Application Note

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Separation and Analysis of Polysorbate 80 in the Presence of Human Serum Immunoglobulin G Using Agilent Bond Elut Lipid Extraction SPE Cartridges

Authors

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Abstract

Protein aggregation and surface adsorption are a major concern in the production of biotherapeutic proteins. Aggregation is known to occur under high-stress conditions (for example, interfacial stress), including high concentrations. Therefore, protein formulations often include nonionic surfactant stabilizers, such as polysorbate 80 (PS 80) and polysorbate 20 (PS 20) to minimize adsorption to surfaces and prevent protein aggregation due to interfacial stress.

In this application note, a convenient method was developed to separate, analyze, and quantify PS 80 in diluted aqueous polysorbate solutions and in human serum immunoglobulin G (IgG) solutions, using Agilent Bond Elut Lipid Extraction cartridges. Method linearity, repeatability, accuracy, limit of detection (LOD), limit of quantitation (LOQ), and specificity were evaluated using LC/DAD and LC/ELSD detection. LOD of PS 80 was found to be 0.1 and 0.03 mg/mL with LC/DAD and LC/ELSD detection, respectively. LOQ of PS 80 was 0.2 mg/mL and 0.04 mg/mL by LC/DAD and LC/ELSD detection, respectively. Linear regression with R^2 over 0.99 was obtained on LC/DAD, while polynomial regression with R^2 over 0.99 was obtained on LC/ELSD. Quantitation accuracy of PS 80 ranges within 86 to 106% with RSD of 2 to 6%. The two orthogonal methods exhibited no interfering peaks (signal-to-noise (S/N) ratio >3) within the retention time window where PS 80 eluted. The calibration range for the LC/DAD method was from 0.2 to 0.6 mg/mL PS 80, whereas for the LC/ELSD method, the range was 0.04 to 0.6 mg/mL PS 80.

Introduction

Polysorbate 80 is a complex, heterogeneous nonionic surfactant consisting of a varying number of polyethylene oxide (POE) groups attached to a hydrophilic sorbitan and isosorbide core. It has a mixture of variable lengths of hydrophobic fatty acid alkyl chains, mainly oleic acid (Figure 1). While it is impossible to accurately determine the molecular weight of PS 80, it is considerably smaller than biologics, such as immunoglobulin G (IgG) and monoclonal antibodies (mAbs), with an average mass of 1,310 Da. More than 80% of commercially available mAbs contain either PS 80 or PS 20 in their formulations.[1-4]

Polysorbates are preferred over other stabilizers due to their high surface activity, high hydrophilic-lipophilic balance, low critical micelle concentration (CMC), and proven safety profiles.[1-4]

The presence of polysorbates stabilizes proteins by reducing adsorption to surface and preventing protein aggregation due to interfacial stress; however, polysorbates degrade over time by oxidation and hydrolysis. PS degradation by auto-oxidation and hydrolysis in biotherapeutic formulations has been a significant concern. Auto-oxidation of PSs generates aldehydes, ketones, peroxides, and short-chain esterified POE sorbitan/isosorbide species, while hydrolysis generates free fatty acids that may then form visible and subvisible particulates in aqueous media. Degradation of PS compromises product quality because interfacial protection is lost, and particles are formed. It is therefore important to monitor the PS quality in neat solutions, aqueous diluted PS solutions, and biopharmaceutical formulations. It is also beneficial to know concentration, composition, purity, and functionality of polysorbates

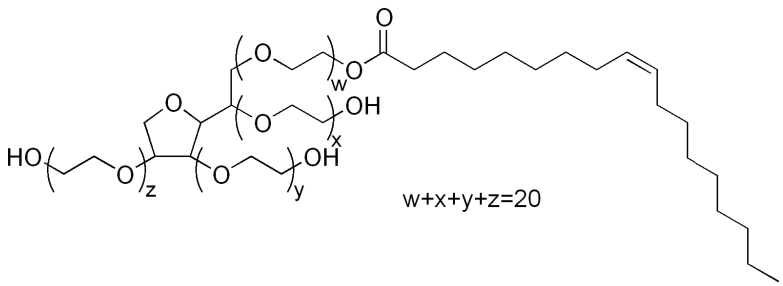

Figure 1. Chemical structure of Polysorbate 80 (PS 80, shown with oleic acid as major fatty acid ester).

