Polysorbate 80 profiling by HPLC with mass and charged aerosol detection

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Application benefits

- HPLC-CAD method can detect batch-to-batch discrepancies
- Polysorbate 80 quality issues in raw material are detected before the surfactant is used in the drug formulation
- Optimized reversed-phase gradient enables fingerprinting of polysorbate 80
- Charged aerosol detector (CAD) provides high-sensitivity detection
- Inverse gradient enables uniform response under gradient conditions and eliminates the need of individual standards
- Mass detection with ISQ EM enables identity assignment of the main species in polysorbate 80
- The CAD and ISQ EM mass detector are easy to operate, even by operators without previous experience with nebulizer or MS based detectors

Goal

Provide an HPLC method suitable for fingerprinting of polysorbate 80 samples and detect variability between different suppliers, grades, and production batches.

Introduction

Polysorbate (PS) is a non-ionic surfactant widely used in pharmaceutical, biopharmaceutical, cosmetic, and beverage formulations. Several types of polysorbate are available, with PS 20, PS 60, and PS 80 being most frequently used in pharmaceutical products. All commercially available PS are complex mixtures of several hundred molecules, including low-level components. This complexity is a consequence of the inherently heterogeneous raw materials used for the synthesis and the synthetic pathway that leads to the final product. Considering PS 80, the product is obtained by esterification of oleic acid with sorbitan polyoxyehthylene (POE) (Figure 1). The oleic acid originates from natural sources and contains other fatty acid impurities including, but not limited to, palmitic, linoleic, and stearic acids. These impurities will participate in the esterification reactions, thereby increasing complexity. The additional presence of the precursor and side product of sorbitan, respectively sorbitol and isosorbide, along with different degrees of ethoxylation of main and by-products, results in a mixture of hundreds of components.

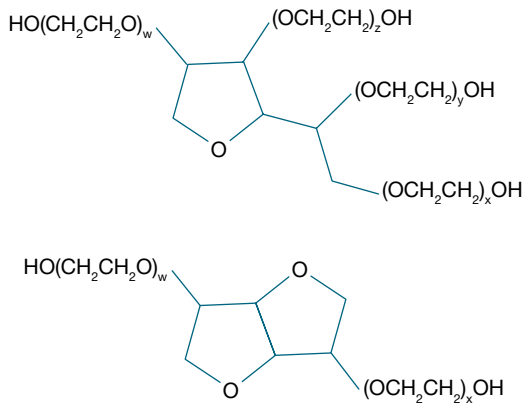

Figure 1. Structure of POE sorbitan (top) and POE isosorbide (bottom). These species are found in polysorbates along with their fatty acid esters. The nature of the fatty acid, the degree of esterification, and the number of oxyethylene units contribute to the overall complexity of polysorbates.

The control of PS as chemical raw material for (bio)pharmaceutical formulations is difficult because of the complexity described above. Nevertheless, such control is needed since variations from lot to lot are expected to occur. Variation of the relative population of esters, and polyoxyethylated polyols, can affect the behavior of PS 80 as an excipient in biotherapeutic formulations. The link between the composition of PS and its properties is still not fully understood,[1] although there have been hypotheses put forth that attempted to shed some light on this topic. For instance, a variation in the ester population may affect the critical micelle concentration, which in turn can affect the solubility of free fatty acids[2] present in the PS as degradation impurities. More often, the different behaviors resulting from lot-to-lot variability of PS cannot be easily explained. Nonetheless, understanding the quality of PS 80 by analyzing the raw material is a potential time- and cost-saving approach, as it would decrease the need of costly root-cause analyses when formulations obtained with a particular batch of PS do not meet the required standards. A full quantitative characterization of a PS sample is, with the current standard approach, an extremely complex task that consumes considerable analytical resources. While quantification of every component of a PS is not required, a simple analytical technique capable of profiling the main features of the PS sample and enabling lot-to-lot comparison is highly desirable.

In this work, we propose an HPLC-based approach to monitor the characteristics of PS 80 samples that provides operational simplicity while generating a high degree of information. The reversed-phase method is highly optimized to ensure sufficient separation of different compound classes within a reasonable run time. For detection, the Thermo Scientific™ Vanquish™ Charged Aerosol Detector, which is the instrument of choice for detection of PS and other surfactants,[3,4,5,6] is used.

The Thermo Scientific™ Vanquish™ Duo UHPLC System for Inverse Gradient seamlessly incorporates two independent flow-delivery systems that simultaneously deliver the analytical and compensation gradients. Setting up the compensation flow is facilitated by a user-friendly wizard in Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS). The inverse gradient option allows CAD-based quantitation without standards even under gradient conditions.[7]

The Thermo Scientific™ ISQ™ EM single quadrupole mass detector features intuitive setting of the detection parameters through the AutoSpray algorithm. It provides a mass range up to *m/z* 2000 allowing for the mass detection of high-molecular weight PS species such as singly-charged di-ester and doubly charged tri- and tetra-ester ions. Obtained masses of the variety of PS species allows the deduction of the identity of main components, complementing the quantitative, mass selective CAD analysis with qualitative information.

Experimental

Chemicals

- Deionized water, 18.2 MΩ·cm resistivity or higher
- Fisher Scientific™ Acetonitrile, Optima™ LC/MS grade (P/N A955-212)
- Fisher Scientific™ Formic acid, Optima™ LC/MS grade (P/N A117-50)
- Fisher Scientific™ Isopropanol, Optima™ LC/MS grade (P/N A461-212)
- Fisher Scientific™ Ammonium formate, Optima™ LC/MS grade (P/N A115-50)

Three PS 80 samples were purchased from Croda Inc. and one sample from Avantor (Table 1).

