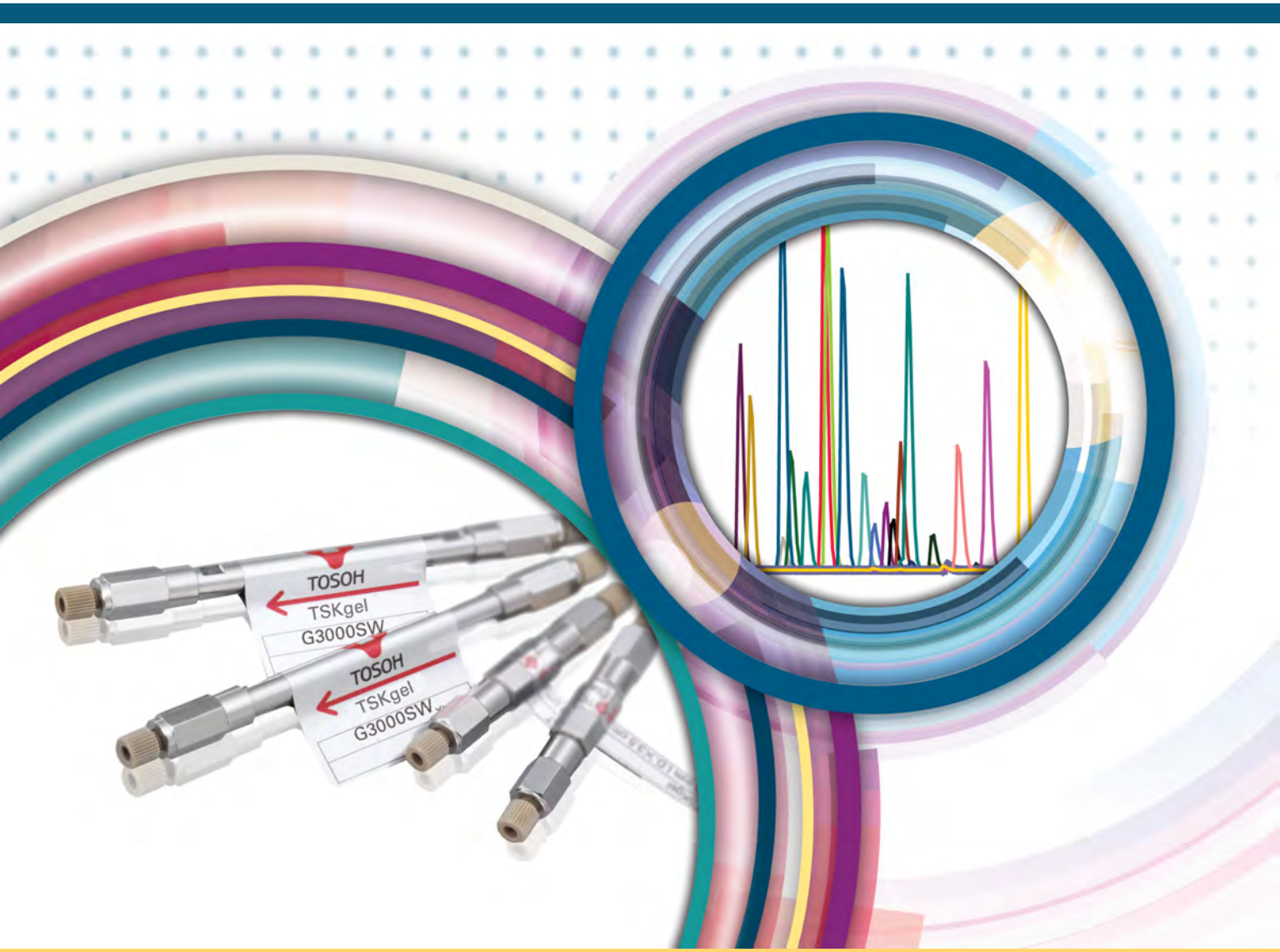
THE APPLICATION NOTEBOOK

TOSOH BIOSCIENCE



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MEDICAL/BIOLOGICAL

Use of Reversed Phase and HILIC in the 2D-LC Separation of Cationic, Anionic, and Hydrotropic Surfactants

Justin Steve and Atis Chakrabarti, PhD, Tosoh Bioscience LLC

Surfactants are frequently found in pharmaceutical and biopharmaceutical drug applications as well as in common household products. Because they can be polar, non-polar, or amphoteric, the structural diversity of the surfactants and complexity of the sample matrix can make their separation and identification by HPLC challenging. Historically, this has been achieved using reversed phase chromatography supplemented with ion exchange chromatography. When performed individually, these techniques can be used to characterize the surfactant profile. This application note illustrates the effectiveness of a reversed phase high throughput column, TSKgel® ODS-140HTP, in series with a TSKgel NH_2-100 HILIC column, for the separation of polar and non-polar surfactants in a single injection using 2D-LC.

Materials and Methods

Columns:	TSKgel ODS-140HTP, 2.3 µm, 2.1 mm ID × 5 cm
	TSKgel NH_2-100, 3 µm, 2 mm ID × 15 cm
Instrument:	Agilent 1200 HPLC system run by Chemstation (ver. B.04.02)
Mobile phase:	A. CH_3CN
	B. 100 mmol/L ammonium acetate, pH 5.4
Gradient:	40–60% A, 2.7 min, hold at 85% A, 5.4 min
Flow rate:	0.5 mL/min
Detection:	UV @ 280 nm, 254 nm, and 210 nm
	FLD λex 280 nm, λem 350 nm
Temperature:	30 °C
Injection vol.:	1 µL
Sample:	Triton™ X
	Triton N
	sodium xylenesulfonate
	sodium dodecylbenzene sulfonate

Results and Discussion

Figure 1 depicts the separation of four surfactants using the TSKgel ODS-140HTP and TSKgel NH_2-100 columns in series. The use of a shallow, non-linear gradient of 40–60% CH_3CN in 2.7 min, followed by an isocratic hold at 85% CH_3CN till 5.4 min, allowed for high resolution of the sodium dodecylbenzene sulfonate impurities and good retention of sodium xylene sulfonate as well. This figure illustrates the utility of 2D-LC methodology for separating a diverse mixture of surfactants.

