# SHIMADZU
## Excellence in Science

# Application News

## No.L486

High Performance Liquid Chromatography

# Analysis of Polysorbate 80 in IgG Aqueous Solution by Online SPE Using Shim-pack MAYI Column – Part 1

Various analyses are required in the evaluation of drugs. When analyzing samples that contain macromolecular proteins at high concentrations by reversed phase HPLC, degradation of the column packing is a concern when using a typical ODS column, therefore requiring prior removal of proteins. As the Shim-pack MAYI series, with its packing pore outer surface coated with a hydrophilic polymer, offers a line-up of online SPE columns that can quickly eliminate proteins, when combined with a column switching HPLC, a variety of components can be analyzed in a seamless flow from deproteinization to analysis.

Examples of applications related to analysis of drugs in plasma and serum using the MAYI series have previously been reported in Application News No. L285, 286, 293, 305, 307, 315 and 327. By using the Co-Sense for BA bio-sample analysis system, even higher sensitivity, higher precision measurement can be achieved.

Here, we introduce an example of analysis of the polysorbate 80 surfactant, widely used as an additive to prevent protein aggregation and adsorption, and to increase protein solubility in a protein formulation.

### ■ Principle of Shim-pack MAYI Column

Fig. 1 shows the structure of the packing used in the Shim-pack MAYI column. While macromolecular proteins are blocked and cannot enter the pores, smaller molecules infiltrate the chemically modified pores to be retained on the column.
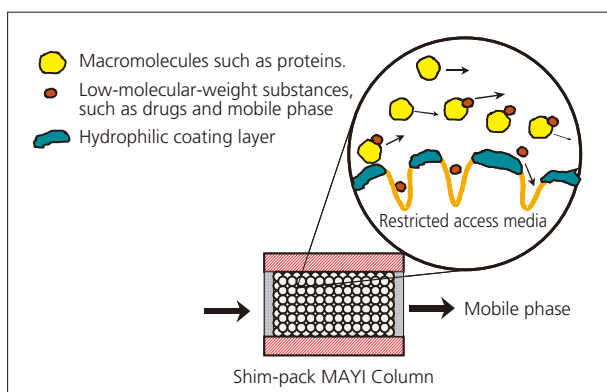


- Macromolecules such as proteins.
- Low-molecular-weight substances, such as drugs and mobile phase
- Hydrophilic coating layer

Restricted access media

Mobile phase

Shim-pack MAYI Column

**Fig. 1  Principle of Deproteinization with Shim-pack MAYI Column**

By incorporating this column in the column switching HPLC flow line shown in Fig. 2, proteins introduced into the pretreatment column from the autosampler are directly discharged out of the system after passing through the column.
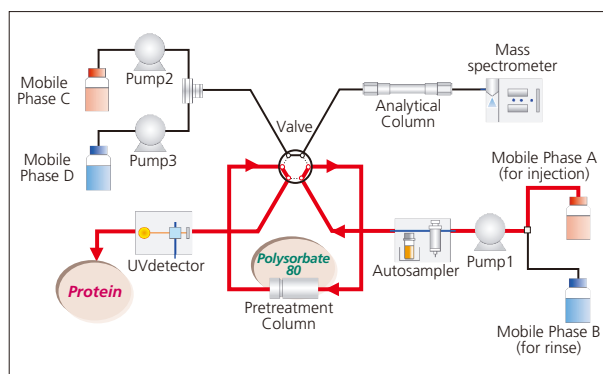


**Fig. 2  Flow Diagram**

By using a UV detector (wavelength 280 nm) to monitor an IgG model sample (described below), a chromatogram such as that shown in Fig. 3 is obtained, confirming that protein (IgG) is rapidly discharged.
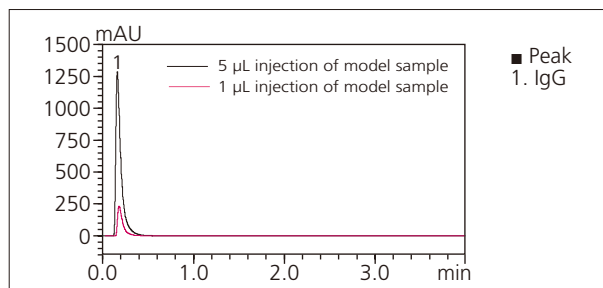