Table 1. PS 80 samples

Vendor	Product name	Product code	Identification name used in the document
Croda Inc.	Super Refined™ Polysorbate 80-LQ-(MH)	SR48833	SA1
	Super Refined™ Polysorbate 80 POA-(LQ)-(MH)	SR40925	SA2
	Tween™ 80 HP-LQ-(MH)	SD43361	SA3
Avantor	Polysorbate 80, N. F. Multi-Compendial J.T. Baker TWEEN 80 HP-LQ-(MH)	4117-02	SB4

Sample handling

- Fisher Scientific™ Fisherbrand™ Mini Vortex Mixer (P/N 14-955-152)
- Glass Vials (amber, 2 mL), Fisher Scientific™ (P/N 03-391-6)
- Vial Caps with Septum (Silicone/PTFE), Fisher Scientific™ (P/N 13-622-292)
- Thermo Scientific™ Orion™ 3-Star Benchtop pH Meter (P/N 13-644-928)
- Thermo Scientific™ Finnpipette™ F1 Variable Volume Pipettes (P/N 14-386-309N)

The Fisher Scientific product codes can be unique to different countries; the codes given above should be compatible across the EU and USA.

Instrumentation

- Thermo Scientific™ Vanquish™ Flex UHPLC system and Thermo Scientific Vanquish Flex Duo UHPLC system consisting of:
  - Vanquish Dual Pump F (used in CAD experiments) (P/N VF-P32-A)
  - Vanquish Quaternary Pump F (used in ISQ EM experiments) (P/N VF-P20-A)
  - Vanquish System Base (P/N VF-S01-A-02)
  - Vanquish Sampler FT (P/N VF-A10-A)
  - Vanquish Column Compartment H (P/N VH-C10-A-02)
  - Thermo Scientific™ Vanquish™ Charged Aerosol Detector H (P/N VH-D20-A)
- Thermo Scientific™ ISQ™ EM single quadrupole mass spectrometer (P/N ISQEM-ESI)
- Vanquish Duo for Inverse Gradient Kit (P/N 6036.2010)

Sample and solvents preparation

PS 80 samples were prepared in 25 mL volumetric flasks diluted with deionized water at a final concentration of 2.5 mg/mL for sample SA1, which was used for the dilutions, and at 1 mg/mL for all the other samples.

Eluent A (Table 2) was prepared by dissolving ammonium formate in deionized water at the concentration of 5 mM. Afterwards, the pH of the eluent was adjusted to pH 4.8 with formic acid.

Method parameters

Table 2. LC method

Parameter	Value			
Column	Thermo Scientific™ Accucore™ C18 150 × 2.1 mm; 2.6 µm, P/N 17126-152130			
Mobile phase	A – 5 mM ammonium formate, pH 4.8 B – 50/50 isopropanol/acetonitrile (v/v)			
Gradient	*Time (min)*	*%B*	*Time (min)*	*%B*
	0	9	26	85
	3	9	35	100
	10	22	45	100
	10	57	46	9
	21	69	56	9
	21	84		
Flow rate	0.4 mL/min			
Autosampler temp.	6 °C			
Column temp.	50 °C forced air mode, fan speed 5 50 °C active pre-heater			
Injection volume	10 µL			
Injection wash solvent	10/90 water/isopropanol (v/v)			

Table 3. CAD detector settings

Parameter	Value
Evaporator temperature	50 °C
Data collection rate	20 Hz
Filter	3.6 s
Power function	1.5

Table 4. ISQ EM mass detector settings

Parameter	Value
Ionization mode	HESI
Source setting	Easy mode. Setting for sensitivity was 1; setting for mobile phase volatility was 3; setting for thermally labile sample was 1
Method type	Scan mode, profile
Polarity Mass range full scan Source CID voltage Dwell time	**Positive** *m/z* 350–2000 0 V 0.5 s

Software

Chromeleon 7.3 CDS was used for data acquisition and analysis. Thermo Scientific™ FreeStyle™ 1.6 application was used to calculate theoretical values of monoisotopic and most abundant isotope masses.

Results and discussion

The HPLC method was developed and optimized using CAD to maximize the number of observed peaks while maintaining a reasonable run time below one hour. The profiles obtained by CAD for PS 80 from different vendors and batches are shown in Figure 2 and Figure 3, where both standard and inverse gradient methods were applied. The chromatograms clearly showed four different groups of peak clusters. This grouping will be used for further discussion and comparison of elution profiles. Even though the profile of the different PS 80 samples is similar, differences in the relative intensity are present. For both inverse gradient and standard gradient, the main peaks in Groups 2 and 3 differ in height depending on the sample: the peak heights for SB4 and SA3 are the lowest, and quite comparable to each other; the most intense peaks in Group 2 and Group 3 are detected for SA2. More differences between samples are present. For instance, one peak in Group 3 and two peaks in Group 4 are detected only for samples SA2 and SA1 (Figure 2).