in neat, diluted aqueous PS solutions and biopharmaceutical solutions at various stages of the biotherapeutic drug development cycle, as well as the final drug product.[4-7]

The analysis of PSs is usually performed by HPLC/UV or ELSD/CAD.[1-4,8,9] Challenges to analyzing PSs include their inherent complexity and heterogeneity, weak absorption in the UV region, and potential matrix interference. In this study, PS 80 was analyzed using LC/DAD detection, and the quantitation was performed on LC evaporative light scattering detection (ELSD) detection. Polysorbate concentration (either PS 20 or PS 80) in protein biotherapeutics is between 0.01 and 1.0 mg/mL; however, the more common concentration ranges between 0.01 and 0.6 mg/mL.[1-3]

Agilent Bond Elut Lipid Extraction cartridges were used for sample preparation prior to instrument analysis. Bond Elut Lipid Extraction products are based on Agilent proprietary EMR—Lipid technology. The sorbent technology provides highly selective and efficient interactions with molecules that have straight aliphatic chain structure, such as lipid molecules, based on the combined size exclusion and hydrophobic interactions. This technology has been frequently used for lipid removal,[10-11] and then extended for lipid extraction.[12] In this study, the products were extended to the application for polysorbate surfactant molecules, which also contains the long and multiple straight chains.

Experimental

Equipment and materials

HPLC grade solvents and reagents were obtained from Sigma-Aldrich (St Louis, Missouri, USA) or VWR Scientific (Bridgeport, New Jersey, USA). PS 80 and human serum immunoglobulin G (IgG, reagent grade ≥95%) were purchased from Sigma-Aldrich (St Louis, Missouri, USA). Formic acid (p/n G2453-85060, reagent grade, 99.9%) was from Agilent. Water was purified using a Milli-Q A10 (Millipore).

Sample preparation equipment included:

- Pipettes and repeater (Eppendorf, NY, USA)
- Agilent positive pressure manifold 48 processor (PPM-48) (p/n 5191-4101)
- 1 mL cartridge rack for the PPM-48 (p/n 5191-4102)
- Waste rack and waste bin for the PPM-48 (p/n 5191-4112)
- Agilent Bond Elut Lipid Extraction cartridge, 1 mL (p/n 5610-2041)

LC columns

- Agilent InfinityLab Poroshell 120 EC-C18, 2.7 µm, 3.0 × 100 mm (p/n 695975-302)
- Agilent InfinityLab Poroshell 120 EC-C18, 2.7 µm, 3.0 × 50 mm (p/n 699975-302)

Instrumentation

Polysorbate samples were analyzed using both a diode array detector (DAD) and an evaporative light scattering detector (ELSD). These were coupled to Agilent 1260 Infinity II LC systems operated by OpenLab or ChemStation software.

Agilent 1260 Infinity II Bio-inert LC instrument comprising:

- Agilent 1260 Infinity II Bio-inert Pump, G5654A
- Agilent 1260 Infinity II Bio-inert Multisampler, G5668A, with sample cooler
- Agilent 1260 Infinity II Multicolumn Thermostat, G7116A, with bio-inert heat exchanger
- Agilent 1260 Infinity II Diode Array Detector, G7115A, with bio-inert flow cell
- Agilent Evaporative Light Scattering Detector, G7102A, 1290 Infinity II

Table 1 lists the instrument methods on both LC/DAD and LC/ELSD for PS 80 detection. Figure 2 and Figure 3 illustrate the chromatograms of PS 80 by LC/UV (DAD) and LC/ELSD analysis, respectively.

Table 1. Instrument methods settings for PS detection.

Parameter	Value
HPLC/UV	
Column	Agilent, InfinityLab Poroshell 120 EC-C18, 2.7 µm, 3.0 × 100 mm (p/n 695975-302)
Mobile Phase A	0.1% phosphoric acid in water
Mobile Phase B	0.1% phosphoric acid in acetonitrile
Flow Rate	0.40 mL/min
Column Temperature	Ambient
Injection Volume	20 µL
Total Run Time	20 minutes
Isocratic	80% mobile phase B
DAD	
Wavelength	195 to 400 nm (full scan)