Due to the success in resolving the surfactant standards in a single run as explained above, the usefulness of this technique in detecting the corresponding UV-absorbing surfactant ingredients in an actual commercial household product was evaluated. Figure 2 illustrates the characterization of the surfactant profile of Armor All™ Wash and Wax using the 2D-LC methodology described above. As shown, the use of the TSKgel ODS-140HTP and TSKgel NH_2-100 columns in series yielded excellent separation and retention of the anionic surfactant sodium dodecylbenzene sulfonate and the

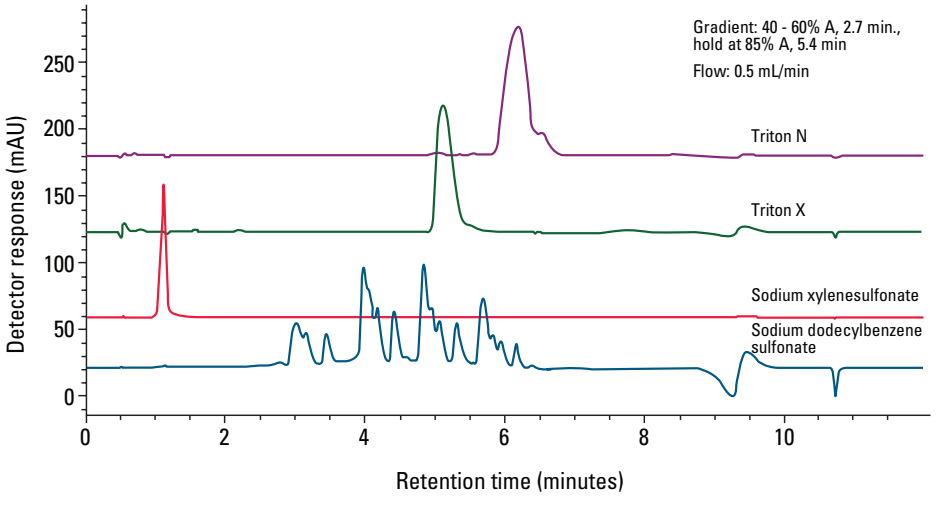

Figure 1: 2D-LC separation of surfactants using the TSKgel ODS-140HTP and the TSKgel NH_2-100 columns.

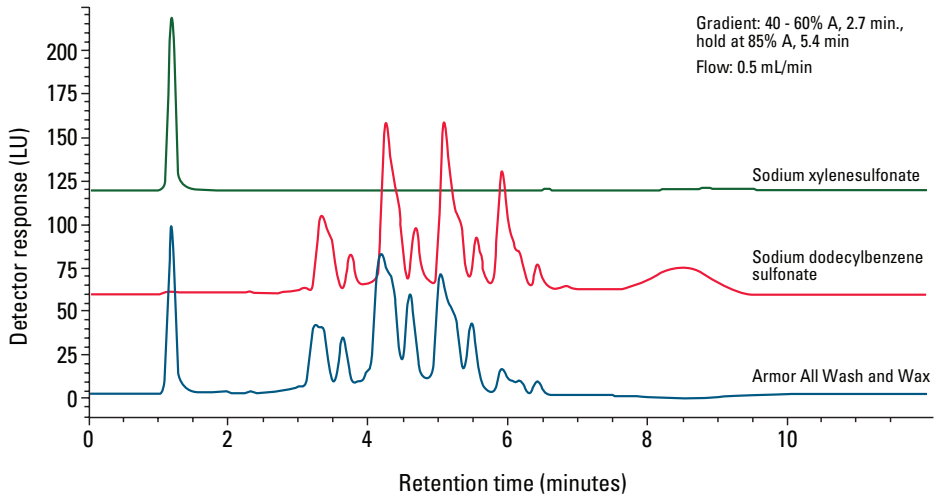

Figure 2: Characterization of surfactant profile in Armor All Wash and Wax using 2D-LC with the TSKgel ODS-140HTP and TSKgel NH_2-100 columns.

hydrotropic surfactant sodium xylene sulfonate present in the Armor All formulation. Additionally, the use of fluorescence detection (λex: 225 nm, λem: 300–400 nm) allowed for increased sensitivity of the low level surfactants found in the product.

Conclusions

The purpose of this study was to illustrate the effectiveness of 2D RP/HILIC for the separation of anionic, cationic, and hydrotropic surfactants. The use of the TSKgel ODS-140HTP column in series with the TSKgel NH_2-100 column allows for the simultaneous retention and separation of both polar an non-polar hydrotropic, nonionic, and anionic surfactants in a single injection. The characterization of surfactants in a common household product is also successfully performed with the TSKgel ODS-140HTP and TSKgel NH_2-100 columns, allowing for strong retention and good peak shape of various surfactants with no interference from other matrix compounds.

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Separation of Nucleobases Using TSKgel® SuperSW mAb HTP Column in HILIC Mode

Justin Steve and Atis Chakrabarti, PhD, Tosoh Bioscience LLC

Hydrophilic interaction liquid chromatography (HILIC) is one of the fastest growing modes of separation, in which any polar chromatographic surface can be used. Chemically bonded diol coated phases, as found in TSKgel SW size exclusion chromatography (SEC) columns, demonstrate high polarity and hydrogen bonding properties and do not contain ionizable groups other than the unreacted residual silanols, making them appropriate for HILIC mode.

For many years, SEC columns have been used to separate various nucleic acid species such as DNA, RNA, and tRNA as well as their constituent bases, adenine, guanine, thymine, cytosine, and uracil. In medicine, several primary nucleobases are the basis for the nucleoside analogues and other synthetic analogs which are used as anticancer and antiviral agents. Nucleobase modifications are the basis of oligonucleotide-based therapeutics, making their purification very important.

The TSKgel SuperSW mAb HTP column is a newly introduced SEC column designed for the high throughput separation of monoclonal antibodies from their high and low molecular mass variants. TSKgel SuperSW mAb HTP has a diol coating to minimize secondary interactions which may occur in SEC separations. This note demonstrates the benefits of using a TSKgel SuperSW mAb HTP column in HILIC mode for the superior resolution of four nucleobases, as opposed to using the column in SEC mode or using a HILIC column.

Materials and Methods

Instrumentation:	Agilent 1100 HPLC system run by Chemstation (ver B.04.02)
Columns:	TSKgel SuperSW mAb HTP, 4 µm, 4.6 mm i.d. × 15 cm TSKgel Amide-80, 5 µm, 2.0 mm i.d. × 10 cm
Mobile phase:	A: acetonitrile (HILIC mode) B: 15 mmol/L ammonium bicarbonate, pH 7.4 (HILIC mode)
Mobile phase:	100 mmol/L phosphate/100 mmol/L sodium sulfate, pH 6.7 + 0.05% NaN_3 (SEC mode)
Gradient:	Isocratic
Flow rate:	0.4 mL/min
Detection:	UV @ 280 nm
Injection vol.:	1 µL
Temperature:	ambient
Samples:	uracil (1.5 mg/mL), adenine (1.5 mg/mL), cytosine (1.5 mg/mL), cytidine (1.5 mg/mL) from Sigma Aldrich

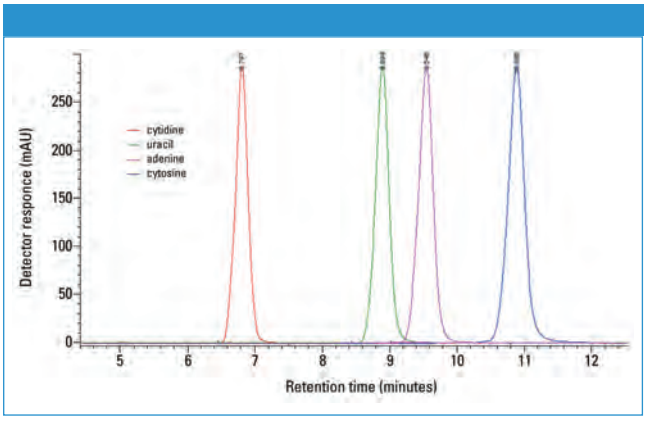

Figure 1: Separation of four nucleobases using TSKgel SuperSW mAb HTP column in HILIC mode at pH 7.4.