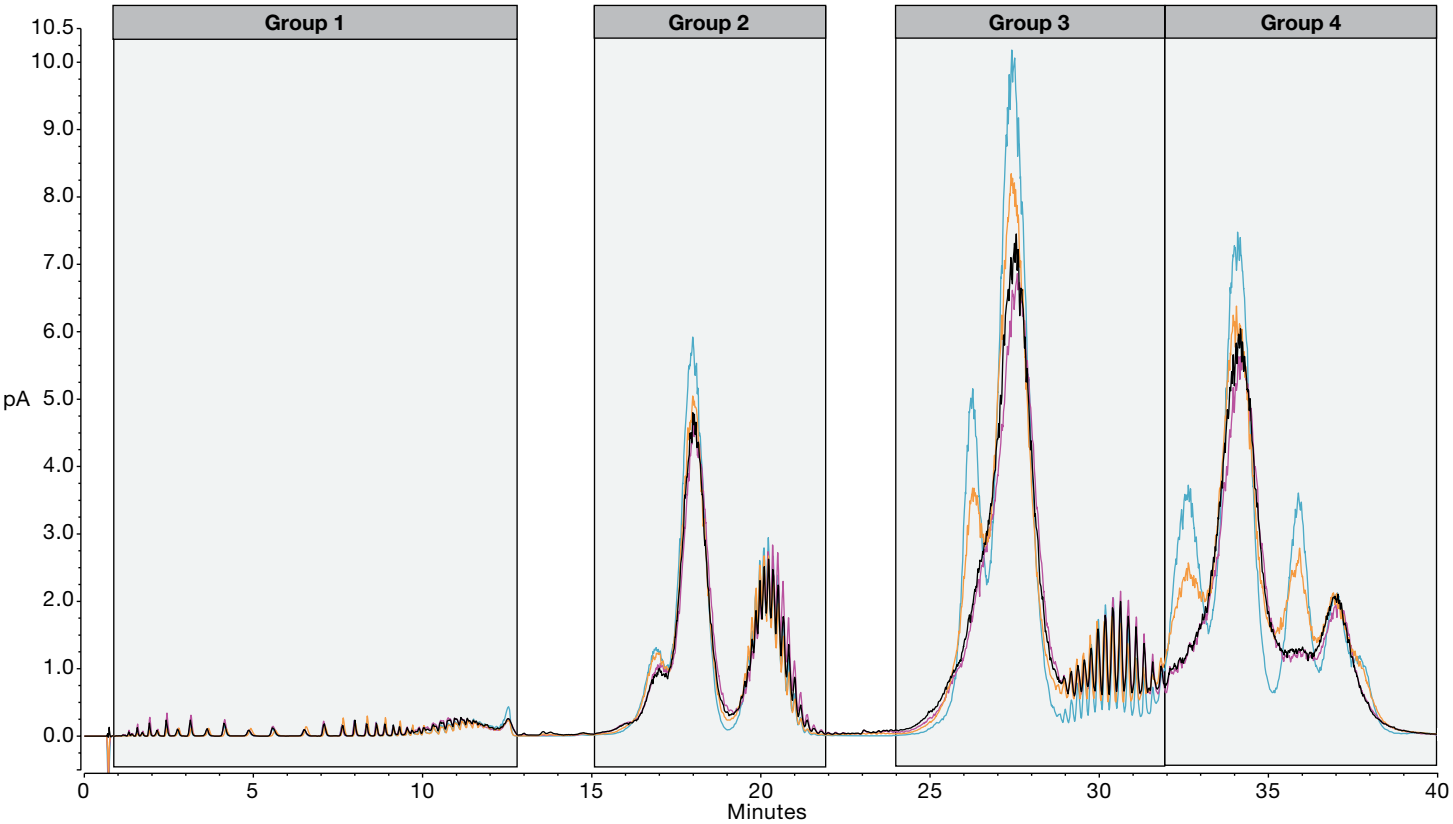

Figure 2. Overlaid chromatogram of the PS 80 samples described in Table 1. CAD detection with standard gradient. Sample concentration 1 mg/mL. Other conditions are described in Table 2 and Table 3. Signals are corrected by matrix injection (matrix: 10 µL water). Orange trace: sample SA1; blue trace: sample SA2; pink trace: sample SA3; black trace: sample SB4.