Parameter	Value
HPLC/ELSD	
Column	Agilent, Poroshell 120 EC-C18, 2.7 µm, 3.0 × 50 mm (p/n 699975-302)
Mobile Phase A	Water with 0.2% formic acid (FA)
Mobile Phase B	1:1 acetonitrile/isopropanol with 0.2% FA
Flow Rate	0.5 mL/min
Column Temperature	25 °C
Injection Volume	20 µL
Total Run Time	10 minutes
Gradient	Time(min) %B 0.00 28 3.00 58 4.50 88 7.50 88 8.50 28 10.00 28
ELSD	
Temperature	80 °C
Gas Flow	1.00 (SLM)
Data Rate	40 Hz
Smoothing	30 (3.0 seconds)

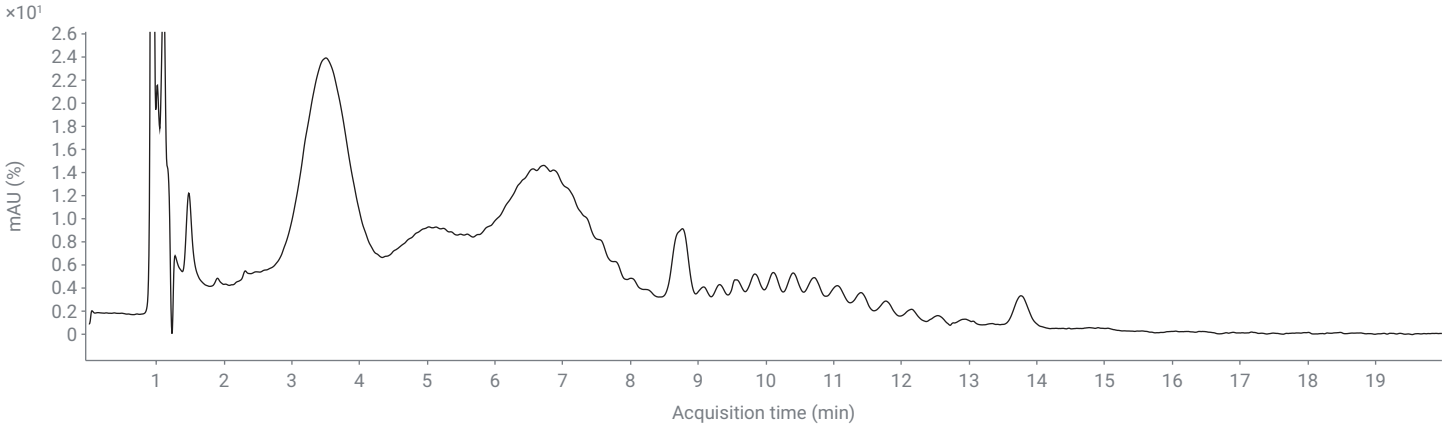

Figure 2. The chromatogram of PS 80 solution (1.0 mg/mL) obtained by LC/DAD with wavelength collection from 195 to 400 nm (with 20 µg column loading using isocratic elution).