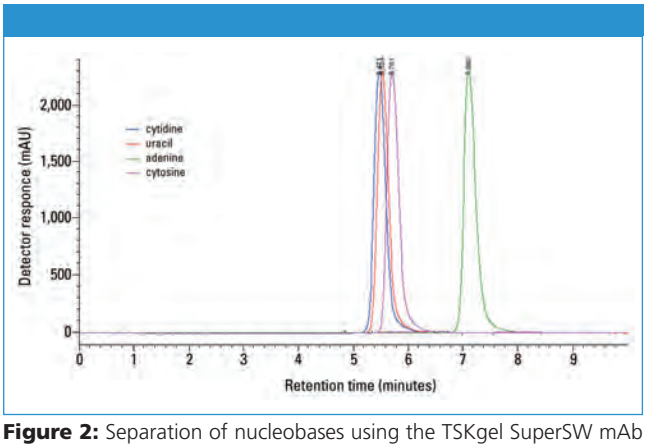

Figure 2: Separation of nucleobases using the TSKgel SuperSW mAb HTP column under conventional SEC conditions.

Results and Discussion

Figure 1 illustrates the separation of four nucleobases using the TSKgel SuperSW mAb HTP column in HILIC mode with 15 mmol/L ammonium bicarbonate, pH 7.4 as mobile phase B. It is important to note that the order of elution of the analytes does not correlate with their molecular mass (as in SEC separations), but instead is based on their relative hydrophilicity.

Figure 2 illustrates the separation of the four nucleobases on the TSKgel SuperSW mAb HTP column using conventional SEC conditions. As expected, due to the similarities in molecular masses between the four compounds, significant interference is observed amongst the peaks of interest, particularly the three pyrimidine derivatives, when separated on the TSKgel SuperSW mAb HTP column under SEC conditions. The late elution of adenine (relative to

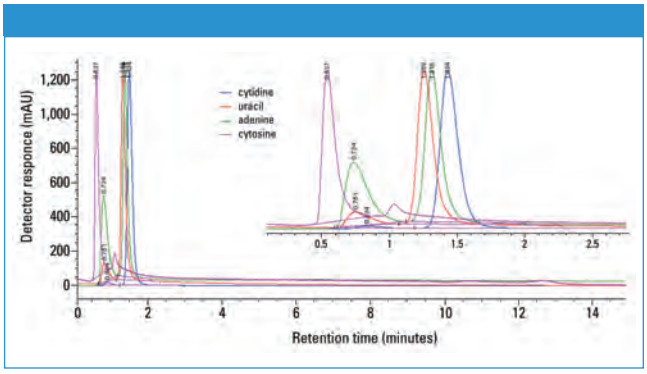

Figure 3: Separation of nucleobases using the TSKgel Amide-80 HILIC column.

the other three compounds) may be attributed to possible interactions between the stationary phase and the derivatized purine compound, leading to a shift towards longer retention time.

In an effort to explore the novelty of the separation of nucleobases using the TSKgel SuperSW mAb HTP column in HILIC mode, the same separation was carried out using a TSKgel Amide-80 HILIC column. The use of the TSKgel Amide-80 column yields very poor separation of the four nucleobases with virtually no retention of any of the components (Figure 3).

Conclusions

This work illustrates the novelty and utility of the TSKgel SuperSW mAb HTP column as a diol-functionalized HILIC column for the high resolution separation of nucleobases on the basis of their relative hydrophilicity, rather than differences in their relative molecular mass. As shown, markedly different separation profiles are observed with the use of the TSKgel Amide-80 HILIC column under identical chromatographic conditions. Additionally, nucleobase separation using the TSKgel SuperSW mAb HTP under conventional SEC conditions yielded poor resolution of all components, making it an ineffective mode of separation for this application. The TSKgel SuperSW mAb HTP column, while designed for SEC separation of monoclonal antibodies, is an extremely effective tool in HILIC mode that should be considered for the fast separation of nucleobases.

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Analysis of Monoclonal Antibody Aggregates by SEC Using MS-Friendly Mobile Phases

Justin Steve and Atis Chakrabarti, PhD, Tosoh Bioscience LLC

The use of mass spectrometry as a means of detection is becoming increasingly more common among research laboratories in the field of proteomics. After more than 15 years of research, LC–MS systems are now more robust, and used more often for routine analyses which are nearly unachievable by any other mode of detection.

Separation of protein aggregates from their native species is often performed using size exclusion chromatography (SEC), as this mode allows for the analysis of various components in a sample on the basis of their hydrodynamic radius in solution. Conventional SEC separations make use of relatively high ionic strength mobile phase compositions in an effort to minimize ionic interactions between the analyte and stationary phase. Due to the substantial amount of salt present in the mobile phase, on-line interfacing with mass spectrometry is not feasible due to the inevitable contamination of the MS ion source by the mobile phase salts. In order to make SEC-MS an applicable technique, volatile, MS-friendly mobile phase compositions must be implemented to avoid damage to the MS system. The challenge of using such mobile phase compositions exists due to the absence of salts which hinder ionic interactions during a separation.

This application note illustrates the effective use of MS-friendly mobile phase compositions in the analysis of monoclonal antibody aggregates using a TSKgel UltraSW Aggregate SEC column. The TSKgel® UltraSW Aggregate column demonstrates high stability and low reactivity via ionic interactions even in low salt and no salt environments most likely due to the diol coating of the silica stationary phase.

Materials and Methods

Instrumentation:	Agilent 1200 HPLC system run by Chemstation® (ver. B.04.02)
Column:	TSKgel UltraSW Aggregate, 3 µm, 7.8 mm ID × 30 cm
Mobile phase:	100 mmol/L PO_4/100 mmol/L SO_4, pH 6.7 20% CH_3CN/0.1% TFA/0.1% FA 100 mmol/L ammonium bicarbonate, pH 7.0
Gradient:	Isocratic
Flow rate:	1 mL/min
Detection:	UV @ 280 nm
Temperature:	25 °C
Injection vol.:	10 µL
Sample:	TBL mAb 1 (4.0 mg/mL)

Results and Discussion

Figure 1 illustrates the separation of mAb 1 using three different mobile phase compositions. All three mobile phase compositions yielded highly similar results for all peak parameters of the mAb 1 monomer. Additionally, slightly later elution of the mAb 1 monomer was observed when separated using the salt-based mobile phase. The observed shift in retention time of the mAb 1 monomer peak represents a %RSD of 0.1% among the three mobile phase conditions. Similarly average peak area of the mAb 1 monomer was found to be highly reproducible as well, yielding a %RSD of 0.53% across the three mobile phase systems evaluated in this work. This illustrates that the observed differences in retention time and peak areas obtained from the three mobile phase systems are not statistically significant.