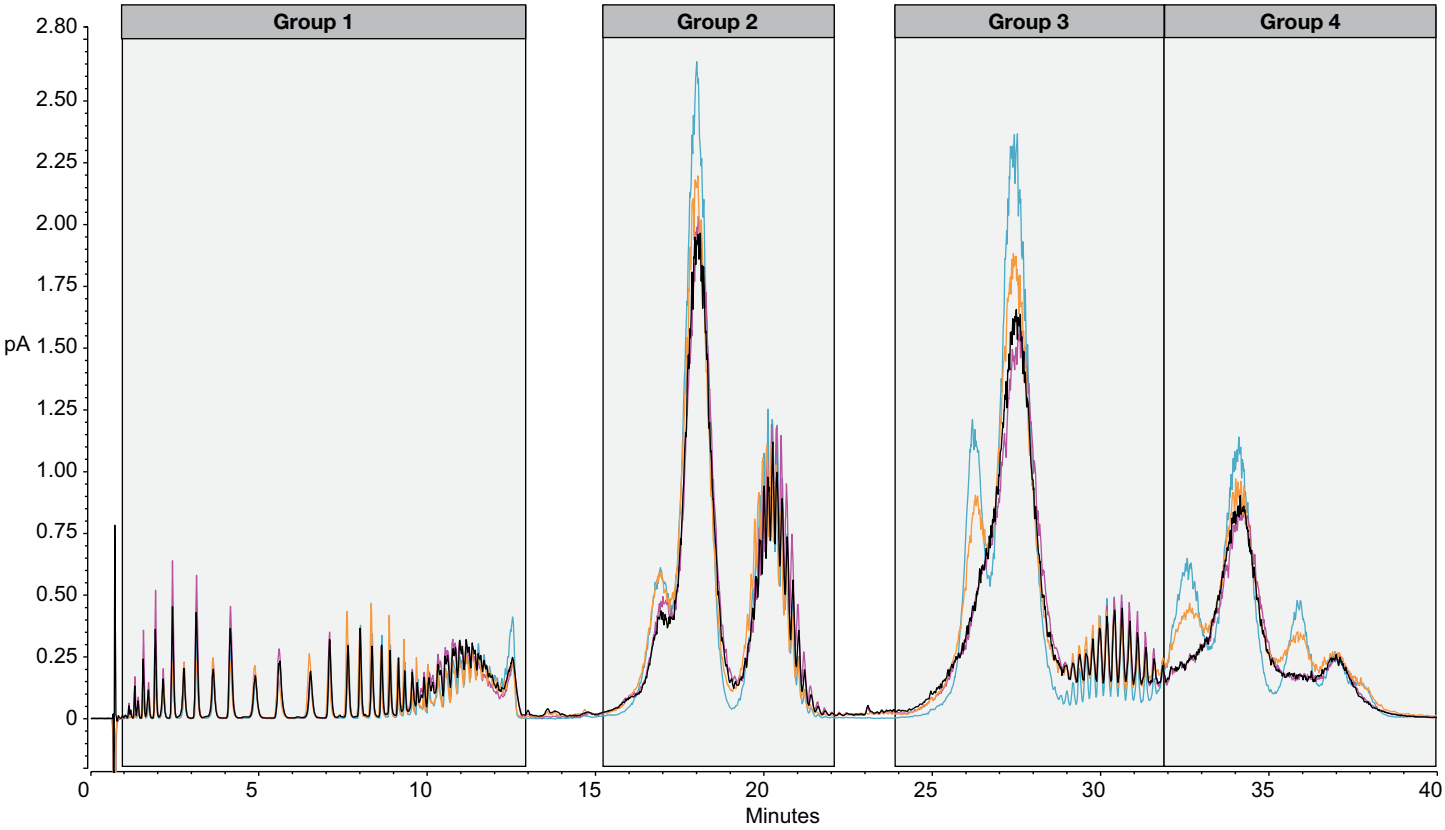

Figure 3A. Overlaid chromatogram of the PS 80 samples described in Table 1. CAD detection with inversed gradient. Sample concentration 1 mg/mL. Other conditions are described in Table 2 and Table 3. Signals are corrected by matrix injection (matrix: 10 µL water). Orange trace: sample SA1; blue trace: sample SA2; pink trace: sample SA3; black trace: sample SB4.

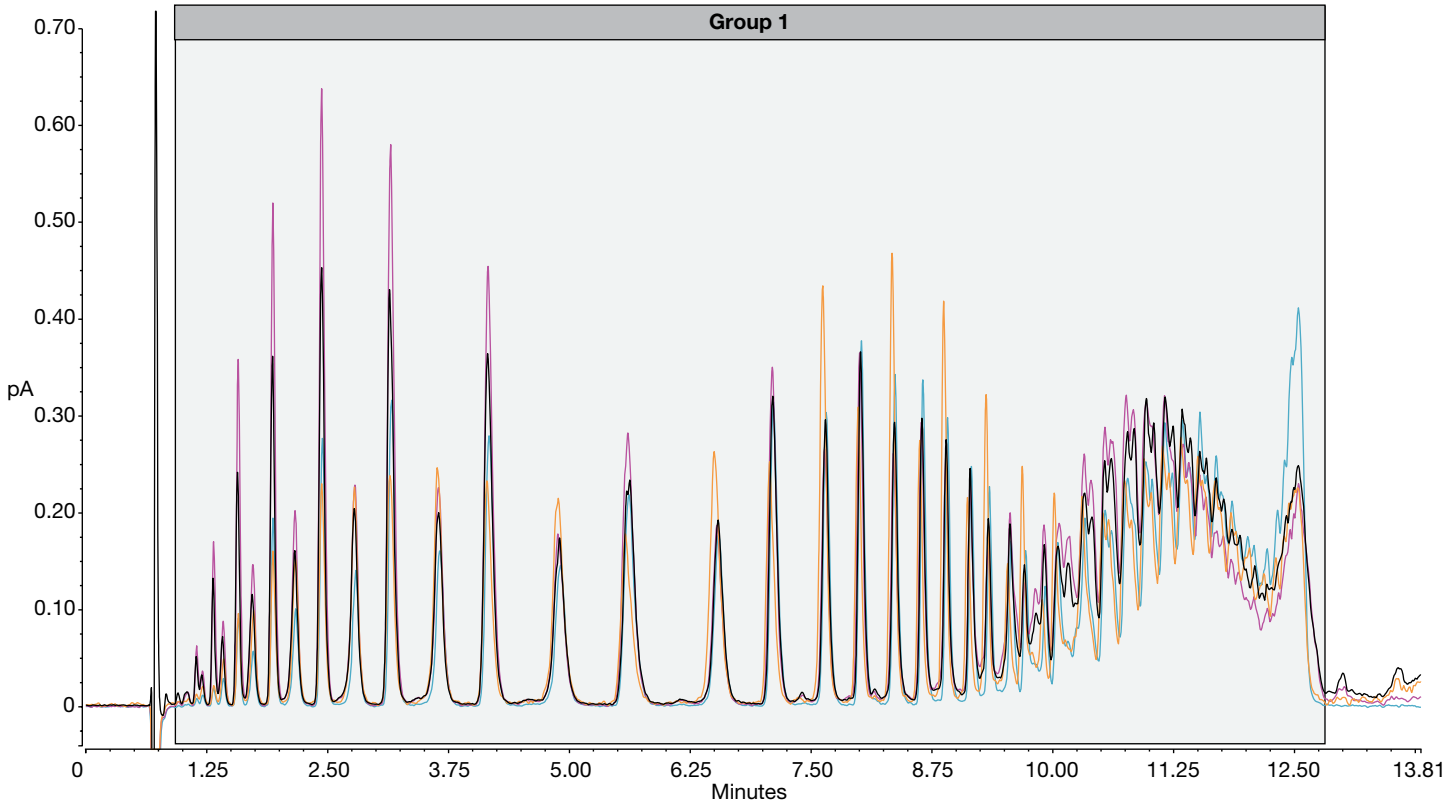

Figure 3B. Detailed view of Figure 3A. Zoom in Group 1. Orange trace: sample SA1; blue trace: sample SA2; pink trace: sample SA3; black trace: sample SB4.

