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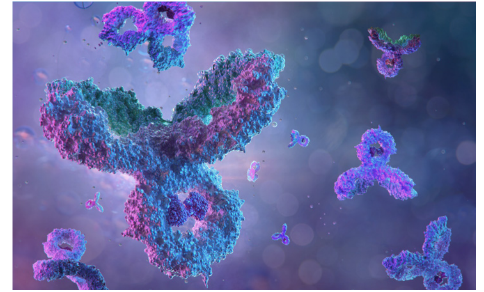
Consumables workflow ordering guide

Polysorbate Degradation Analysis with the AdvanceBio Surfactant Profiling HPLC Column



Introduction

Polysorbates are ubiquitous ingredients in healthcare products, from eye drops and cough syrups to mouthwash and sunscreen. Polysorbates act as emulsifiers, solubilizers, surfactants, and stabilizers in these products. In protein-based therapeutics such as monoclonal antibodies (mAbs), antibody-drug conjugates (ADCs), bispecifics, and fusion proteins, polysorbate 20 and polysorbate 80 are the most common surfactants used. The polysorbates work to prevent protein aggregation and protein-surface interactions to keep the therapeutic stable. Another advantage of polysorbates is their high biocompatibility and low toxicity properties. Polysorbates have a complex structure, and current regulations from the various international pharmacopoeias each have their own purity requirements. Furthermore, certain grades of polysorbate have higher purities as well as lower levels of endotoxins and peroxides.

A major issue associated with polysorbates is their tendency to degrade, compromising the stability of therapeutics. The two primary mechanisms of polysorbate degradation are hydrolysis and oxidation. Hydrolysis can occur chemically, although this is less frequent and typically occurs at extreme pH levels. More commonly, enzymatic hydrolysis occurs by lipases or esterases, which are proteins present in host cells. In 2024, the United States Pharmacopeia began selling lipoprotein lipase as a host cell protein heavy-labeled standard because of its concerning role in polysorbate degradation.[1] Oxidation, on the other hand, is driven by residual peroxides and is accelerated by light exposure or transition metals, even at typical pH and temperature conditions.

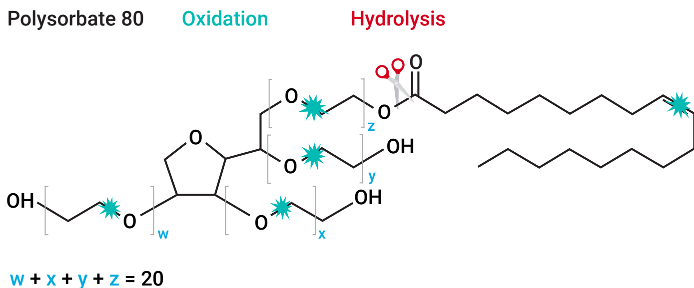


Figure 1. A diagram showing the two mechanisms by which polysorbates degrade: hydrolysis and oxidation. Hydrolysis cleaves the fatty acid, while oxidation can occur in several places on the polysorbate structure.

Despite the lack of current regulatory guidance on surfactant degradation, it is crucial not to overlook its analysis Polysorbates are undergoing increased scrutiny from the US Food and Drug Administration and European Medicines Agency.[2,3] Implementing this type of assay in the early stages of development will help to prevent quality issues later in the pipeline. Biopharma companies also have a growing interest in and awareness of this type of analysis.[4,5] This workflow ordering guide presents an end-to-end workflow, including tips and tricks to easily enable surfactant degradation analysis in laboratories. Performing this analysis early on could help prevent and mitigate protein stability issues at later times and stages.

Best practices

Sample preparation

Sample preparation is one of the biggest pain points of this workflow. The overall goal of sample preparation is to remove the biologic from the polysorbate and polysorbate components. There are two options available, not performing sample preparation at all or protein precipitation.

- In general, sample preparation should always be performed, so avoiding this step is not recommended. Although the Agilent AdvanceBio Surfactant Profiling column is a wide-pore column, injecting a large amount of protein may overload the column and/or interfere with the polysorbate peaks. In contrast, injecting a low concentration (≤ 10 mg/mL) and a low volume (≤ 15 µL) with the gradient under the recommended starting conditions causes the protein to elute in approximately one minute and does not interfere with the polysorbate peaks.
- Protein precipitation is a common approach to protein removal. For example, a 50% methanol and 50% ethanol mixture can be used to precipitate protein, and the supernatant can be taken for analysis. The supernatant should be diluted to closely match starting mobile phase conditions. The advantage of protein precipitation is that it works well for very concentrated protein solutions.