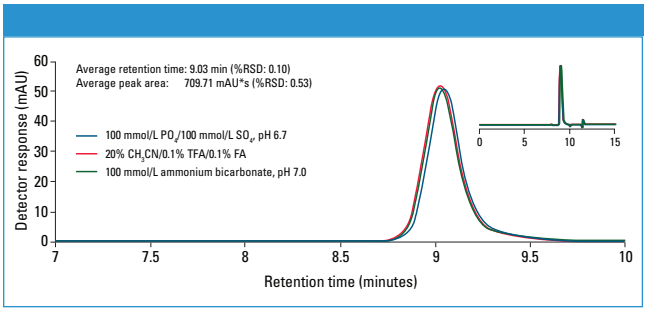

Figure 1: Separation of mAb 1 using volatile and salt-based mobile phase compositions on the TSKgel UltraSW Aggregate column.

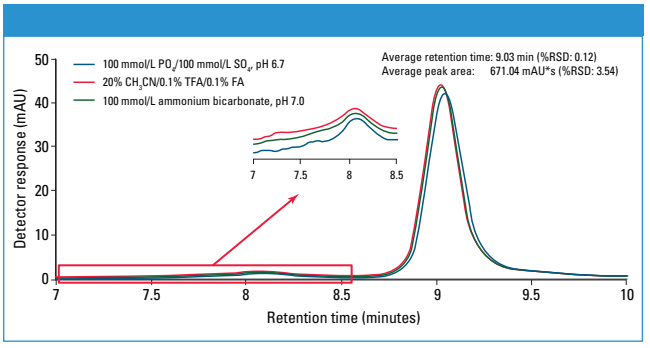

Figure 2: Separation of forced aggregated mAb 1 using volatile and salt-based mobile phase compositions on the TSKgel UltraSW Aggregate column.

As characterization of protein aggregates is of increasing importance in research, and with significant work being directed towards analysis by MS, the mAb 1 antibody was subjected to thermal stress for forced aggregation to evaluate the various mobile phase systems in this context. As shown in Figure 2, aggregates of mAb 1 are clearly separated from the monomeric species using all three mobile phase compositions. Similar to Figure 1, results for critical peak parameters of the mAb 1 monomer are highly reproducible

regardless of the mobile phase composition. Additionally, the total peak area (monomer and aggregate) obtained using the three mobile phase systems was highly similar to one another (avg: 671.04 mAU*s), and yielded a %RSD of 3.54%, illustrating the differences are not statistically significant. It is also of note that the observed shift in retention time of the mAb 1 monomer peak only corresponds to a %RSD of 0.12% for all three mobile phase compositions.

Conclusions

The growing interest in both protein aggregate analysis and mass spectrometry in the field of proteomics demand effective SEC-MS methods utilizing suitable mobile phases. The use of volatile mobile phase systems, such as 20% acetonitrile, 0.1% trifluoroacetic acid, and 0.1% formic acid or 100 mmol/L ammonium bicarbonate at pH 7.0 yield highly reproducible separation of mAb 1 aggregates, with similar or better performance over traditional salt-based mobile phase compositions. These results also show the effectiveness of the diol coating on the TSKgel UltraSW Aggregate column to assist in minimizing ionic interaction which are frequently present between the sample and stationary phase in low salt environments.

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UHPLC Separations Using HPLC Methods and TSKgel® Columns

Tosoh Bioscience LLC

The use of UHPLC systems for small molecule analysis has gained widespread acceptance among researchers in recent years. A UHPLC system is a HPLC system optimized with regards to dead volume, injection performance, and detector sampling rate and is able to tolerate application pressures exceeding 1000 bar. It is therefore advantageous to use UHPLC instrumentation for methods developed on conventional HPLC systems with HPLC columns. The use of HPLC columns with UHPLC systems offers the advantages of cost and time savings over having to purchase and develop new methods with UHPLC columns. This application note demonstrates the excellent performance of conventional TSKgel HPLC columns on a UHPLC system.

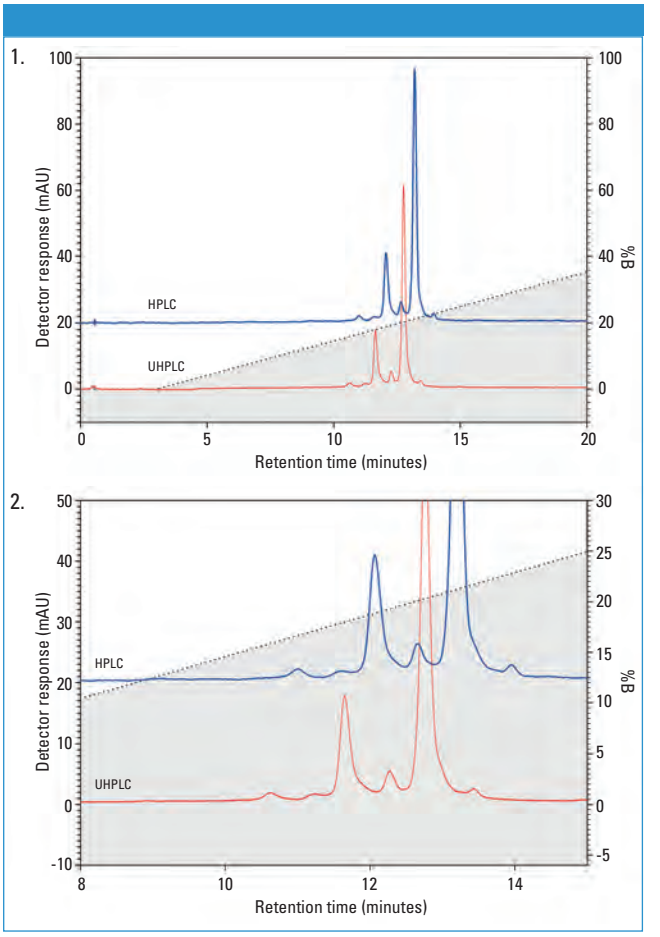
Experimental Conditions

Column:	TSKgel SP-STAT, 7 µm, 4.6 mm i.d. × 10 cm
Systems:	Dionex UltiMate® 3000 HPLC System (equipped with Dionex UVD 170S Detector) Dionex UltiMate 3000RS UHPLC System
Mobile phase:	A: 10 mmol/L sodium phosphate, pH 7.0 B: 10 mmol/L sodium phosphate, pH 7.0, + 1 mol/L sodium chloride
Gradient:	0–50% B in 25 min
Flow rate:	1.0 mL/min
Detection:	UV @ 280 nm
Injection vol.:	5 µL
Sample:	monoclonal IgG, 1 mg/mL

Column:	TSKgel G2000SWXL, 5 µm, 7.8 mm i.d. × 30 cm
Systems:	Dionex UltiMate 3000 HPLC System (equipped with Dionex UVD 170S Detector) Dionex UltiMate 3000RS UHPLC System
Mobile phase:	0.1 mol/L sodium phosphate, pH 6.7 + 0.1 mol/L sodium sulfate
Flow rate:	1.0 mL/min
Detection:	UV @ 280 nm
Injection vol.:	20 µL
Samples:	para-aminobenzoic acid, thyroglobulin, γ-globulin, ovalbumin, ribonuclease A, 2 mg/mL each

Results and Discussion

Figure 1 shows the analysis of a monoclonal IgG using a TSKgel SP-STAT cation exchange column. The same column, sample, and method were used to verify the column-system compatibility on both the HPLC (blue chromatogram) and UHPLC (red chromatogram) systems. The column performs better in the UHPLC system than it does when connected to the HPLC system. The number of theoretical plates is 6% higher for the UHPLC setup.