The ISQ EM single quadrupole mass detector was used to elucidate the nature of the species eluting in the different groups. Figure 4A represents the total ion chromatogram (TIC) obtained in positive ionization mode. The peak annotation is based on the analysis of the averaged spectra across selected time windows (Figure 4B to Figure 4F). Identified species based on the annotated mass spectra are consistent with the main species expected in a PS 80 sample, namely POE sorbitan oleic acid esters, and POE isosorbide oleic acid esters. The components were detected as singly, doubly, or triply ammoniated adducts, depending on the species. The expected mass differences of one ethylene oxide unit for the singly, doubly, and triply charged ions are *m/z* 44.0, 22.0, and 14.7, which fit to the mass shifts observed in the spectra. In Table 5 some of the observed ions are listed and compared to the expected *m/z* based on calculated masses.

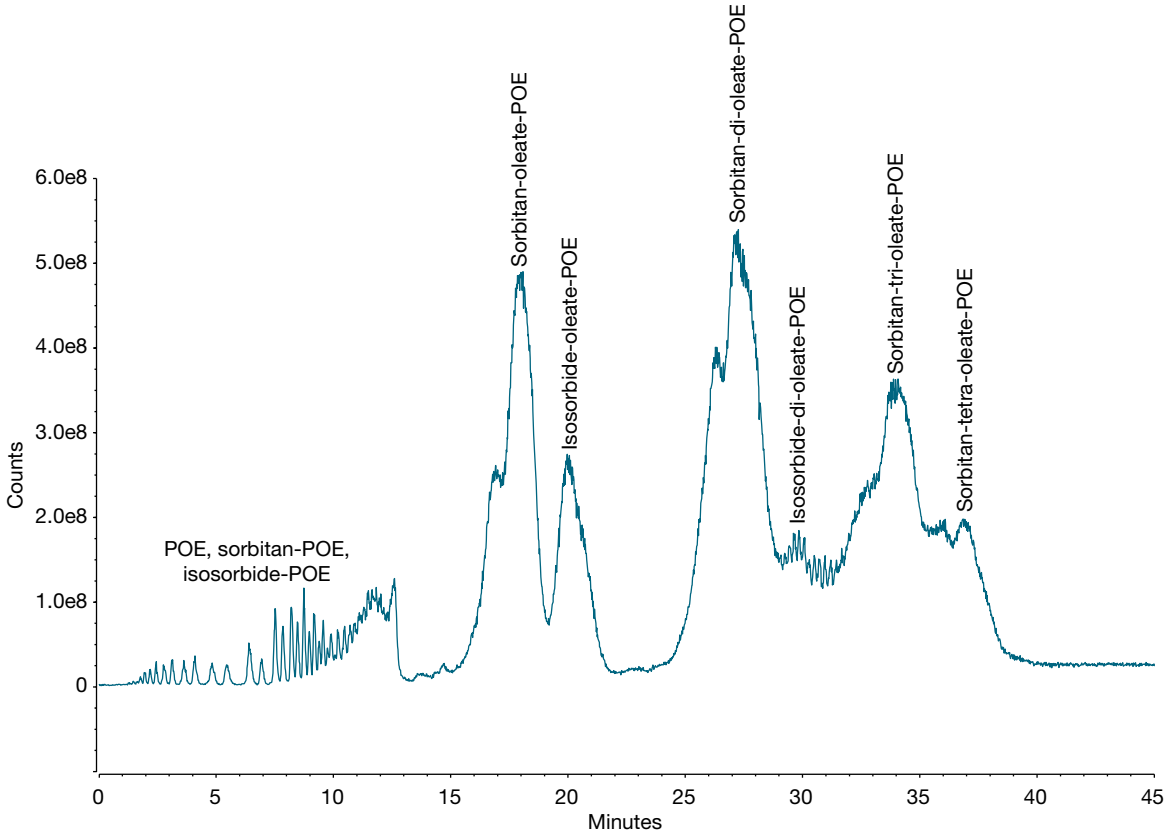

Figure 4A. TIC of PS 80 0.5 mg/mL (SA1). Conditions are described in Table 2 and Table 4.

Table 5. Examples of detected ions with tentative identity assignment

Component	Formula	Detected ion	Theoretical *m/z* based on most abundant isotope mass	Theoretical *m/z* based on monoisotopic mass	Theoretical *m/z* based on average mass	Observed *m/z*	Δ *m/z* (observed – theoretical average)
Sorbitan-oleate-POE_{27}	$C_{78}H_{152}O_{33}$	$[M+2NH_4]^{2+}$	826.5	826.5	827.1	826.8	-0.3
Sorbitan-oleate-POE_{33}	$C_{90}H_{176}O_{39}$	$[M+3NH_4]^{3+}$	645.1	645.1	645.5	645.3	-0.2
Sorbitan-di-oleate-POE_{33}	$C_{108}H_{208}O_{40}$	$[M+3NH_4]^{3+}$	733.5	733.2	733.6	733.5	-0.1
Sorbitan-di-oleate-POE_{26}	$C_{94}H_{180}O_{33}$	$[M+2NH_4]^{2+}$	937.2	936.7	937.3	937.0	-0.3
Isosorbide-oleate-POE_{12}	$C_{48}H_{90}O_{17}$	$[M+NH_4]^{+}$	956.7	956.7	957.3	956.7	-0.6
Isosorbide-oleate-POE_{16}	$C_{56}H_{106}O_{21}$	$[M+2NH_4]^{2+}$	575.4	575.4	575.8	575.6	-0.2
Isosorbide-di-oleate-POE_{12}	$C_{66}H_{122}O_{18}$	$[M+NH_4]^{+}$	1220.9	1220.9	1221.7	1221.1	-0.6
Isosorbide-di-oleate-POE_{15}	$C_{72}H_{134}O_{21}$	$[M+2NH_4]^{2+}$	685.5	685.5	686.0	685.7	-0.3
Sorbitan-tri-oleate-POE_{28}	$C_{116}H_{220}O_{36}$	$[M+2NH_4]^{2+}$	1113.3	1112.8	1113.5	1113.6	+0.1
Sorbitan-tetra-oleate-POE_{27}	$C_{132}H_{248}O_{36}$	$[M+2NH_4]^{2+}$	1223.4	1222.9	1223.7	1223.7	+0.0