Recommended starting conditions

Table 1. Suggested LC method for analysis of surfactant profiling using the 2.1 x 50 mm Agilent AdvanceBio Surfactant Profiling column.

Parameter	Value
Flow Rate	0.25 mL/min
Mobile Phase	A: 10 mM ammonium acetate B: Methanol
Column Temperature	25 °C
Gradient	0–0.2 min; 0% B 0.2–0.6 min; 0–50% B 0.6–1.5 min; 50% B 1.5–5.1 min; 50–95% B 5.1–7 min; 95% B 7–8 min; 95–0% B 8–10 min; 0% B
Needle Wash	20:80 methanol:water, flush for 10 seconds
1290 Infinity II ELSD Conditions	Evaporator Temperature: 30 °C Nebulizer Temperature: 30 °C Gas Flow Rate: 1.20 SLM

Optimizing chromatographic conditions

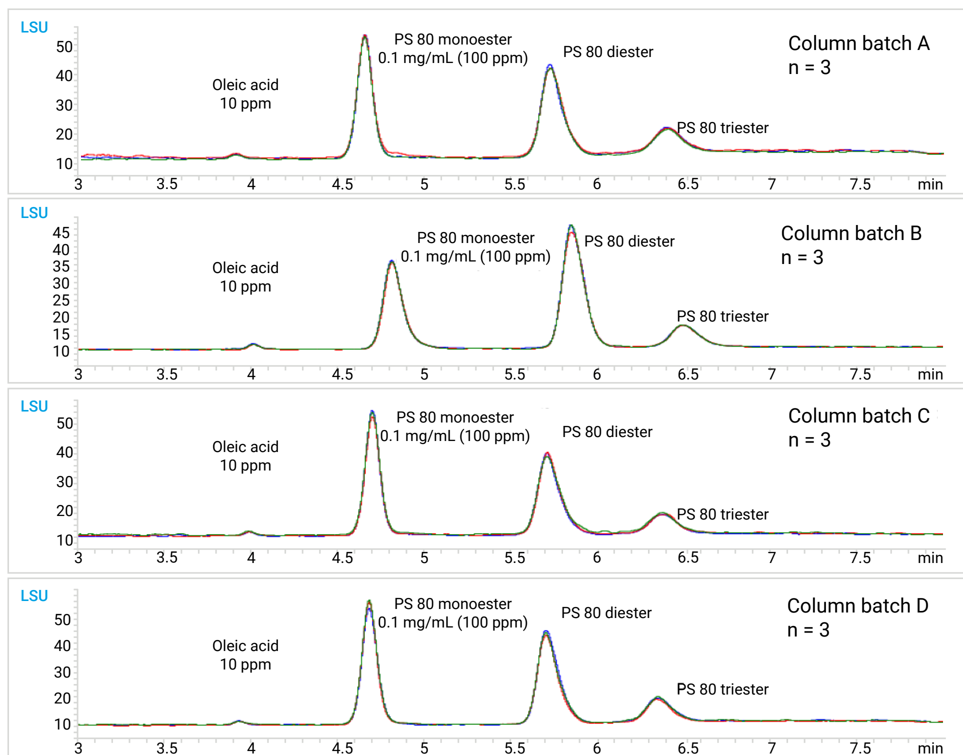
- Ammonium acetate is a hygroscopic salt. If possible, buy in small, high purity batches to ensure that weight measurements are consistent.
- Mobile phase pH should be maintained for consistent column chemistry and reproducible separations.
- Polysorbates can be "sticky", so the needle wash is highly recommended to avoid carryover (Table 1). If free fatty acids are causing carryover, consider increasing the methanol content of the methanol/water wash.
- Lower the flow ramp rate from the default to 1 mL/min or lower. The gradual increase in flow rate will prolong column lifetime and help prevent sudden overpressuring. This setting can be found in the Advanced section for LC pump control in the Agilent software.
- Set the maximum pressure limit in the LC method to match that of the column. This pressure should be 600 bar for the AdvanceBio Surfactant Profiling column, except for the 2.1 x 50 mm and 4.6 x 50 mm dimension columns, which have a limit of 400 bar.
- Monitor column and guard column performance by choosing a few specifications and tracking them regularly.