Figures 1 and 2: Analysis of a monoclonal IgG using a TSKgel SP-STAT column and a Zoomed Elution Profile of the mAb charge variants on the TSKgel SP-STAT column.

Figure 2 shows a zoomed elution profile of the mAb charge variants on the TSKgel SP-STAT column, which provides better insight into what extent the elution profile benefits from using a UHPLC system. The peak width is smaller for the UHPLC chromatogram. The decreased system dead volume resulted in the peak elution to occur earlier than it did on the HPLC system.

Figure 3 shows a standard protein mixture analyzed using a TSKgel G2000SWXL size exclusion column. The number of theoretical plates exceeds 32,000 for para-aminobenzoic acid when connected to the UHPLC system (blue chromatogram), while the column connected to the HPLC system (red chromatogram) only

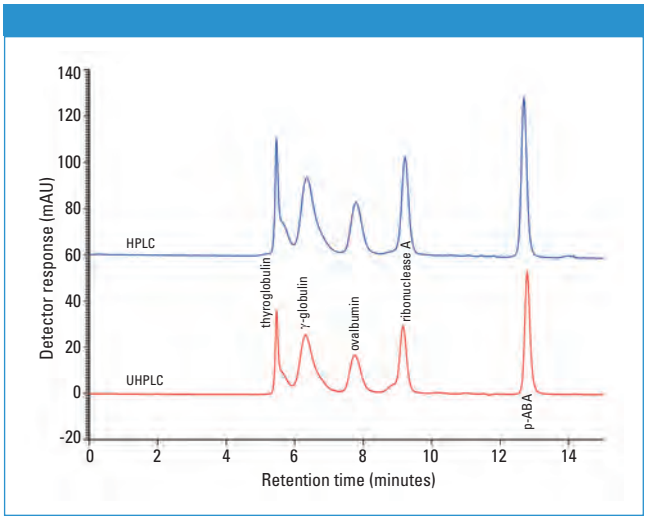


Figure 3: Separation of a standard protein mixture using a TSKgel G2000SW$_{XL}$ column.

reaches 29,000 for the same component.

Of course, resolution is an important factor when considering chromatographic performance. The HPLC data shows a resolution of 2.1 for the separation of γ-globulin and ovalbumin, and 10.2 for ribonuclease A and para-aminobenzoic acid. For the UHPLC data, the resolution factors increase to 2.2 and 10.9 for the respective peak pairs.

Conclusions

The results of both analyses using a TSKgel SP-STAT ion exchange column and a TSKgel G2000SW$_{XL}$ size exclusion column indicate excellent separation with high resolution when used on a UHPLC system. As a UHPLC system is simply an optimized HPLC system, bioseparation method transfer from HPLC to UHPLC is hardly more complicated than from one HPLC instrument to another. The applied pressure in these applications was far from exceeding the limit of a conventional HPLC system. This is the case in most bioseparation applications, since many biological sample molecules themselves cannot withstand very high pressure. Transferring established HPLC methods using HPLC columns to a UHPLC system is an economical and time-saving way to take advantage of this latest wave of development in HPLC technology.

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Separation of a Monoclonal Antibody Monomer from Its Impurities Using New TSKgel® SW mAb Columns

Atis Chakrabarti, PhD and Justin Steve, Tosoh Bioscience LLC

The analysis of monoclonal antibodies (mAb) is growing in importance in the field of biotherapeutics for the treatment of a variety of diseases. Quality control of therapeutic mAb is essential, as the introduction of species to the body other than the monomer may induce toxic side effects. Therefore, the pure antibody monomer must be very well resolved from its dimer and higher molar mass aggregates, as well as the antibody fragments. Size exclusion chromatography (SEC) is the best choice for determining mAb monomers and their impurities, including aggregates, oligomers, and mAb fragments.

Tosoh Bioscience has answered the call for dedicated SEC columns for the high resolution separation of mAb with the new silica-based 4 µm TSKgel SuperSW mAb HR column, for high resolution separation of the monomer and dimer, and the 3 µm TSKgel UltraSW Aggregate column for the separation and quantification of mAb aggregates and oligomers. This application note demonstrates the superb performance of these new columns for the analysis of monoclonal antibodies.

Experimental Conditions

Column:	TSKgel SuperSW mAb HR, 4 µm, 7.8 mm i.d. × 30 cm TSKgel G3000SWXL, 5 µm, 7.8 mm i.d. × 30 cm
Mobile phase:	200 mmol/L potassium phosphate buffer + 0.05% NaN_3, pH 6.7
Flow rate:	1.0 mL/min
Detection:	UV @ 280 nm
Temperature:	25 °C
Injection vol.:	10 µL
Sample:	10 g/L IgG digested with papain for 0–24 h

Column:	TSKgel UltraSW Aggregate, 3 µm, 7.8 mm i.d. × 30 cm
Mobile phase:	100 mmol/L potassium phosphate buffer, 100 mmol/L sodium sulfate, pH 6.7 + 0.05% NaN_3
Flow rate:	1.0 mL/min
Detection:	UV @ 280 nm
Temperature:	60 °C
Injection vol.:	20 µL
Sample:	BI-mAb-02 (4.6 mg/mL)

Results and Discussion

IgG monomer, dimer, and fragments digested by papain over a 24 h period were analyzed using the TSKgel SuperSW mAb HR column (Figure 1). The results exhibit the superior resolving power of this column for monomer/fragment and monomer/dimer separation (Rs = 2.87 and 2.02 respectively).

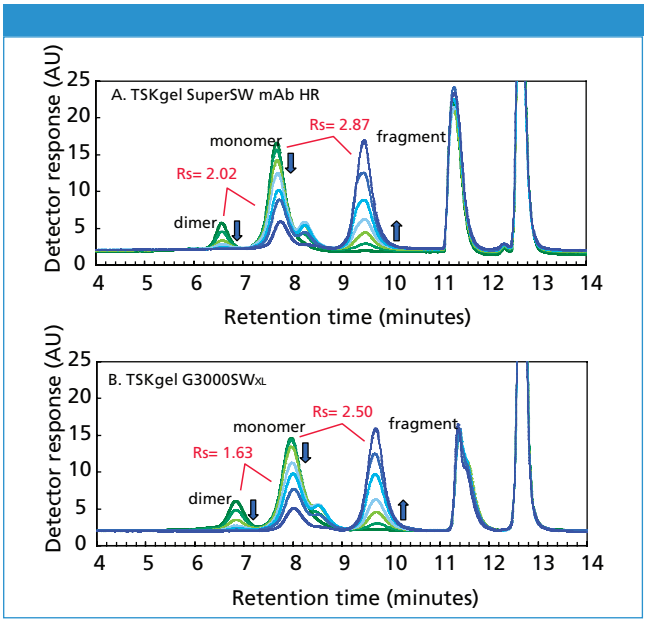

Figure 1: Separation of IgG monomer, dimer, and fragments from papain digested IgG by TSKgel SuperSW mAb HR and TSKgel G3000SWXL columns.