Since six ethylene oxide repeat units are isobaric to one oleate unit, it should be noted that the spectra alone could not provide any information on the degree of esterification. However, it was assumed that the hydrophobicity within a class of component increases with oleic acid content. Based on this assumption, higher order esters would elute later in the chromatogram relative to lower order ones.

In Group 1, free POE, POE sorbitan, and POE isosorbide are detected; the identity assignment for most of the peaks of Group 1 is straightforward since most of them represent single components, and consequently the spectra are easy to interpret with confidence (data not shown).

Comparing the TIC in Figure 4A to the CAD chromatograms, it is clear that the peaks were clustered in groups based on degree of esterification. All groups indeed include species with the same degree of esterification, apart from Group 4, which combines tri and tetra esters. TIC, CAD with standard gradient, and CAD with inverse gradient are all suitable for profiling PS 80. When the CAD detection is considered, the substantial difference between standard and inverse gradient is the fact that the quantitation with the latter provides the real mass-balance across all components within each sample. When the standard gradient is applied, the profiling is valuable for distinguishing one sample from another, but the method overestimates the amount of late eluting di-, tri-, and tetra-esters while underestimating non-esterified components.

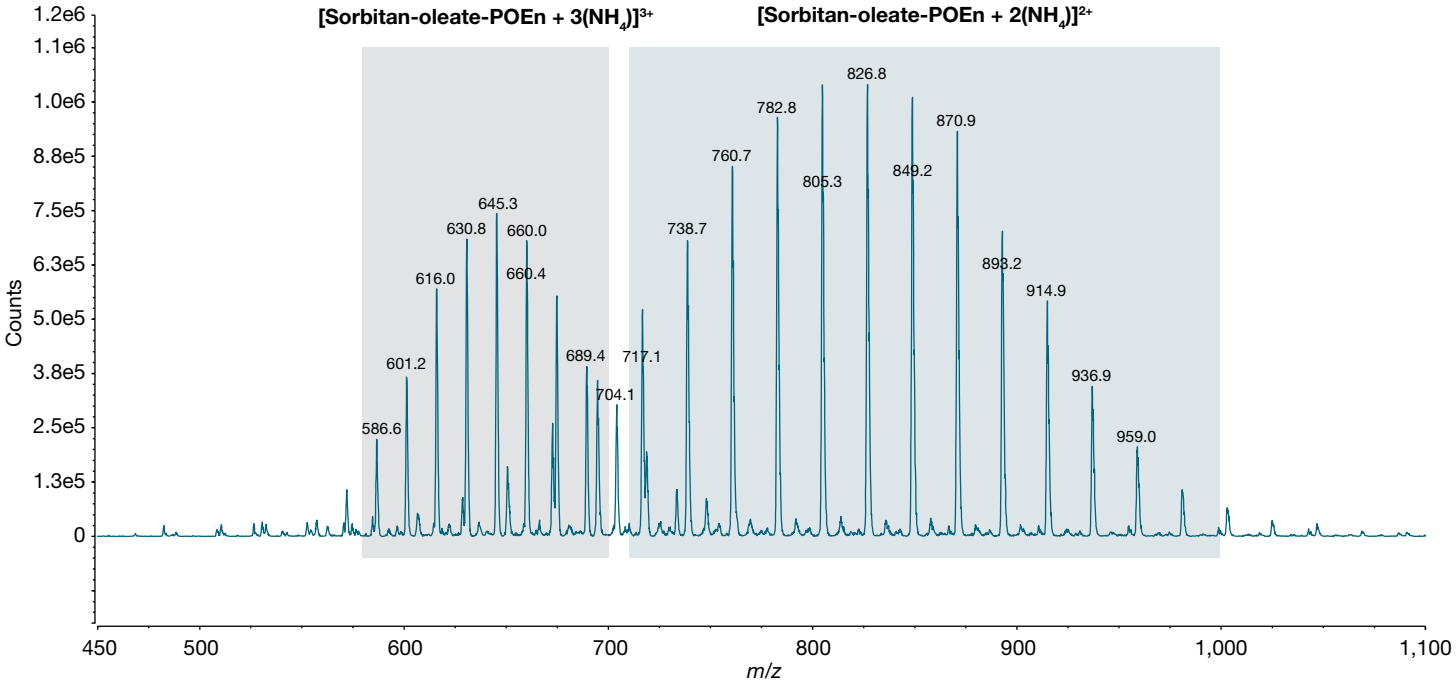

Figure 4B. Mass spectrum representing the averaged spectra across the retention time window 17.3–19.1 min, capturing the peak with apex at 17.9 min (Figure 4A). The signal ions are inferred as sorbitan-oleate-POEn doubly and triply ammoniated adducts. For doubly charged adducts polymer distribution, the detected species contain number of oxyethylene units n between 22 and 35; for the triple charged adducts polymer distribution n is between 29 and 35.