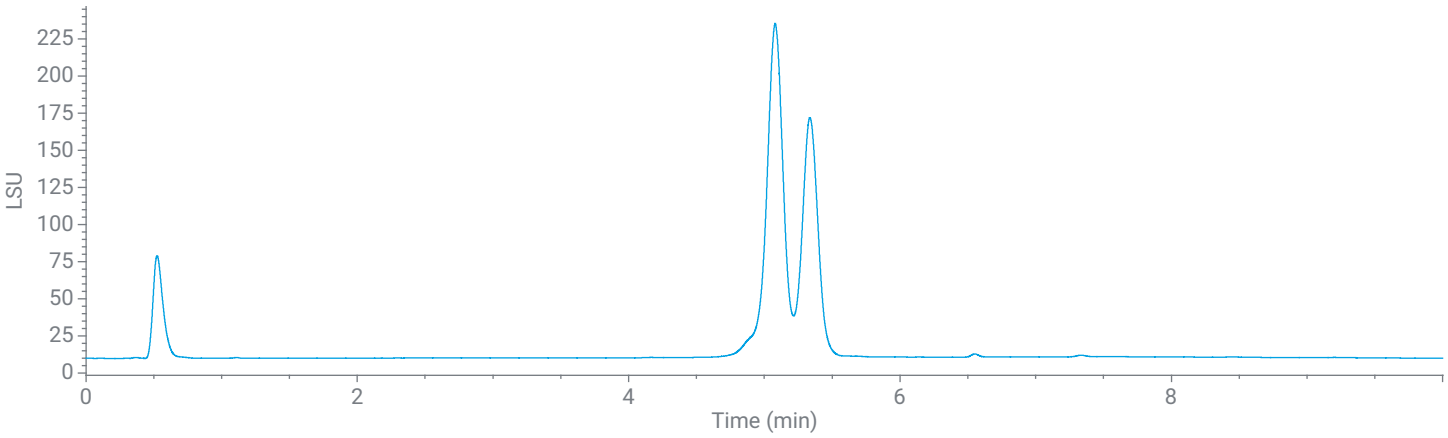

Figure 3. The chromatogram of PS 80 solution (0.5 mg/mL) obtained by LC/ELSD analysis at 80 °C (with 10 µg column loading using gradient elution).

Reagent preparation

PS 20 and PS 80 stock sample solutions (at 1.0 mg/mL) were prepared in water by weighing and transferring 25.3 mg of PS 80 into a 25 mL volumetric flask and diluting to volume with Milli-Q water. The resulting solution was then sonicated for approximately 10 minutes and inverted to mix thoroughly. This stock solution was used to prepare corresponding polysorbate solutions at 0.01, 0.03, 0.04, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 mg/mL in water.

For the phosphate buffer (100 mM at pH 7.0) preparation, sodium phosphate dibasic anhydrous (10.7218 g) and sodium phosphate monobasic anhydrous (2.9584 g) were weighed and added to a 1 L Erlenmeyer flask. Milli-Q water (approximately 800 mL) was added and the contents were fully dissolved by stirring. The pH of the solution was adjusted to 7.0 using 1 N HCl solution. The resulting solution was quantitatively transferred to a 1 L volumetric flask, diluted to volume with Milli-Q water and mixed thoroughly. The phosphate buffer was then filtered through a 0.45 µm filter to a 1 L HPLC bottle to obtain the final buffer solution.

To prepare the human serum IgG (5 mg/mL) stock solution, a lyophilized cake of human serum IgG (25.05 mg) was weighed and transferred into a 5 mL Eppendorf tube. Next, 5 mL of phosphate buffer was added to the tube. The Eppendorf tube was gently inverted multiple times and vortexed gently for 10 seconds and mixed thoroughly.

The 1.012 mg/mL PS 80 stock solution and phosphate buffer were used to prepare spiked human serum IgG (1.0 mg/mL) samples from 0.01 mg to 0.60 mg/mL PS 80 spike levels, as shown in Table 2.

Mobile phase A for LC/DAD method was prepared by adding 1.0 mL of 85% phosphoric acid to 1 L of Milli-Q water whereas mobile phase B for LC/DAD method was made by mixing 1.0 mL of 85% phosphoric acid with 1 L of acetonitrile. Mobile phase A for LC/ELSD method was prepared by adding 2 mL of formic acid in 1 L of Milli-Q water, whereas mobile phase B for LC/ELSD method was prepared by adding 2 mL of formic acid into a mixture of 500 mL of acetonitrile and 500 mL of isopropanol in a 1 L HPLC bottle.

Table 2. PS 80 spiked human serum IgG sample preparation.

Vol. of PS 80 at 1 mg/mL (µL)	Vol. of IgG at 5 mg/mL (µL)	Vol. of PB at 100 mM (µL)	Total Vol. (µL)	PS 80 Final Conc. (mg/mL)	IgG Final Conc. (mg/mL)
0	0	1000	1000	Control 1	NA
0	200	800	1000	Control 2	1
10	200	790	1000	0.01	1
30	200	770	1000	0.03	1
40	200	760	1000	0.04	1
50	200	750	1000	0.05	1
100	200	700	1000	0.1	1
200	200	600	1000	0.2	1
300	200	500	1000	0.3	1
400	200	400	1000	0.4	1
500	200	300	1000	0.5	1
600	200	200	1000	0.6	1

Note: 800 µL of the final spike solution from each prepared volume was used for offline analysis.

Sample preparation

Precision (repeatability and intermediate precision), accuracy, specificity, linearity, range, limit of detection (LOD), and limit of quantification (LOQ) were evaluated for PS 80 analysis using Agilent Bond Elut Lipid Extraction cartridges. Samples were prepared in triplicates at all spike levels, except at 0.30 mg/mL PS 80, where six replicates were prepared to determine repeatability. After preparation of samples (see Table 1), 800 µL of each sample was prepared following the SPE workflow detailed in Figure 4.

