Application Note

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Shining Light on Polysorbate Hydrolysis by LC/ELSD

Featuring the Agilent AdvanceBio Surfactant Profiling HPLC column

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Abstract

Polysorbates are surfactants commonly used as stabilizers in protein-based therapeutics. Polysorbate hydrolysis is becoming of increasing concern, particularly as high-concentration formulations are becoming more popular. High-concentration formulations also increase the concentration of host cell proteins that can hydrolyze polysorbate. This application note addresses the challenge of monitoring polysorbate hydrolysis, presenting a fast, high-resolution method for separating free fatty acids from polysorbate monoesters. The method demonstrates high reproducibility, low carryover, and can be used for additional applications such as relative quantitation of polysorbates and free fatty acid analysis.

Introduction

Most monoclonal antibodies (mAbs) and other therapeutic proteins, including multispecific antibodies, antibody-drug conjugates, and fusion proteins, contain a stabilizer or surfactant in their formulation buffer to prevent aggregation and protein-surface interactions. Surfactants become more important as therapeutic concentrations increase, which increases the probability of aggregation. Polysorbates, particularly polysorbate 20 (PS 20) and polysorbate 80 (PS 80), work effectively even at low concentrations and are highly biocompatible, making them the surfactants of choice for protein therapeutics. With their lack of a chromophore, polysorbates can be detected using a universal detector, such as an evaporative light scattering detector (ELSD).

While polysorbates offer many advantages as surfactants in biotherapeutic formulation buffers, they are not without issues. Polysorbates can degrade by hydrolysis or oxidation. The focus of this application note is on degradation through hydrolysis. Hydrolysis can occur chemically or enzymatically. Enzymatic hydrolysis is more common because of host cell proteins that cleave the ester bond, releasing the fatty acids. The host cell proteins, typically lipases or esterases, are difficult to remove completely during purification, making hydrolysis a significant risk. In response, the US Pharmacopoeia (USP) has started selling stable isotope-labeled lipase peptide standards.[1] High concentration mAb formulations make purification and removal of host cell proteins even more challenging. Polysorbate degradation compromises the surfactant's ability to stabilize proteins[2] and can lead to the formation of proteinaceous or free fatty acid particles.[3,4]

An increasing number of protein therapeutic manufacturers are recognizing this issue and implementing polysorbate characterization methods.[5,6] However, separating free fatty acids from polysorbate monoesters using traditional C18 or C8 HPLC columns can be challenging, often requiring lengthy methods exceeding 30 minutes for baseline separation. Mixed-mode columns are ineffective for this analysis as they do not retain polysorbate degradation components, which elute in the void. This application note presents a new HPLC column and solution for monitoring polysorbate hydrolysis, offering a fast, easy-to-implement method with high resolution between free fatty acid and polysorbate monoester peaks.

Experimental

Materials

All chemicals were purchased from Sigma-Aldrich or Thermo Fisher Scientific.

Instrumentation

An Agilent 1290 Infinity II UHPLC system was coupled to an Agilent 1290 Infinity II ELSD.

- Agilent 1290 Infinity II high-speed pump (part number G7120A)
- Agilent 1290 Infinity II multisampler (part number G7167B)
- Agilent 1290 Infinity II thermostatted column compartment (part number G7116B)
- Agilent 1290 Infinity II evaporative light scattering detector (part number G7102A)

Method conditions

Table 1. LC/ELSD parameters used for polysorbate analysis.

Parameter	Value
Column	AdvanceBio Surfactant Profiling 300 Å, 3.5 µm, 2.1 × 50 mm (p/n 865750-907) with guard (p/n 821126-927)
Mobile Phase A	10 mM Ammonium acetate
Mobile Phase B	Methanol
Flow Rate	0.25 mL/min
Injection Volume	10 µL Unless otherwise specified
Column Temperature	30 °C
Hydrolysis Gradient	Time (min) %B 0 to 0.2 0 0.2 to 0.6 0 to 50 0.6 to 1.5 50 1.5 to 5.1 50 to 95 5.1 to 7 95 7 to 8 95 to 0 8 to 10 0
Free Fatty Acid Gradient	Time (min) %B 0 to 0.2 0 0.2 to 0.6 0 to 50 0.6 to 1.0 50 1.0 to 1.5 50 to 55 1.5 to 2.5 55 2.5 to 4 55 to 60 4 to 6.8 60 to 95 6.8 to 8 95 8 to 9 95 to 0 9 to 11 0
Needle Wash	20:80 Methanol:water
ELSD Evaporator Temperature	30 °C
ELSD Nebulizer Temperature	30 °C
Gas Flow Rate	1.20 SLM