The results also show that the TSKgel SuperSW mAb HR column has superior performance of mAb separation in comparison to the TSKgel G3000SWXL column. While TSKgel G3000SWXL has set the standard for the separation of general proteins for more than 25 years, the new TSKgel SuperSW mAb HR column is more specifically suited for the analysis of mAb, as seen in the results of the analysis of IgG.

A heat denaturation study of a monoclonal antibody was conducted using a TSKgel UltraSW Aggregate column. The column was used to monitor the denaturation of the antibody as a function of time at pH 5.5 and 60 °C. Heating for 1 h at 60 °C results in almost complete breakdown of the monoclonal antibody and the formation of very large aggregates that extend to the exclusion volume of the column. As seen in Figure 2, the efficient separation of aggregates from the monomer, induced by heat denaturation, could be achieved using the TSKgel UltraSW Aggregate column. Also shown in Figure 2, an "unknown" aggregate peak of intermediate molar mass between the monomer and dimer and several higher order aggregate peaks, in addition to the presumed dimer peak at 8.5 min, was seen.

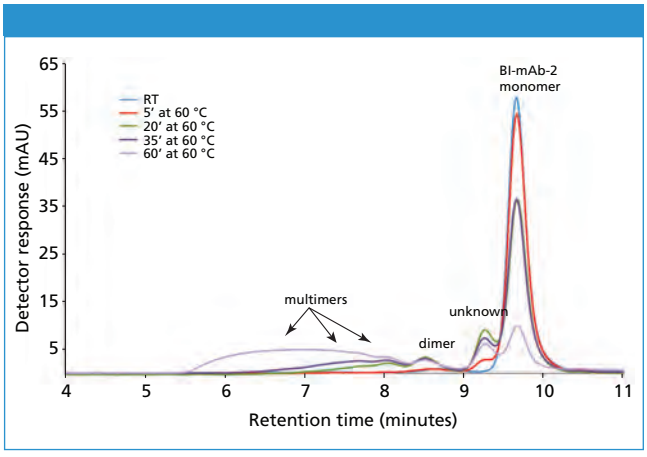

Figure 2. Heat denaturation study of monoclonal antibody (BI-mAb-02) using a TSKgel UltraSW Aggregate column.

Conclusions

The results of both analyses show the superb performance of the new TSKgel SuperSW mAb HR and UltraSW Aggregate columns for the analysis of monoclonal antibodies. The TSKgel SuperSW mAb HR column exhibited superior resolving power for IgG monomer, dimer, and fragments, while the TSKgel UltraSW Aggregate column demonstrated efficient separation of aggregates from the monomer peak. These new additions to the TSKgel SW-type column line are an excellent choice for your mAb analysis: TSKgel SuperSW mAb HR for high resolution monomer, dimer, and fragment analysis, TSKgel UltraSW Aggregate for superior resolution of aggregates.

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Analysis of Monoclonal Antibody and Protein Aggregates Induced by Denaturation Using a Novel Size Exclusion Chromatography Column

Atis Chakrabarti, PhD, and Justin Steve, Tosoh Bioscience LLC

Degradation studies of biotherapeutic proteins are necessary to test their stability. The best way to test the suitability of a method is the use of real-time stability samples containing all relevant degradation products that might occur over time. Certain factors, such as product development timeline, process characteristics, excipients, and other environmental factors, however, make the use of a forced degradation study necessary. The biological phenomenon of protein aggregation is a major issue in therapeutic protein development, since the presence of these impurities reduces the potency of the drug formulation, even if non-toxic. Monoclonal antibody proteins, widely being used in the field of biotherapeutics, with the potential to replace small molecules in the future, must be free from these aggregate impurities.

In order to fully evaluate the aggregates, a size exclusion column is needed which has a large enough molecular exclusion limit, so that the higher order aggregates are not excluded in the void but separated as a function of hydrodynamic volume. This application note will show the superior resolving power of the TSKgel® UltraSW Aggregate column for the analysis of monoclonal antibody and metalloprotein aggregates formed under forced denatured conditions, including acid and thermal denaturation.

Experimental Conditions

Figure 1

Column:	TSKgel UltraSW Aggregate, 3 µm, 30 nm, 7.8 mm ID × 30 cm
Mobile phase:	100 mmol/L potassium phosphate buffer, 100 mmol/L sodium sulfate, pH 6.7 + 0.05% NaN_3
Flow rate:	1.0 mL/min
Detection:	UV @ 280 nm
Temperature:	ambient
Injection vol.:	10 µL
Sample:	mAb-02 from Boehringer-Ingelheim (gift from Tosoh Bioscience GmbH)
Concentration:	4.5 g/L in glycine/Na phosphate, pH 6.0

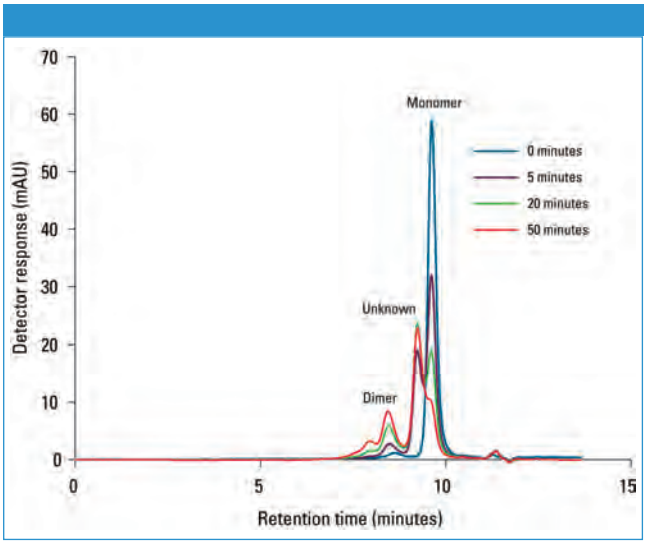


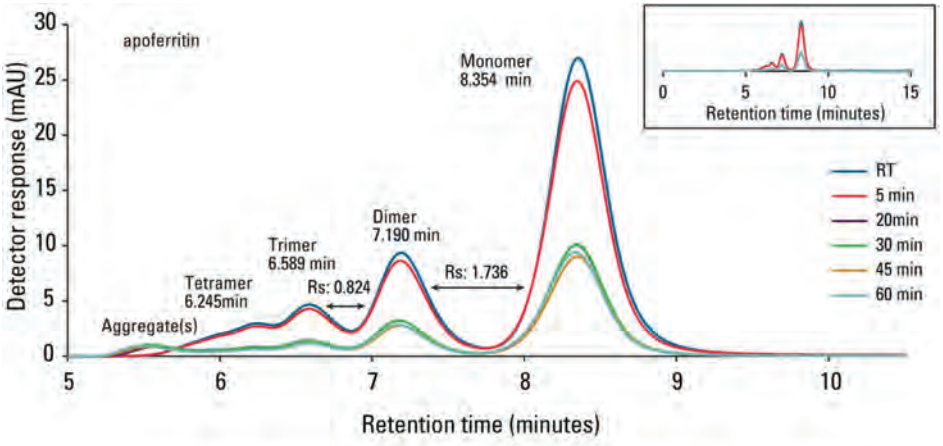
Figure 1: Analysis of mAb aggregates formed by forced denaturation by acid at pH 4.7.