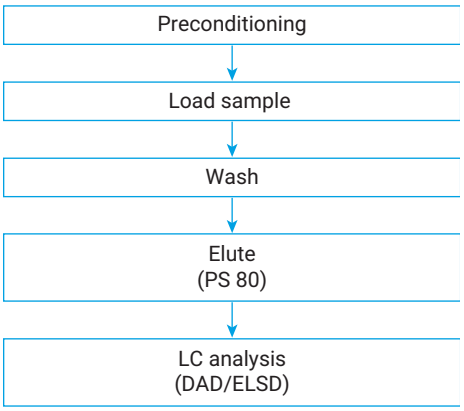

Figure 4. Workflow for sample preparation.

First, cartridges were prewashed with 80% acetonitrile (ACN) in water (1 mL × 4) followed by water (1 mL × 2). Next the sample in aqueous or buffered solution was loaded and eluted using the positive pressure manifold at the rate of 1 drop per 2 to 3 seconds. Several water washes were then performed (1 mL × ≥3) to get rid of nonvolatile salts in buffers and residual macromolecules. The eluent from loading and washing steps can be collected and combined for macromolecules like proteins analysis, when needed. Finally, PS 80 was eluted using 80% ACN in water (load volume of sample ≥500 µL × 1). The eluent was collected and mixed thoroughly. PS 80 was then analyzed and quantified using the LC/DAD and LC/ELSD detection. A total peak area of PS 80 was used for quantification. The retention time window of the PS 80 total peak area for LC/DAD analysis was from 2.5 to 15 minutes, whereas the retention time window of PS 80 total peak area for LC/ELSD analysis was ~4.5 to 5.5 minutes.

Results and discussion

SPE method development and consideration

Bond Elut Lipid Extraction SPE features the use of EMR—Lipid sorbent, which demonstrates selective and efficient interaction with lipid molecules. This interaction is based on the combined mechanism of size exclusion and hydrophobic interaction. Molecules with long and unbranched aliphatic chains (lipids) are selectively captured by the sorbent, while bulky molecules (small molecule analytes) pass through unretained. EMR—Lipid technology has been demonstrated successfully for lipid removal and extraction in biological and food matrices.[10-12]

Many surfactant molecules bear similar lipid structural features with long and unbranched chains. These structural features provide the potential of interactions between such molecules and EMR—Lipid sorbent. They therefore selectively interact with EMR—Lipid sorbent. However, since the ether groups in these surfactant molecules change the polarity of chains, they impact the hydrophobic interactions for chains with EMR—Lipid sorbent. Modification to this method, especially for sample loading, is critical for successful surfactant molecule retention. Experiments determined that polysorbates are retained by the EMR sorbent when 100% aqueous buffer is loaded, unlike more hydrophobic lipids (fatty acids, phospholipids) in previous studies.[10-12] The loading conditions should not contain more than 10% MeCN to avoid unwanted breakthrough of polysorbates. The trapped polysorbates can then be eluted using 80/20 acetonitrile/water mixture.

PS 80 analysis

Due to the poor UV absorbance of PS 80, ELSD detection provides better sensitivity than DAD detection. The limit of detection (LOD, S/N >3) for PS 80 was 0.10 mg/mL on DAD detection, whereas it was 0.03 mg/mL on ELSD detection. Capacity of the Bond Elut Lipid Extraction cartridges for PS 80 was evaluated by loading 1.0 mL of 1.0 mg/mL PS 80 solution and analyzing the eluent using DAD and ELSD detectors. No detectable PS 80 was found in the eluent after sample loading and washing steps.

Calibration curve linearity of PS 80 was then evaluated using the average peak area at each spiking level on both detectors. Calibration curves were generated using PS 80 standard solutions, prepared through the same sample preparation workflow (Figure 4).

The PS 80 calibration curve exhibits good linearity with LC/DAD analysis and shows a coefficient of determination (R^2) of ≥0.99. But with LC/ELSD analysis, the polynomial regression was used for calibration curve within a relative broad range, 0.04 to 0.60 ng/mL. The polynomial relationship of PS 80 peak area versus concentration is common with ELSD detection.[2] However, the polynomial calibration curves may create quantitation difficulties, resulting in less accurate results. Therefore, the polynomial curve was used for estimation for sample concentration initially. For more accurate quantitation on ELSD, linear calibration curves within relative narrow range need to be generated. In our study, two linear calibration curves for the range of 0.04 to 0.1 mg/mL and 0.2 mg/mL to 0.6 mg/mL were generated and used for accurate quantitation on ELSD (Figure 6).