Sample preparation

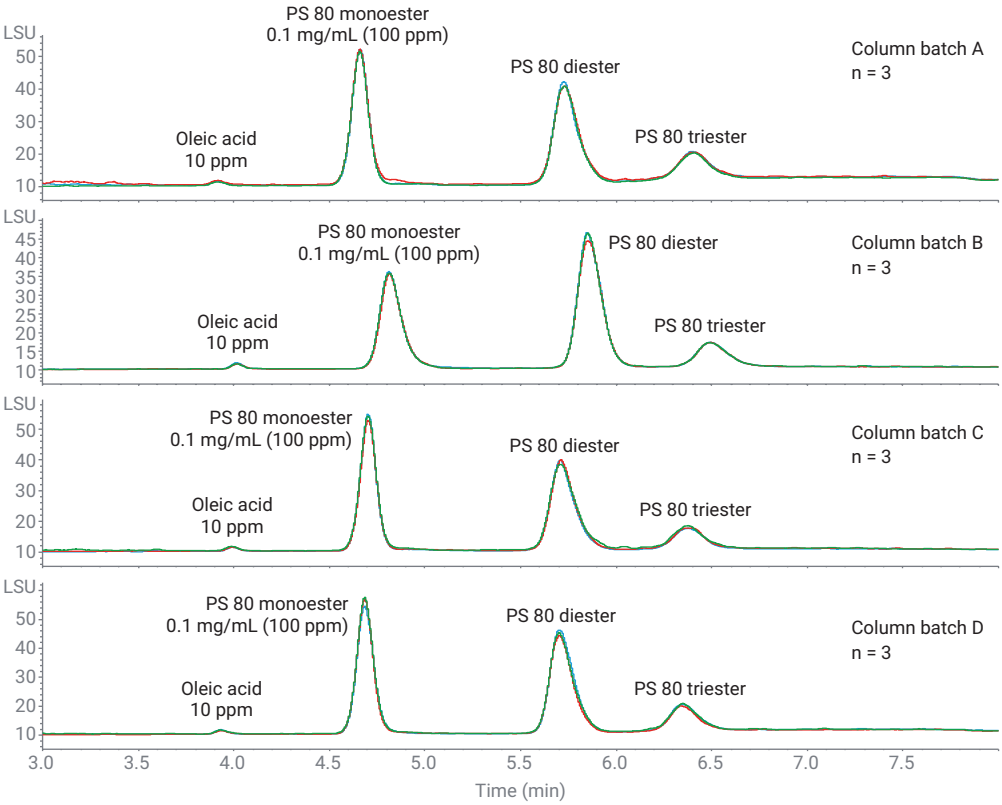
The formulation buffer consisted of a final concentration of 1.525 mg/mL sodium phosphate dibasic, 0.8625 mg/mL sodium phosphate monobasic, 6.1625 mg/mL sodium chloride, 1.3 mg/mL citric acid, and 0.3 mg/mL sodium citrate tribasic, adjusted to pH 6.0 with sodium hydroxide. PS 20 and 80 were spiked into the formulation buffer at 100 ppm, oleic acid was spiked at 10 ppm, and lauric acid was spiked at 5 ppm for the reproducibility and carryover studies. The fatty acid and quantitation sample preparation protocols are described in the Results and discussion section.

Results and discussion

Column quality and robustness

When developing any new method, ensuring reproducibility is crucial, whether it involves injection-to-injection or batch-to-batch consistency. For this specific method, it is imperative that the free fatty acids can be fully separated from the polysorbate monoester peak, and ideally, this should be done in as little time as possible.

Figure 1 demonstrates these requirements. PS 80 and its major fatty acid, oleic acid, show high resolution in a 10-minute run. In addition, three replicates were run from four different media batches, and the retention times, peak areas, and resolution were measured. The retention times for both oleic acid and the PS 80 monoester were consistent over four batches, with a variation of less than 1.5% relative standard deviation (RSD). The reproducibility of the oleic acid peak area over 12 replicates was 7.9%, and the %RSD for the PS 80 monoester between the batches was 12.4. The data show that batch B was mostly responsible for this variation. Although Agilent LC columns are maintained to high-quality standards, some variations within a certain range are normal. These variations do not have a strong effect on retention time or resolution, the latter of which has an impressive average of 4.97 resolution between oleic acid and the PS 80 monoester with a %RSD of 3.1.