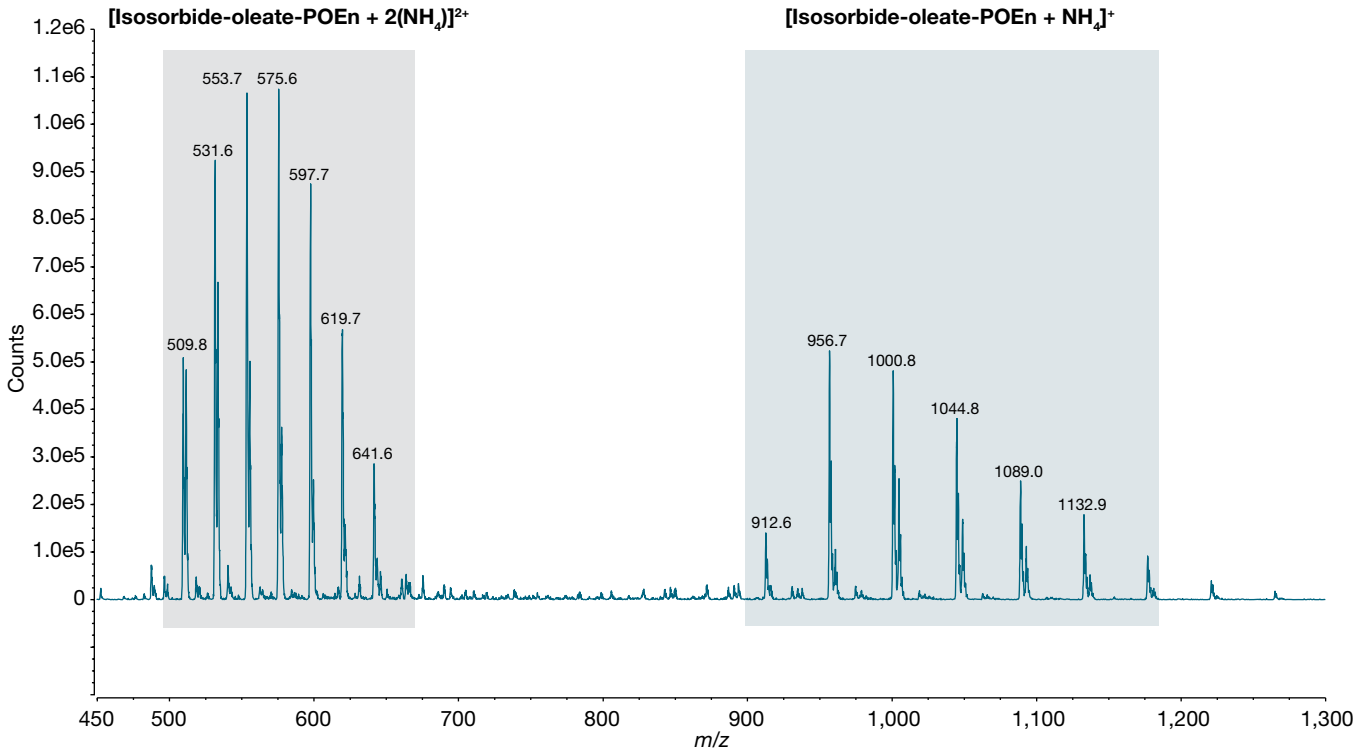

Figure 4C. Mass spectrum representing the averaged spectra across the retention time window 19.4–20.3 min, capturing the peak with apex at 20.0 min (Figure 4A). The signal ions are inferred as isosorbide-oleate-POEn single and double ammoniated adducts. For singly charged adducts polymer distribution, the detected species contain a number of oxyethylene units n between 11 and 17; for the doubly charged adducts polymer distribution n is between 13 and 19.