Precision, accuracy, and repeatability for PS 80 analysis were calculated using the linear calibration curves on both detectors. Overall the calibration curves (Figure 5A and Figure 6) exhibited good linearity for PS 80 detection (R^2 ≥0.99). Quantitation accuracy of PS 80 at all spike levels ranged from 86 to 106% (see Table 3). Comparing the two detection methods, LC/ELSD provides better sensitivity at low concentrations, therefore allowing for broader calibration ranges in quantitation. The LC/DAD provides better linear calibration curves, but due to limited sensitivity at low levels, the calibration range is narrower than LC/ELSD.

Method repeatability for PS 80 was evaluated at 0.3 mg/mL with human IgG. The %RSD for the repeatability of PS 80 at a 0.3 mg/mL spike level was 3% with LC/ELSD analysis, while %RSD with LC/DAD analysis was 5%.

Method specificity was evaluated by analyzing two sets of three control blanks, control 1 and 2, with and without IgG in the sample. There were no interfering peaks (S/N >3) within the retention time window where PS 80 elutes in both control blanks. The final calibration ranges for these two analytical methods were determined as 0.2 to 0.6 mg/mL PS 80 for LC/DAD, and 0.04 to 0.6 mg/mL PS 80 for LC/ELSD.

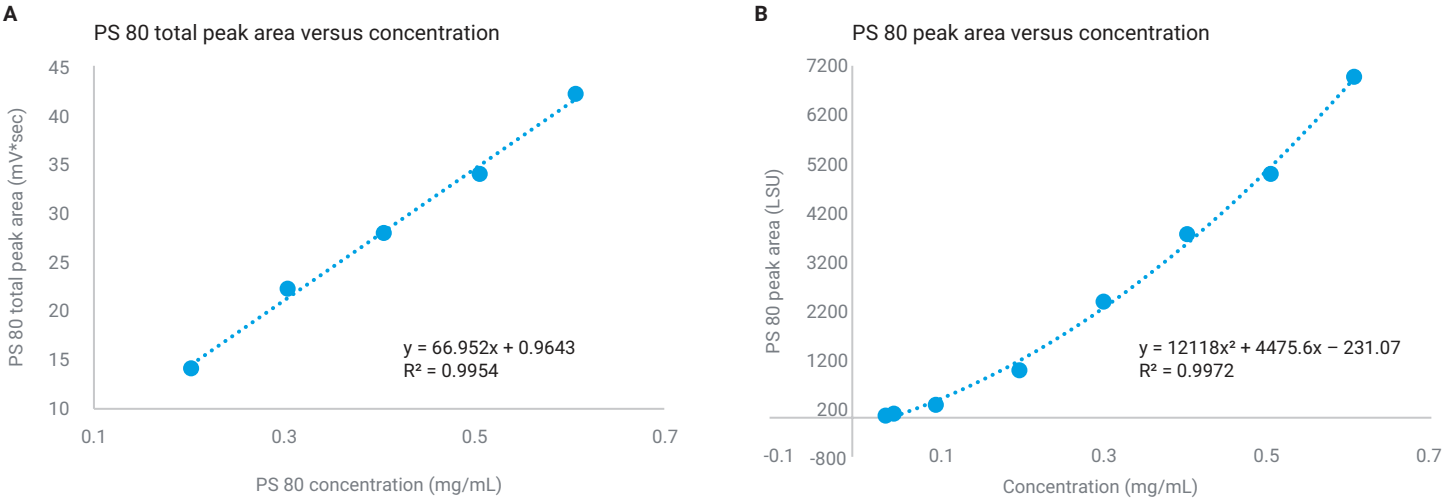

Figure 5. Calibration curves of PS 80 (A) for the range of 0.2 to 0.6 ng/mL using LC/DAD analysis, and (B) for the range of 0.04 to 0.60 ng/mL by LC/ELSD analysis. Results are the average of three replicates.