Interbatch retention time (n = 12)

Analyte	Average	%RSD
Oleic Acid	3.97	1.1
PS 80 Mono	4.72	1.3

Interbatch peak area (n = 12)

Analyte	Average	%RSD
Oleic Acid	7.27	7.9
PS 80 Mono	263.1	12.4

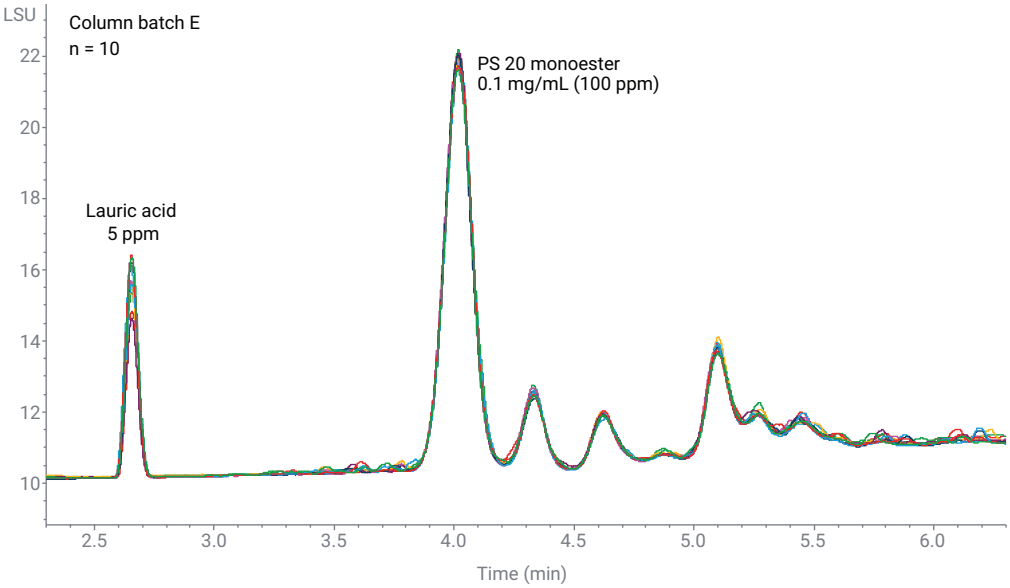
Interbatch resolution (n = 12)

Analyte	Average	%RSD
Oleate-PS 80	4.97	3.1

Figure 1. Batch-to-batch reproducibility for the Agilent AdvanceBio Surfactant Profiling column yields excellent precision for retention time, resolution, and peak area (n = 12).

This method was designed such that the ELSD settings could be universal for PS 20 and 80, as both are commonly used in biotherapeutics. Figure 2 shows an example of PS 20 hydrolysis, with lauric acid as the main fatty acid, demonstrating reproducibility from injection-to-injection with a batch different from those shown in Figure 1. The reproducibility values for retention time are excellent, as is the resolution between lauric acid and the PS 20 monoester (8.7).

This batch also has good reproducibility, with a %RSD of 1.4. Similar to oleic acid, lauric acid spiked at a low amount (5 ppm) demonstrates peak area reproducibility of 12.8%, which is excellent as this value is close to the limit of detection (LOD). The PS 20 monoester peak area has a %RSD of 1.3, which demonstrates excellent reproducibility over 10 replicate injections.



Retention time

Analyte	Average	%RSD
Lauric Acid	2.66	0.08
PS 20 Mono	4.02	0.05

Peak area

Analyte	Average	%RSD
Lauric Acid	19.99	12.8
PS 20 Mono	95.15	1.3

Resolution (lauric acid and PS 20 monoester)

Analyte	Average	%RSD
Laurate-PS 20	8.68	1.4

Figure 2. This method using an Agilent AdvanceBio Surfactant Profiling column is also applicable to polysorbate 20 analysis without any gradient adjustments or ELSD changes. Retention time, resolution, and peak area exhibit excellent run-to-run reproducibility.