Evaporative light scattering detector (ELSD)

- Consider 10 mM ammonium acetate or lower in the aqueous phase, as the ELSD nebulizer will become dirtier or clogged faster with a higher salt concentration.
- In the event of a noisy baseline or loss in sensitivity, clean the nebulizer and/or evaporator tube. Refer to your ELSD manual for instructions.[7]
- Use a high performing LC pump to minimize nebulization issues, which will in turn improve reproducibility.

Mass spectrometry

- Divert the LC stream to waste when outside of the retention times of interest, especially during a high organic rinse at the end of the method and, if possible, as the void volume elutes.
- Use HPLC-grade or higher solvents.
- Establish a regular cleaning routine for the MS source.
- If the sample contains phosphate buffer, perform either a buffer exchange or inject very low amounts, as phosphate will suppress the mass spec signal.



Inter-batch retention time (n =12)		
Analyte	Average	%RSD
Oleic Acid	3.97	1.1
PS 80 Mono	4.72	1.3

Inter-batch peak Area (n = 12)		
Analyte	Average	%RSD
Oleic Acid	7.27	7.9
PS 80 Mono	263.1	12.4

Inter-batch resolution (n=12) (Oleic acid & PS 80 monoester)		
Analyte	Average	%RSD
Oleate-PS 80	4.97	3.1

Figure 2. The Agilent AdvanceBio Surfactant Profiling column demonstrates strong batch-to-batch reproducibility. One of the key factors to strong reproducibility is ensuring that the mobile phase is prepared in a consistent manner.[6]

Getting started

The AdvanceBio Surfactant Profiling column has been uniquely designed to characterize surfactants and their degradation products with a high-resolution and high-throughput method.

Selecting optimal column dimensions

- The 50 mm length column is ideal when screening for polysorbate hydrolysis. The method was designed to provide high resolution between the free fatty acid and polysorbate monoester peak in a 10-minute run.
- The 50 mm length column is available in 2.1 and 4.6 mm inner diameters (ids). The 2.1 mm id is ideal for increased sensitivity, and the 4.6 mm id is best if larger volume injections are required.
- The 100 and 150 mm length columns are ideal for increased resolution. These would be most appropriate for polysorbate oxidation analysis, as the degradation profile is more complex with many different degradant by-products.
- Guard columns will increase analytical column lifetime and should match the analytical column's id.

Selecting the right detector

- Polysorbates lack a chromophore, so UV analysis is not ideal for this application.
- ELSD or charged aerosol detector (CAD) are often the detectors of choice for polysorbate analysis.
- Mass spectrometry, particularly high-resolution accurate mass instrumentation, is appropriate for elucidating structure analysis with tandem mass spectrometry and for providing accurate mass measurements, which can be especially useful in more complicated oxidation analysis.