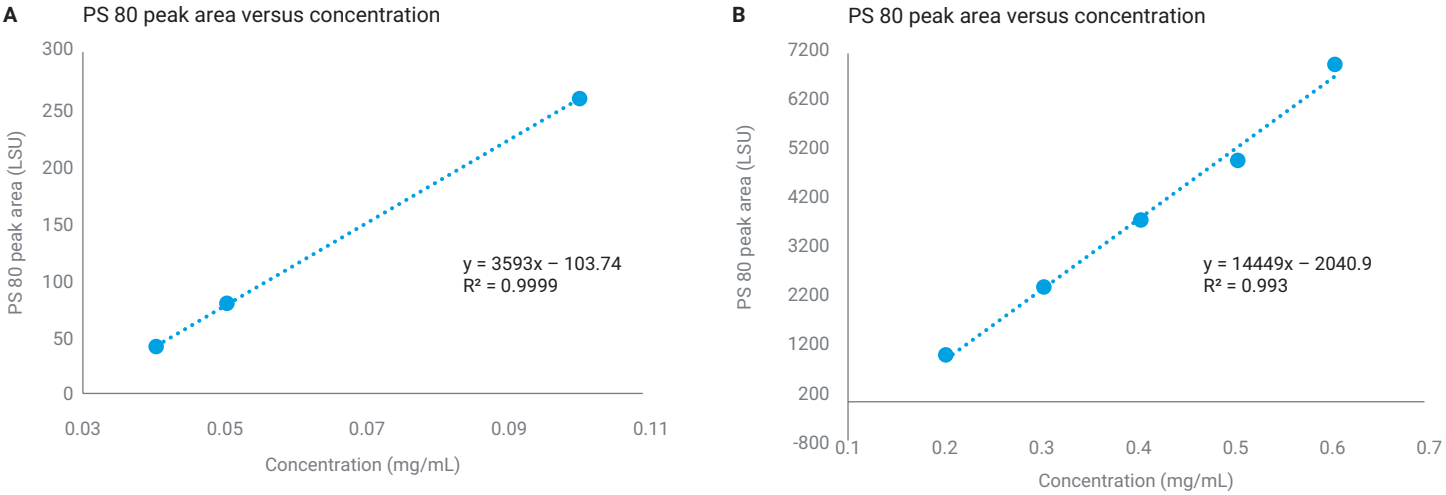

Figure 6. Separated calibration linearity curves for PS 80 calculated concentrations using LC/ELSD analysis (Figure 5B). Linear calibration curve of the PS 80 response (A) for the range of 0.04 to 0.1 mg/mL, and (B) 0.2 to 0.6 mg/mL.

Table 3. PS 80 accuracy and precision with LC/DAD and LC/ELSD analyses.

PS 80 Spiking Level (mg/mL)	LC/DAD Detection			LC/ELSD Detection		
	Calc. Average Conc. (mg/mL)	Accuracy (%)	RSD (%) n=3*	Calc. Average Conc. (mg/mL)	Accuracy (%)	RSD (%) n=3*
0.0405	NA	NA	NA	0.0427	105.4	2.9
0.0506	NA	NA	NA	0.0523	103.3	2.8
0.101	NA	NA	NA	0.101	100.0	1.8
0.202	0.213	105.5	4.9	0.206	102.1	2.2
0.304	0.318	104.6	5.0	0.289	94.9	2.8
0.405	0.386	95.3	2.6	0.349	86.1	3.5
0.506	0.478	94.5	6.3	0.441	87.2	3.5
0.607	0.542	89.4	4.5	0.536	88.3	4.7

*** At 0.3 ng/mL level, n=6**
NA = Not applicable due to limited sensitivity of LC/DAD detection.

Future studies and conclusions

Preliminary studies were performed for other related nonionic surfactants, including Solutol HS 15, Triton X-100, and Poloxamers, using Agilent Bond Elut Lipid Extraction cartridges. The preliminary studies demonstrated that the methodology can extended to the extraction of other nonionic surfactants (*vide supra*). Further studies with these nonionic surfactants are in progress.

Agilent Bond Elut Lipid Extraction cartridges have successfully been used to prepare polysorbates (PS 80) in diluted aqueous solutions and in human serum IgG spike solutions. Two analytical methods using LC/DAD and LC/ELSD detections have been successfully developed to analyze and quantify PS 80. Method accuracy and precision, linearity and range, LOD, LOQ, and specificity were evaluated. This sample preparation method using Agilent Bond Elut cartridges eliminates the interreference of proteins in the quantitation and characterization of PS 80.

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