To increase the robustness of the method and to optimize throughput, having little to no carryover is essential. This is important, as free fatty acids can be "sticky" compounds. Using the needle wash step suggested in the Experimental section, no carryover was observed for PS 20, PS 80, or their main free fatty acids (Figure 3). In both cases, the amount of free fatty acids injected was minimal. If the free fatty acids are more abundant and are causing carryover issues, the needle wash can be adjusted to increase the amount of methanol, potentially up to 70%. Note that this adjustment was not necessary for the free fatty acid experiment described in the following section but may be necessary if a free fatty acid analysis is of interest. Fortunately, the AdvanceBio Surfactant Profiling column is an excellent choice for this analysis.

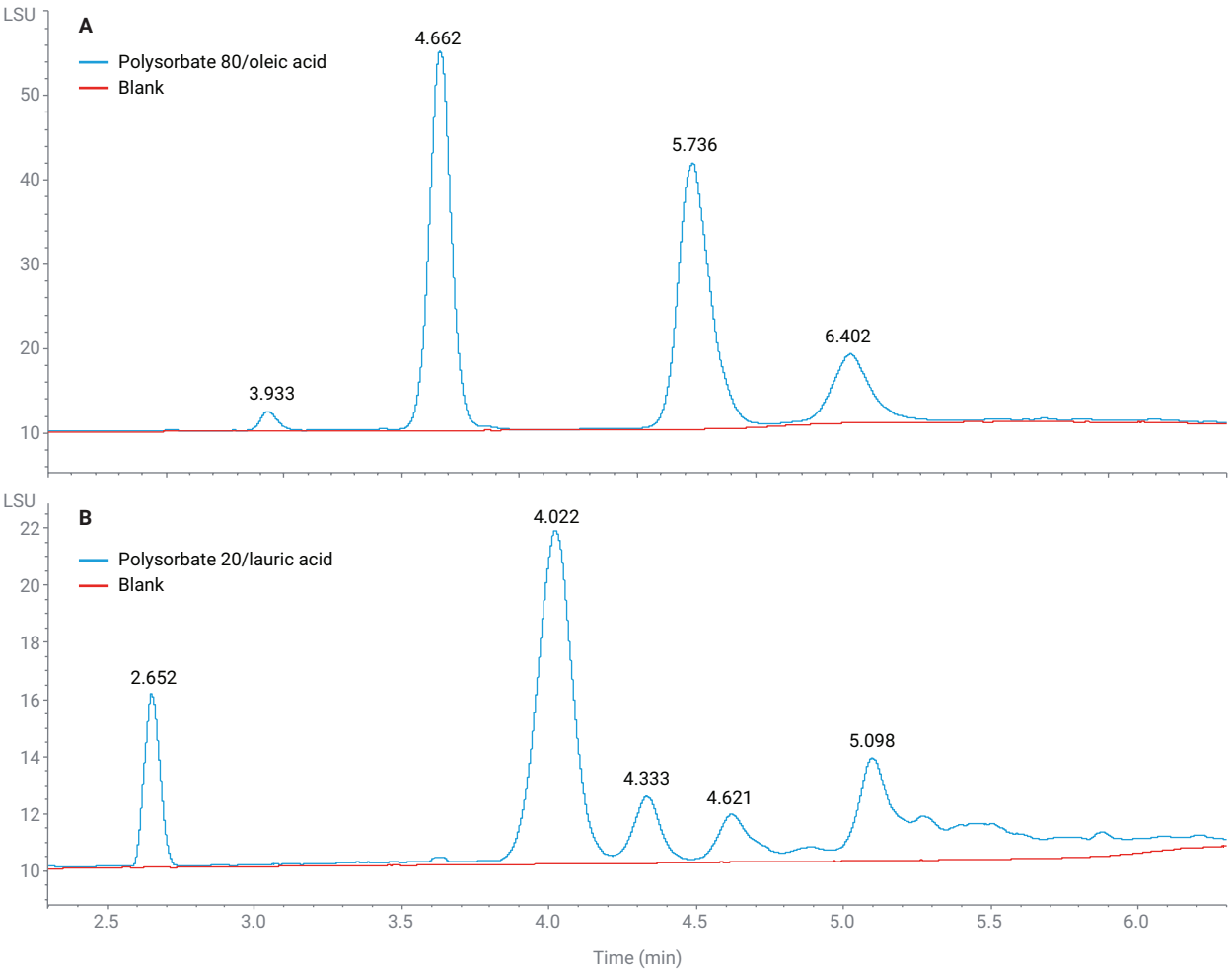


Figure 3. No carryover is observed for polysorbate 80 (A) nor polysorbate 20 (B) using an Agilent AdvanceBio Surfactant Profiling column. The free fatty acids also show no carryover.