- 5 µL injection of model sample
- 1 µL injection of model sample
- ■ Peak 1. IgG

**Fig. 3  Confirmation of Protein Elution from Shim-pack MAYI Column**

**Table 1  Analytical Conditions (Sample Loading)**

| | |
|---|---|
| Column | : Shim-pack MAYI-ODS (5 mm L. × 2.0 mm I.D., 50 µm) |
| Mobile Phase | : A: 10 mmol/L Ammonium Formate in Water B: 2-Propanol |
| Time Program | : Solvent switching A (0 - 1.5 min) → B (1.5 - 3.5 min) → A (3.5 - 9 min) |
| Flowrate | : 0.6 mL/min |
| Extraction Time | : 1 min |
| Injection Vol. | : 1 µL |
| Column Temp. | : 40 ˚C |
| Detection | : UV280 nm (Semi-micro cell) |

On the other hand, a Shim-pack MAYI-ODS column, with packing pores that are chemically modified with C18 (octadecyl group), was used to extract polysorbate 80 on the pretreatment column side. After discharging the protein (in this case, 1 minute later), the valve is switched to direct the pretreatment column to the analysis channel, while the sample introduction flow line is rinsed to prepare for the next analysis, all operations that were programmed beforehand for automated execution.

■ **Analysis of Standard Solution**

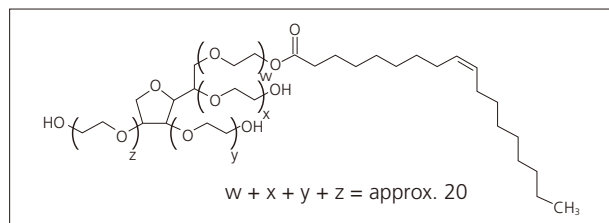The structural formula for polysorbate 80 (polyoxyethylene sorbitan monooleate) is shown in Fig. 4.

**Fig. 4 Typical Structure of Polysorbate 80**

$w + x + y + z = \text{approx. } 20$

Due to the weak UV absorption of polysorbate, a mass spectrometer was used for detection in the analytical flow line. The analytical conditions are shown in Table 2, and the TIC chromatogram of a standard sample (100 µg/mL) is shown in Fig. 5. Generally, polysorbate includes a large number of by-products, and because some of these are very strongly retained, 2-propanol was used as the final mobile phase.

**Table 2 Analytical Conditions**

| | |
|---|---|
| Column | : Kinetex 5u C18 100 Å |
| | (50 mm L. × 2.1 mm I.D., 5 µm) |
| Mobile Phase | : C: 10 mmol/L Ammonium Formate in Water |
| | D: 2-Propanol |
| Time Program | : D.Conc. 5 % (0 - 1 min) → 100 % (6 - 7 min) |
| | → 5 % (7.01 - 9 min) |
| Flowrate | : 0.3 mL/min |
| Column Temp. | : 40 °C |
| Detection | : LCMS-2020 |
| Ionization Mode | : ESI Positive |
| Applied Voltage | : 4.5 kV |
| Nebulizer Gas Flow | : 1.5 mL/min |
| Drying Gas Flow | : 15 L/min |
| DL Temp. | : 250 °C |
| Block Heater Temp. | : 400 °C |
| Scan Range | : m/z 300 - 2000 |
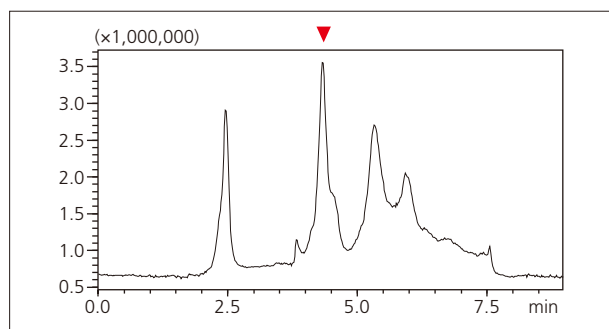
**Fig. 5 TIC Chromatogram of 100 µg/mL Polysorbate 80 Standard**