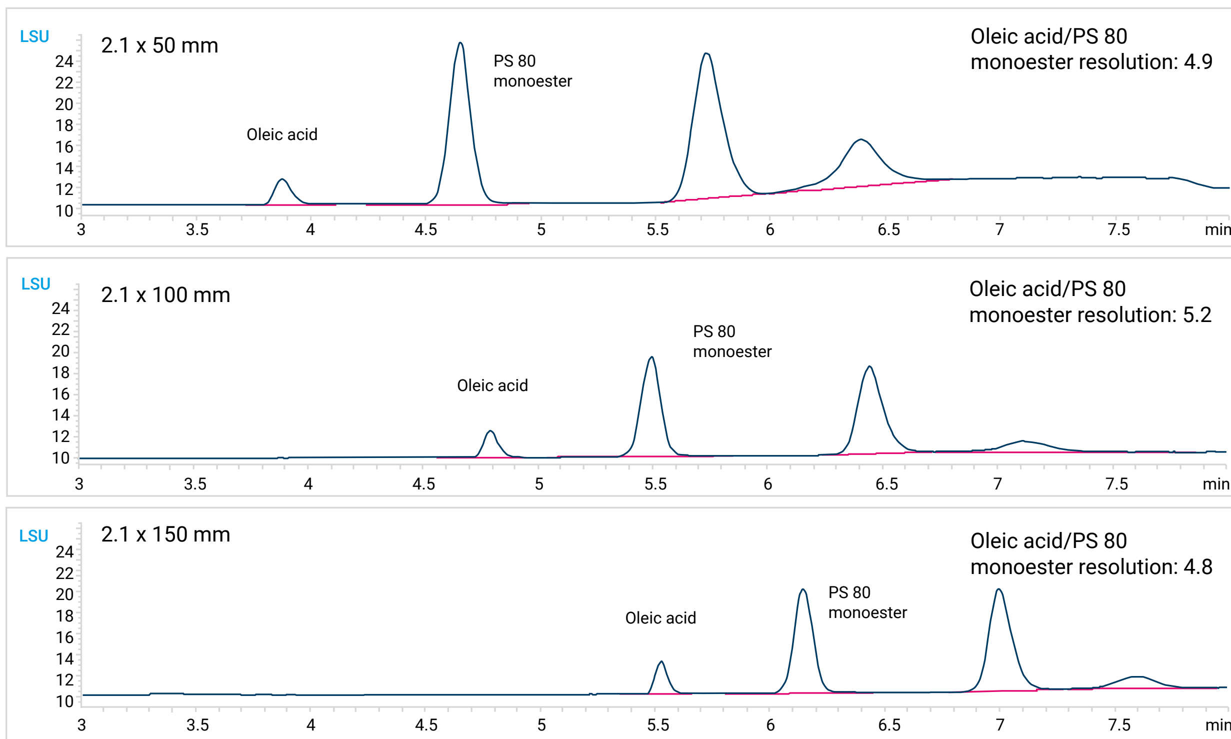


Figure 3. While longer columns increase resolution, the 2.1 x 50 mm column is more than sufficient to resolve the free fatty acid and polysorbate monoester peaks. Using a 100 mm column slightly increases the resolution, but the difference is not significant.[8]

Easy selection and ordering information

To order items listed in the tables from the Agilent online store, add items to your Favorite Products list by clicking on the MyList # header links. Then, enter the quantities for the products you need, add the products to your Cart, and proceed to checkout. Your list will remain under Favorite Products for your use with future orders.

If this is your first time using Favorite Products, you will be asked to enter your email address for account verification. If you have an existing Agilent account, you will be able to log in. However, if you do not have a registered Agilent account, you will need to register for one. This feature is valid only in regions that are eCommerce enabled. All items can also be ordered through your regular sales and distributor channels.

MyList 1: AdvanceBio Surfactant Profiling HPLC Columns

Description	Part No.
AdvanceBio Surfactant Profiling, 2.1 x 50 mm	865750-907
AdvanceBio Surfactant Profiling, 2.1 x 100 mm	861775-907
AdvanceBio Surfactant Profiling, 2.1 x 150 mm	863750-907
AdvanceBio Surfactant Profiling, 2.1 mm fast guard (3 pack)	821126-927
AdvanceBio Surfactant Profiling, 4.6 x 50 mm	865973-907
AdvanceBio Surfactant Profiling, 4.6 mm fast guard (3 pack)	820951-927

MyList 2: Protein/Peptide Standards

Description	Part No.
Agilent NIST mAb, 25 µL	5191-5744
Agilent NIST mAb, 4 x 25 µL	5191-5745