Method applications

Free fatty acid analysis can be performed by gas chromatography, but this requires derivatization. Running this method with LC/ELSD requires no additional sample preparation and can be run with a slightly modified gradient to the polysorbate hydrolysis method. In this experiment, PS 80 and its free fatty acids were added to formulation buffer to mimic realistic conditions. In addition, the free fatty acids were injected at four different volumes (0, 2, 5, and 10 µL), and each was spiked in a relative abundance to the USP standards. Oleic acid made up the largest amount at 58%, and linolenic acid made up the least amount at 4%. Saturated fatty acids have a greater response in the ELSD than unsaturated fatty acids (Figure 4). Free fatty acid analysis serves as an alternate method to screen for polysorbate degradation, and the AdvanceBio Surfactant Profiling column facilitates rapid analysis.

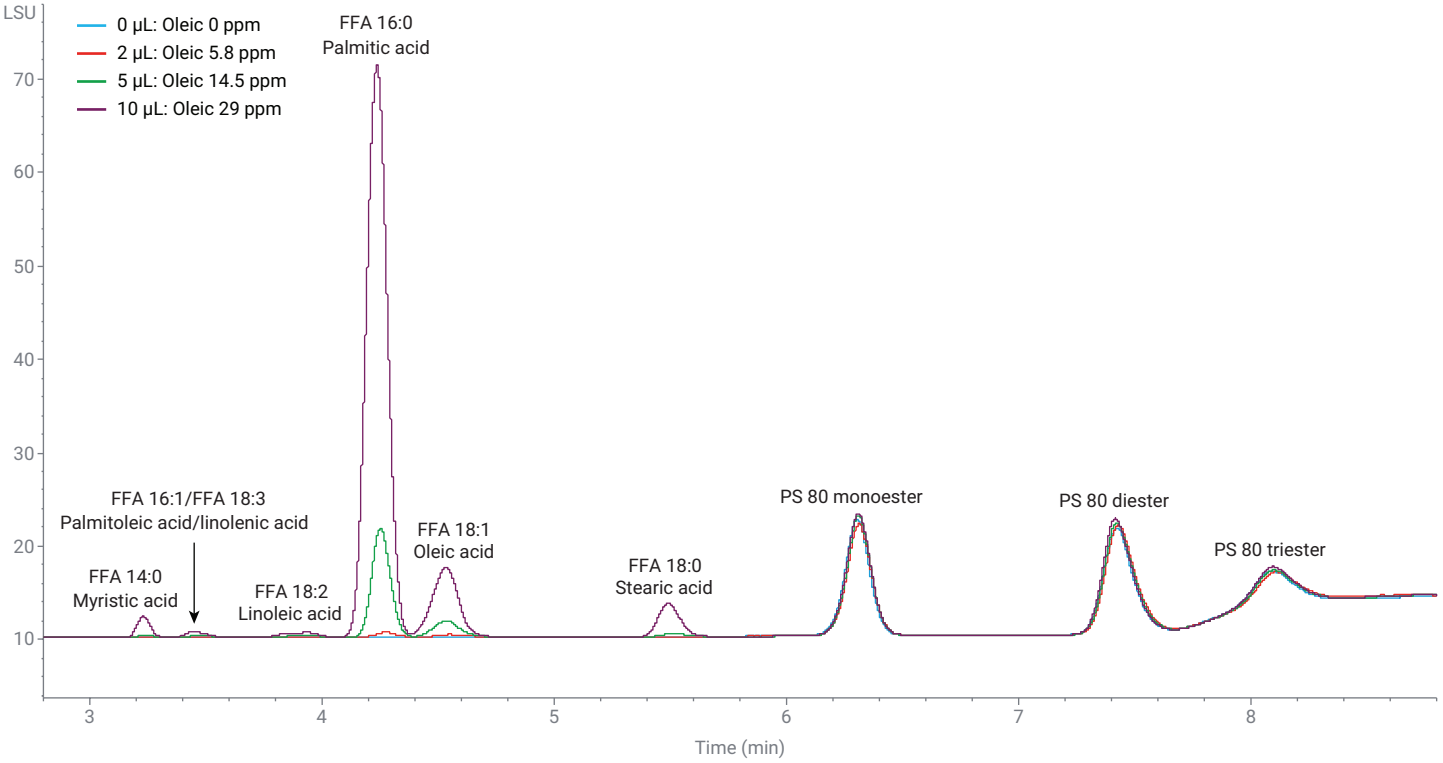


Figure 4. Free fatty acid analysis of polysorbate 80 using an Agilent AdvanceBio Surfactant Profiling column. Six out of the seven fatty acids are resolved with a slightly modified gradient. Fatty acids were spiked at three different volumes, all in relative amounts according to the USP standards.