Figure 2

Column:	TSKgel UltraSW Aggregate, 3 µm, 30 nm, 7.8 mm ID × 30 cm
Mobile phase:	50 mmol/L potassium phosphate (monobasic), 50 mmol/L sodium phosphate (dibasic) 100 mmol/L sodium sulfate, 0.05% NaN_3, pH 6.7
Flow rate:	1.0 mL/min
Detection:	UV @ 280 nm
Temperature:	30 °C
Injection vol.:	10 µL
Sample:	Apoferritin Sigma, 5.0 mg/mL in 50% glycerol and 0.075 mol/L sodium chloride, stored at -20 °C

Results and Discussion

Figure 1 shows the use of a TSKgel UltraSW Aggregate column for the analysis of mAb aggregates formed by the forced acid denaturation of a monoclonal antibody. After reducing the pH of the mAb-02 sample solution to 4.7 by dilute phosphoric acid, aliquots were analyzed at 5, 20, and 50 min and the response was compared to that of the original sample solution. The degradation of the monoclonal antibody creates a larger MW entity (unknown) that elutes directly after the dimer and before the monomer. Continued decay increases both peaks, but more so for the dimer. Clearly, the dimer peak height increases while the peak height of the monomer decreases. Hints of higher order "multimers" show between 7 and 8 min.



Protein	Molecular weight (kDa)			
	Monomer	Dimer	Trimer	Tetramer
apoferritin	450	900	1350	1800

Figure 2: Analysis of heat induced forced denatured, large hydrophobic metalloprotein, apoferritin.

The analysis of a heat denatured, large hydrophobic metalloprotein, apoferritin, is shown in Figure 2. A set of six, 0.3 mL HPLC vials each containing 100 µL stock solution of apoferritin was used for protein thermal denaturation. Thermal denaturation was carried out at 60 °C using an electric heating block. Individual sample vials were tightly capped and exposed to the heat for 5, 20, 30, 45, and 60 min. Samples were analyzed using a TSKgel UltraSW Aggregate column at the end of each incubation time period. The TSKgel UltraSW Aggregate column yielded high resolution between the monomer and dimer. The trimer, tetramer, and higher order aggregates of apoferritin were well separated.

Conclusions

The results of both analyses demonstrate the high resolution separation of the aggregates of a monoclonal antibody and the large metalloprotein, apoferritin, generated by forced denaturation using a TSKgel UltraSW Aggregate column. With the increasing use of monoclonal antibodies in biotherapeutics, the TSKgel UltraSW Aggregate offers superior analysis of aggregates.

The TSKgel UltraSW Aggregate, 3 µm, SEC column with 30 nm pore size is specially designed with controlled pore technology which produces a shallow calibration curve in the molecular weight region of a typical monoclonal antibody. The larger pore size with an estimated exclusion limit of ~2 × 10^6 Da provides improved resolution and quantitation of mAb aggregates and oligomers. The TSKgel UltraSW Aggregate is an excellent choice for the analysis of monoclonal antibody protein aggregates, present in their native state or when induced by forced denaturation.

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Separation of Monoclonal Antibody (mAb) Monomer from its Half-body using Size Exclusion Chromatography

Justin Steve and Atis Chakrabarti, PhD Tosoh Bioscience LLC

Monoclonal antibody (mAb) research continues to grow in an effort to develop effective biotherapeutics for a wide range of diseases. Recent research has shown an interest in mAb half-bodies as therapeutic vectors as they can be further targeted for conjugation, enzyme labeling, or antibody immobilization.

Monoclonal antibody half-bodies can be generated through the genetic engineering of cells or by selective reduction of hinge-region disulfide bonds present in the mAb by mild reducing agents, such as TCEP [tris(2-carboxyethyl)phosphine]. Due to its lack of odor and resistance to oxidation in the presence of air, TCEP is a stable reducing agent commonly used in mAb half-body formation.

A mAb half-body was generated through protein reduction using TCEP and subsequently identified by gel electrophoresis for use in this study. The superior resolution obtained between a monoclonal antibody monomer and half-body species using a TSKgel SuperSW mAb HR column is demonstrated in this application note.

Experimental Conditions

Column:	TSKgel SuperSW mAb HR, 4 µm, 7.8 mm ID × 10 cm
Mobile phase:	0.1 mol/L phosphate/0.1 mol/L sulfate buffer + 0.05% NaN_3
Flow rate:	0.5 mL/min
Detection:	UV @ 280 nm
Temperature:	25 °C
Injection vol.:	10 µL
Sample:	human IgG (4.6 g/L) – Sigma

Results and Discussion

The complex and diverse nature of mAb structures make the reproduction of published methods difficult when using unique mAb samples. For this reason, multiple mAb reduction protocols were investigated for this study, all using TCEP Bond-Breaker® (Thermo Scientific). The use of 150 mmol/L TCEP with human IgG (4.6 g/L) incubated for 20 hours at 37 °C yielded the highest concentration of mAb half-body without excessive reduction of the protein into its low molar mass fragments. Predictably, the molar mass of the mAb half-body was approximately 70 kDa, or half that of the intact mAb.

Figure 1 illustrates the separation of human IgG monomer, half-body and fragment (1/3 mAb) formed using the TCEP reduction method discussed above using a TSKgel SuperSW mAb HR column. High resolution (Rs = 1.13) of the IgG monomer and half-body species was achieved.

SDS-PAGE was used to confirm the identity of the mAb monomer, half-body and fragment collected from the SEC separation on the TSKgel SuperSW mAb HR column. Fractions of each protein

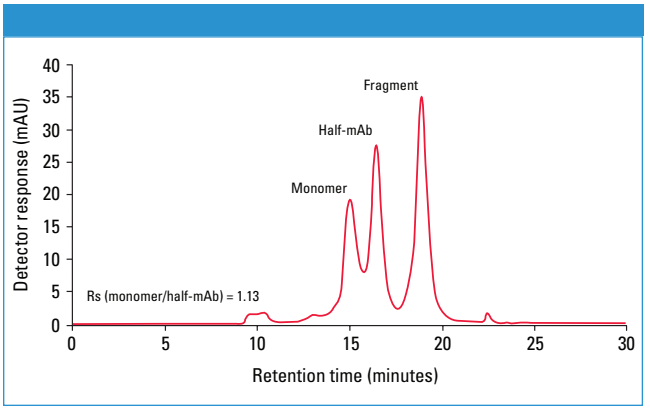

Figure 1: Separation of Human IgG Monomer, Half-body, and Fragments using a TSKgel SuperSW mAb HR column.

