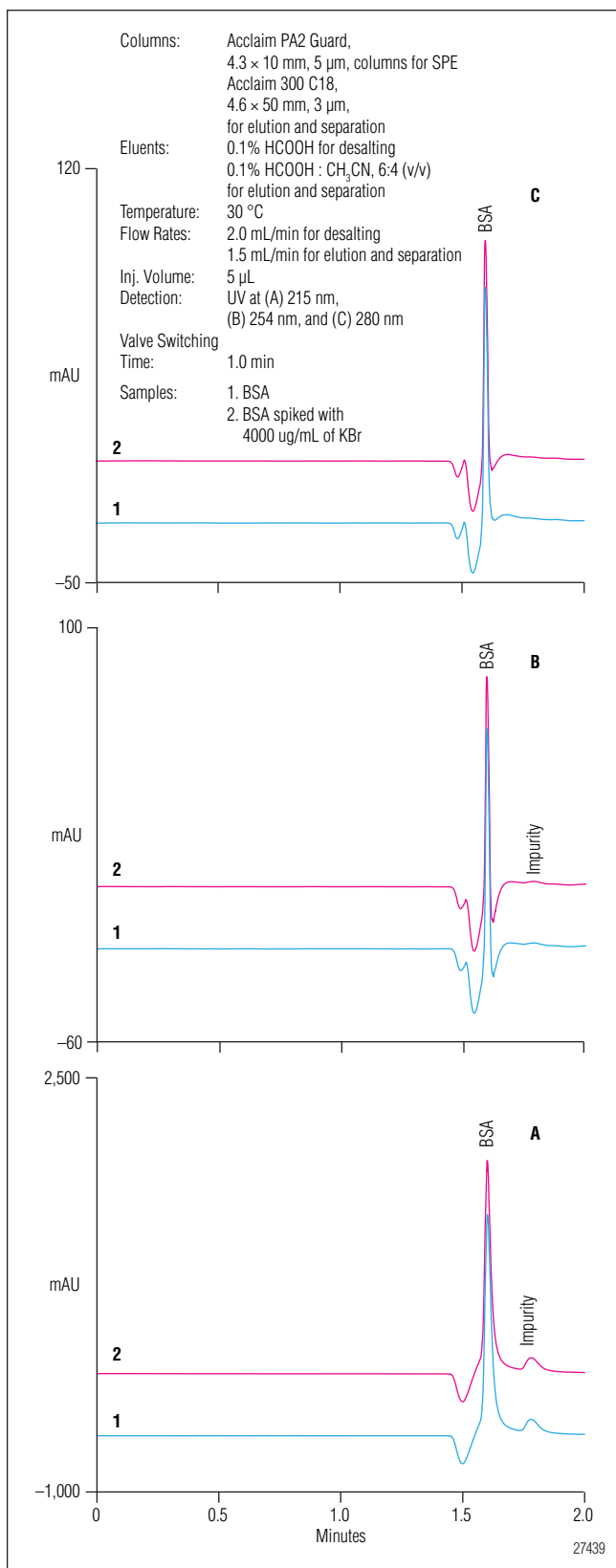


Figure 7. Overlays of chromatograms of BSA and BSA spiked with KBr at A) 215 nm, B) 254 nm, and C) 280 nm.

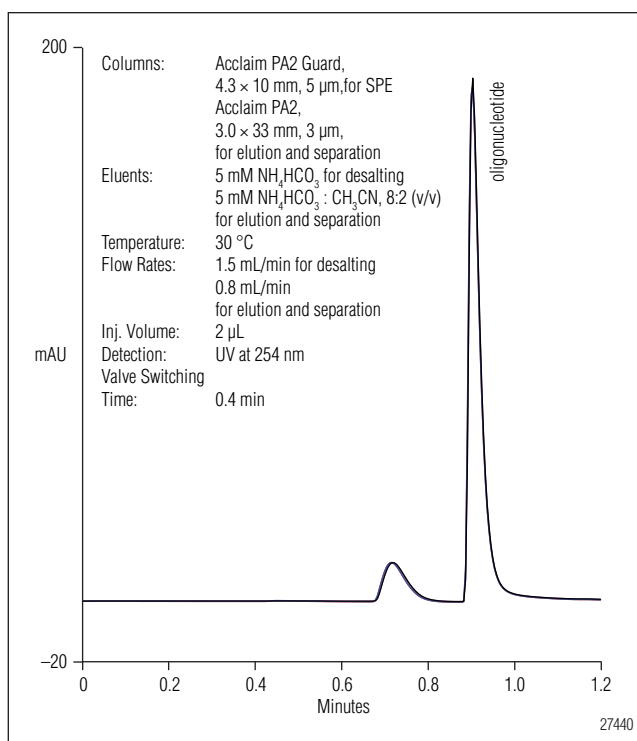


Figure 8. Overlay of chromatograms from four consecutive injections of an oligonucleotide onto two SPE columns (two injections on each SPE column).

CONCLUSION

This application update demonstrates a fast method to desalt oligonucleotide or protein samples for either on-line analysis by HPLC or LC/MS.

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