Regulatory agencies require both qualitative and quantitative analysis of drug product components, so quantifying the amount of polysorbate in the formulation buffer is necessary. While mixed-mode chromatography is the gold standard for this type of analysis, it can also be useful to have an assay that combines quantitative information with qualitative degradation characterization, particularly in development labs. A calibration curve was designed based on the PS 80 content of 126 commercially available mAbs, ranging from 0.001 to 0.2% (w/v).[7] Ten points on the calibration curve ranged from 0.0005 to 0.5% (w/v) and were collected in duplicate. The calibration curve was prepared in formulation buffer with 0.025 mg/mL oleic acid spiked into each sample. Final injection volumes were 20 µL. The calibration curve was linear on a log-log scale, and values calculated from the monoester peak area show high reproducibility (Figure 5). The lowest point on the calibration curve has a %RSD of 20.9, just at the edge of the lower limit of quantitation (Table 2).

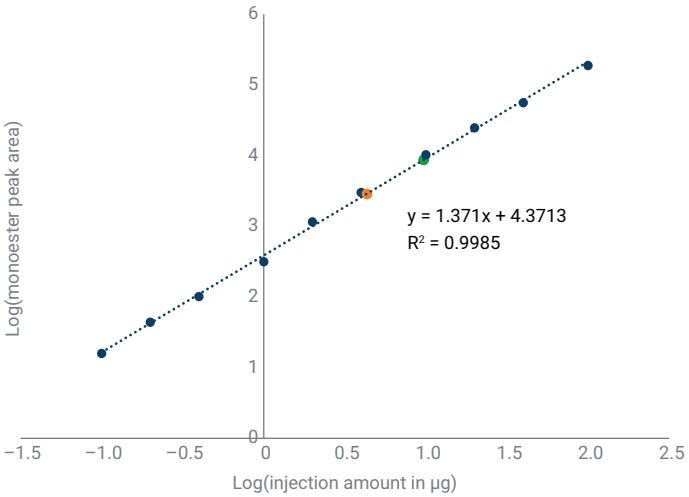


Figure 5. Calibration curve designed based on the polysorbate 80 content of 126 mAbs. Although this assay was designed for qualitative analysis, it can be used quantitatively for scientists who desire to increase the throughput of their lab. Unknowns were proteins spiked with 200 and 500 ppm of PS 80.

The test samples consisted of 5 mg/mL of a commercially available protein in formulation buffer spiked with 0.02 and 0.05% PS 80. These test samples were collected in duplicate. The protein eluted in approximately one minute and did not interfere with the fatty acid or polysorbate peaks. The test samples showed high accuracy and high reproducibility, demonstrating the feasibility of using this method for polysorbate quantitation as a two-in-one method (Table 3).

Table 3. The two test samples run in duplicate show excellent accuracy and precision, establishing proof of concept for a quantitative method.

Test Samples (ppm/%)	200/0.02	500/0.05
Average (ppm)	220	470
Accuracy	110%	94%
%RSD	0.35	7.1

Table 2. Calibration curve precision. The lowest point is just at the limit of quantitation with a %RSD of 20.9. The remaining points are well within the limits of precision.

Calibration (ppm/%)	5/0.0005	10/0.001	20/0.002	50/0.005	100/0.01	200/0.02	500/0.05	1,000/0.1	2,000/0.2	5,000/0.5
%RSD	20.9	12.9	1.2	1.9	0.44	0.36	0.24	0.47	0.54	1.8

Conclusion

Polysorbate degradation, particularly hydrolysis caused by residual host cell proteins, is a significant issue that is receiving increasing attention in the scientific community. This application note presents a 10-minute method capable of readily resolving free fatty acids from the polysorbate monoester, providing an efficient screening tool for polysorbate hydrolysis. This method is robust, reproducible, and simple to implement. Additionally, this assay can be used semiquantitatively and for free fatty acid analysis.

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