The mass spectrum of the peak in the retention time vicinity of 4.4 minutes is shown in Fig. 6. Many peaks are observed because of the included polyoxyethylene, which displays different degrees of polymerization. However, we conducted SIM measurement using the ion at m/z 783 as a marker for detection, which is attributable to the 2NH4+ adduct of polyoxyethylene sorbitan monooleate, containing 25 polyoxyethylene groups. (Fig. 7)
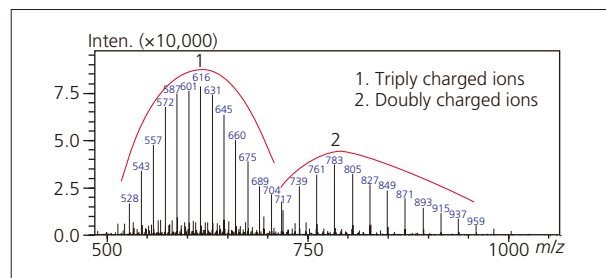
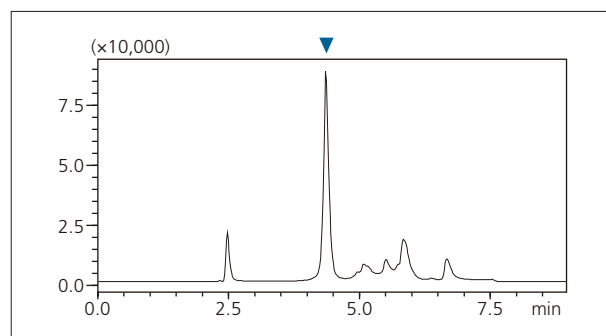**Fig. 6 Mass Spectrum of the Peak at 4.4 min in Fig. 5**

**Fig. 7 SIM Chromatogram of 100 µg/mL Polysorbate 80 Standard**

The results indicated a coefficient of determination ($R^2$) greater than 0.999 over a concentration range of 10 to 200 µg/mL, demonstrating excellent linearity. Following this, these conditions were applied to a protein-containing model sample.
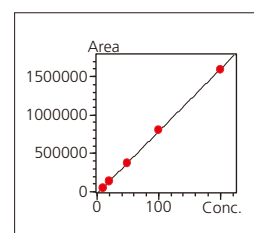
**Fig. 8 Linearity (10-200 µg/mL)**

■ **Analysis of Antibody Model Sample**

Polysorbate 80 was added to 10 mmol/L phosphate buffer solution (pH 6.8) that included 20 mg/mL of IgG, to obtain a concentration of 100 µg/mL, and this was injected into the HPLC as the sample. Utilizing online auto deproteinization, the polysorbate 80 recovery rate was 99 %, demonstrating measurement with excellent repeatability (retention time: 0.034 % RSD, peak area: 1.11 % RSD).
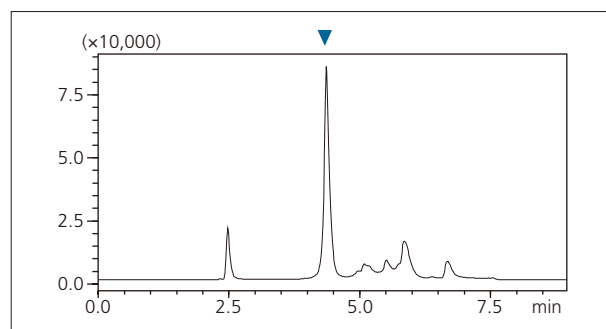
**Fig. 9 SIM Chromatogram of Antibody Model Sample**

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Liquid Chromatograph with online Sample Preparatio…

Shim-pack

> **Shim-pack MAYI Series**
HPLC Column

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