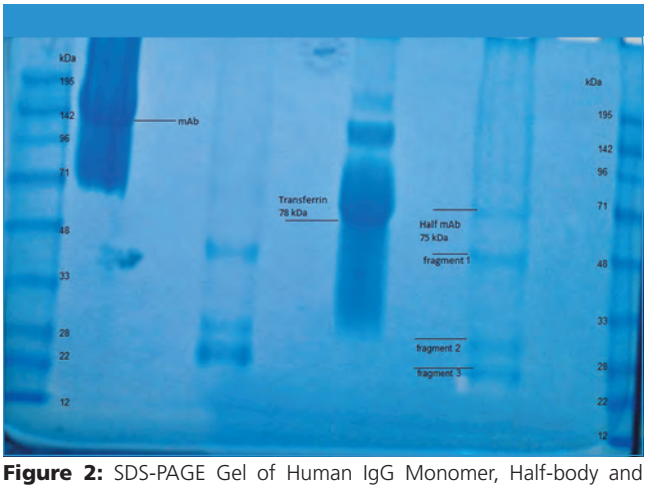

Figure 2: SDS-PAGE Gel of Human IgG Monomer, Half-body and Fragments Separated using a TSKgel SuperSW mAb HR column.

species were collected during the SEC separation and precipitated using acetone. The acetone was then removed and the protein precipitates were reconstituted in 100 µL of SDSPAGE running buffer. The monoclonal antibody, half mAb and the fragment are clearly identified with the SDS-PAGE molar mass marker as well as transferrin (78 kDa) (Figure 2). This clearly shows that the half mAb could be generated using the TCEP reduction method and separated using the TSKgel SuperSW mAb HR column.

Conclusions

After investigation of multiple mAb reduction methods, it was determined that 150 mmol/L TCEP with human IgG incubated for 20 hours at 37 °C yielded a high concentration of IgG half-body.

Separating the reduction products (IgG monomer, half-body and fragment) on the TSKgel SuperSW mAb HR column yielded high resolution (Rs of 1.13).

The TSKgel SuperSW mAb HR is able to achieve high resolution between the mAb and the mAb half-body due to its unique pore-controlled technology optimized for mAb analysis, as well as its smaller 4 µm particle size. Gel electrophoresis confirmed the identity of the reduction products separated using the TSKgel SuperSW mAb HR column. This study shows an excellent method for the separation of half-mAb or mAb half-body using the TSKgel SuperSW mAb HR column.

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Improved SEC Separations Using TSKgel® Columns on a UHPLC System

Tosoh Bioscience LLC

Ultra high performance liquid chromatography (UHPLC) continues to grow in popularity due to highly efficient columns and fast run times, which result in excellent resolution and high throughput. UHPLC instrumentation is an optimized HPLC with low dead volume and has the ability to maintain constant flow rate against the higher back pressure generated by columns packed with small particles.

Recently introduced by Tosoh Bioscience, the TSKgel SuperSW mAb HTP column is able to be used with a UHPLC system, taking advantage of this column's smaller particle size and optimized dimensions. The end result is fast run times with a traditional HPLC column on a UHPLC system.

This note details the rapid analysis of an aggregated monoclonal antibody.

Experimental Conditions

Column:	TSKgel SuperSW mAb HTP, 4 µm, 4.6 mm i.d. × 15 cm
System:	Dionex UltiMate® 3000RS UHPLC System
Mobile phase:	0.1 mol/L sodium phosphate, pH 6.7 + 0.1 mol/L sodium sulfate
Flow rate:	0.35 mL/min 0.5 mL/min 0.7 mL/min
Detection:	UV @ 280 nm
Injection vol.:	5 µL
Sample:	aggregated monoclonal antibody

Results and Discussion

Figure 1 shows what is possible with a short gel filtration column made of HPLC column hardware packed with 4 µm particles when used on a UHPLC system. The new TSKgel SuperSW mAb HTP (4.6 mm i.d. × 15 cm) is optimized for high throughput applications and is compatible on UHPLC systems because of its ability to withstand high flow rates and low pressure drops.

It is possible to shorten analysis time of antibody aggregates from 15 min, using a conventional 30 cm column with 5 µm particles, down to 4 min when using a 15 cm column with 4 µm silica particles. The particle size is still large enough to keep the pressure in a moderate range, avoiding frictional heating inside the column, which might cause further aggregation or fragmentation during the analysis. These kinds of artifacts can be misinterpreted as an indication of a higher degree of impurity than actually exists within the sample vial.

Conclusions

The results of the analysis of a monoclonal antibody using the new TSKgel SuperSW mAb size exclusion column show a fast and high resolution separation using a UHPLC system. The TSKgel SuperSW mAb HTP column can withstand high flow rates and low pressure drops.

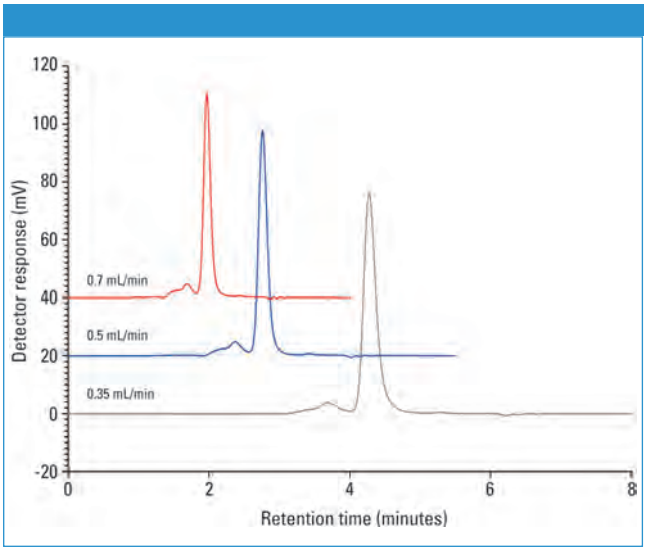

Figure 1: Aggregated mAb sample analyzed on a TSKgel SuperSW mAb HTP column.

The use of HPLC columns with UHPLC systems offers the advantages of cost and time savings over having to purchase and develop new methods with UHPLC columns. Whether you are working with a UHPLC system or a conventional system, TSKgel columns are the first choice for bioseparations.

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CaPure-HA™

15	30.974
277	2.1
44.30	
P	
[Ne]3s²3p³	
1.82	±3,4,5

92	238.029
4134	1.2
1132	
U	
[Rn]5f⁶6d7s²	
19.0	3,4,5,6

75	186.207
5596	1.5
3180	
Re	
[Xe]4f¹⁴5d⁶6s²	
19.0	-1,2,4,6,7

34	78.96
685	2.5
221	
Se	
[Ar]3d¹⁰4s²4p⁴	
4.79	-2,4,6

91	231.036
1600	1.1
Pa	
[Rn]5f²6d7f²	
4.79	4,5

88	2326.025
1536	
700	
Ra	
[Rn]7s²	
5.0	2

22	47.867
3289	1.6
1670	
Ti	
[Ar]3d²4s²	
5.0	3,4

8	15.999
-182.82	3.5
-222.65	
O	
[He]2s²2p⁴	
1.43	-2

7	14.007
-195.65	3.1
-209.86	
N	
[He]2s²2p³	
1.25	2,±3,4,5

16	32.07
444.75	2.4
115.36	
S	
[Ne]3s²3p⁴	
2.07	2,±3,4,5

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