MyList 3: Supplies & Solvents

Description	Part No.
Connections and Tubing	
InfinityLab Quick Connect LC fitting	5067-5965
Quick Connect capillary stainless steel 0.12 x 105 mm	5500-1173
InfinityLab Quick Connect Fitting assembly with pre-fixed 0.12 x 105mm capillary (for connection on column inlet)	5067-5957
InfinityLab Quick Turn Fitting (for connection on column outlet)	5067-5966
Quick Turn Capillary SST 0.12 x 280 (for Quick Turn fitting)	5500-1191
Mounting tool for quick turn fittings	5043-0915
Ultralow dispersion tubing kit for Agilent 1290 Infinity II	5067-5963
Filter	
InfinityLab Quick Change inline filter assembly, for UHPLC Including 5 filter discs (2.1 mm id, 0.2 µm pore size), with 90 mm flexible capillary	5067-1603
InfinityLab Quick Change filter disc, 2.1 mm id, 0.2 µm pore size, 5/pk. for InfinityLab Quick Change inline filter	5067-1610
Sample Containment	
High recovery vial, screw top, with fixed insert, clear, 300 µL insert volume, 100/pk. Vial size: 12 x 32 mm (12 mm cap)	5188-6591
Cap, screw, blue, PTFE/red silicone septa, 100/pk. Cap size: 12 mm	5182-0717
Vial, screw top, polypropylene, certified, 250 µL, 1,000/pk. Vial size: 12 x 32 mm (12 mm cap)	5190-2243
Cap, screw, blue, certified, PTFE/white silicone septa, 500/pk. Cap size: 12 mm	5185-5863
InfinityLab 96-well plate, 0.5 mL, 30/pk	5043-9310
InfinityLab 96-well plate closing mat, 50/pk	5042-1389
Solvents	
InfinityLab water for LC/MS, 1 x 1 L	5191-5121-001
InfinityLab methanol for LC/MS, 1 x 1 L	5191-5111-001
Solvent Handling	
InfinityLab Stay Safe cap starter kit	5043-1222
InfinityLab solvent bottle, clear, 1 L	9301-6524
InfinityLab solvent bottle, amber, 1 L	9301-6526
Solvent bottle, clear, 2 L	9301-6342
Solvent bottle, amber, 2 L	9301-6341
InfinityLab Stay Safe purging bottle	5043-1339
InfinityLab waste can, GL45, 6 L with Stay Safe cap	5043-1221
InfinityLab charcoal filter with time strip, 58 g	5043-1193
Stay Safe starter kit and purging bottle, includes InfinityLab Stay Safe purging bottle (p/n 5043-1339) and Stay Safe caps starter kit (p/n 5043-1222)	5043-1340
Mass Spectrometry	
LC/MS Calibration standard, for ESI-TOF, 100 mL	G1969-85000
API-TOF Reference Mass Solution Kit	G1969-85001
Cloth, lint-free, 23 x 23 cm, 100% cotton, 15/pk	05980-60051
Abrasive mesh, 8000 grit (2 µm)	8660-0852

References

1. Host Cell Protein Contaminants in mAb and Protein Therapy Manufacturing. https://www.usp.org/biologics/host-cell-proteins.
2. Aryal, B.; Lehtimaki, M.; Rao, V. A. Stress Mediated Polysorbate 20 Degradation and Its Potential Impact on Therapeutic Proteins. *Pharm. Res*. **2024**, *41*, 1217-1232. DOI: 10.1007/s11095-024-03700-7.
3. Committee for Medicinal Products for Human Use, European Medicines Agency. Information for the Package Leaflet Regarding Polysorbates Used as Excipients in Medicinal Products for Human Use, EMA/CHMP/190743/2016, Last Revised December 2023. https://www.ema.europa.eu/en/documents/scientific-guideline/information-package-leaflet-regarding-polysorbates-used-excipients-medicinal-products-human-use_en.pdf.
4. Wuchner, K.; Yi, L.; Chery, C.; Nikels, F.; Junge, F.; Crotts, G.; Rinaldi, G.; Starkey, J.; Bechtold-Peters, K.; Shuman, M.; et al. Industry Perspective on the Use and Characterization of Polysorbates for Biopharmaceutical Products Part 1: Survey Report on Current State and Common Practices for Handling and Control of Polysorbates. *J. Pharm. Sci*. **2022**, *111*, 1280-1291. DOI: 10.1016/j.xphs.2022.02.009.
5. Wuchner, K.; Yi, L.; Chery, C.; Nikels, F.; Junge, F.; Crotts, G.; Rinaldi, G.; Starkey, J.; Bechtold-Peters, K.; Shuman, M.; et al. Industry Perspective on the Use and Characterization of Polysorbates for Biopharmaceutical Products Part 2: Survey Report on Control Strategy Preparing for the Future. *J. Pharm. Sci*. **2022**, *111*, 2955-2967. DOI: 10.1016/j.xphs.2022.08.021.
6. He, C.; Hale, W.A.; Coffey, A.; Tripodi, A.P.; McQuay, A. Shining Light on Polysorbate Hydrolysis by LC/ELSD Featuring the AdvanceBio Surfactant Profiling HPLC Column; *Agilent Technologies application note*, publication number 5994-8123EN, **2025**.
7. Agilent InfinityLab LC Series Evaporative Light Scattering Detectors; *Agilent Technologies user manual*, publication number D0013647 Rev. B, **2022**.
8. In-Depth Characterization of Surfactant and Surfactant Degradation in Biotherapeutic Applications: Agilent AdvanceBio Surfactant Profiling HPLC columns; *Agilent Technologies brochure*, publication number 5994-8065EN, **2025**.

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DE-004903

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Published in the USA, March 06, 2025
5994-8101EN

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