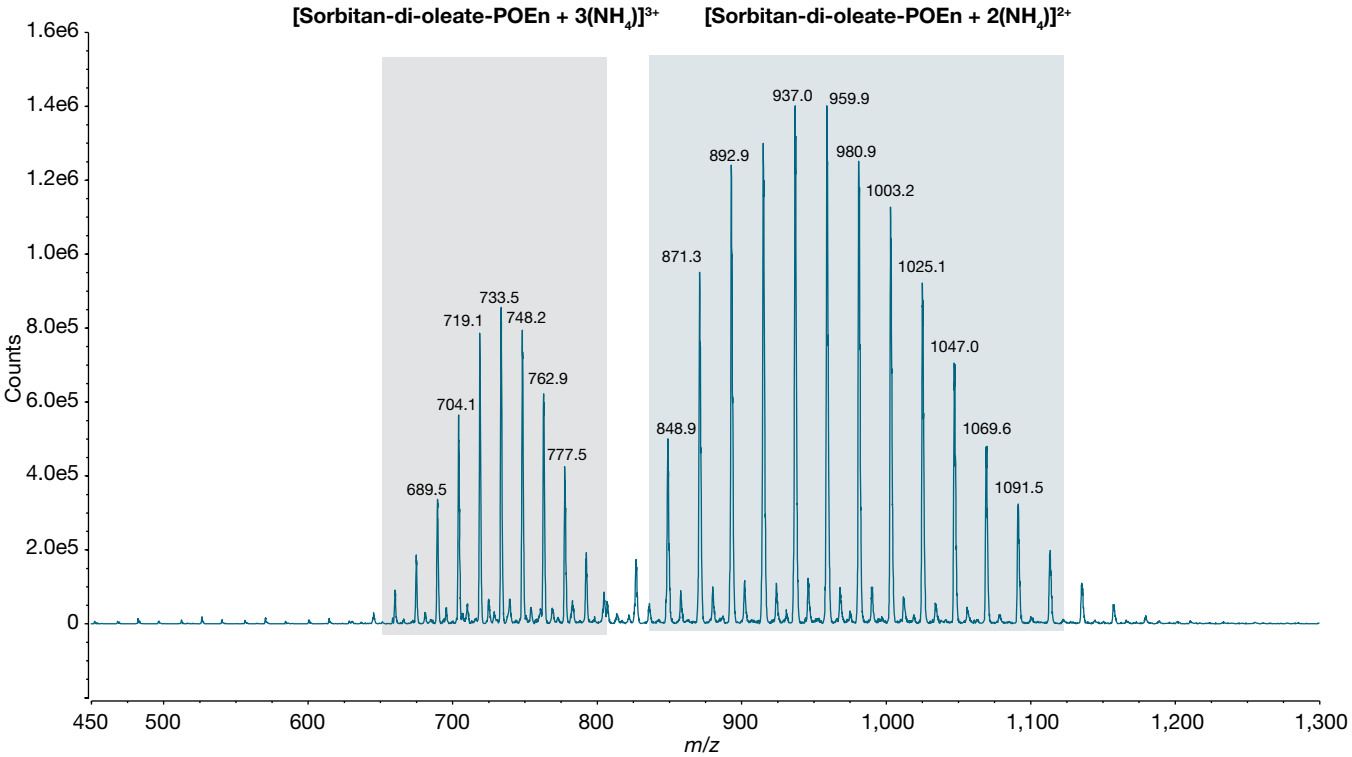

Figure 4D. Mass spectrum representing the averaged spectra across the retention time window 26.9–28.3 min, capturing the peak with apex at 27.2 min (Figure 4A). The signal ions are inferred as sorbitan-di-oleate-POEn double and triple ammoniated adducts. For doubly charged adducts polymer distribution, the detected species contain number of oxyethylene units n between 23 and 36; for the triply charged adducts polymer distribution n is between 27 and 37.

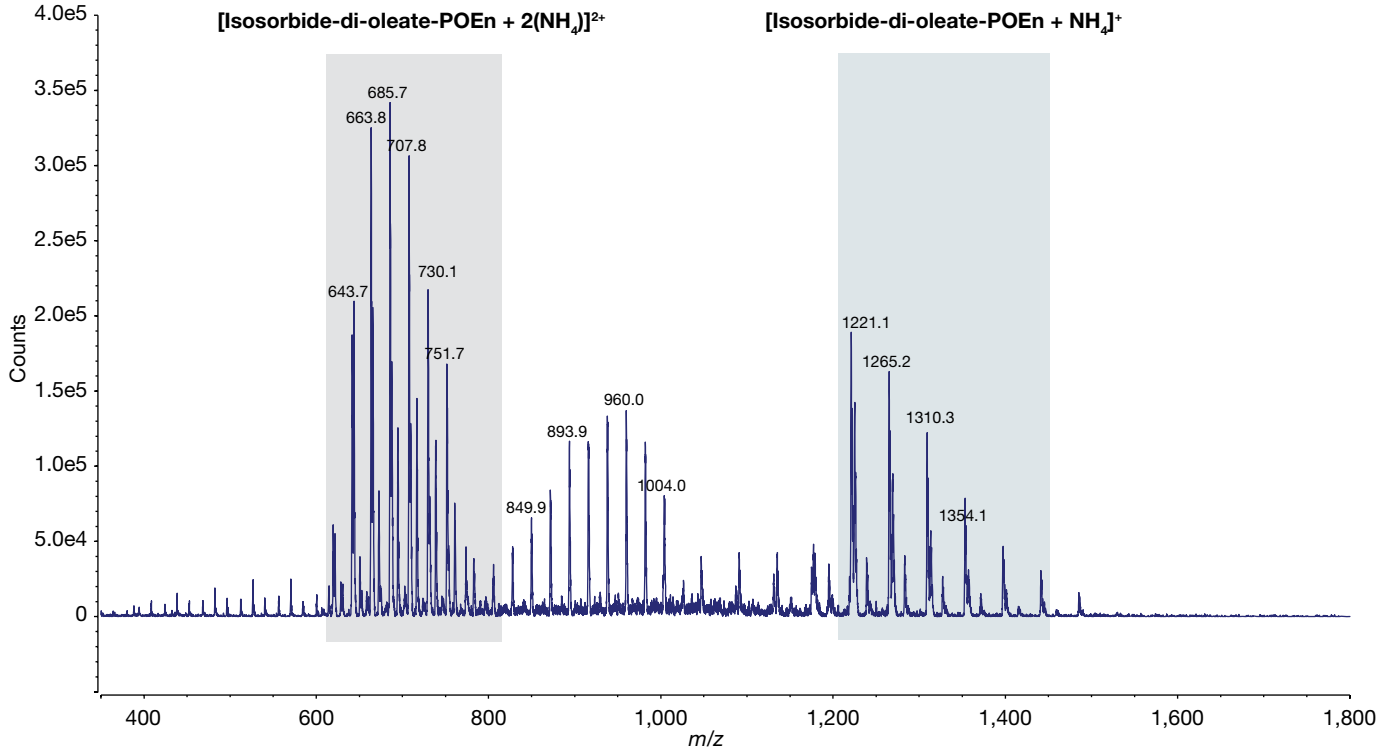

Figure 4E. Mass spectrum representing the averaged spectra across the retention time window 29.0–30.4 min. The signal ions are inferred as isosorbide-di-oleate-POEn single and double ammoniated adducts. For single charged adducts polymer distribution, the detected species contain number of oxyethylene units n between 12 and 17; for the double charged adducts polymer distribution n is between 13 and 18.

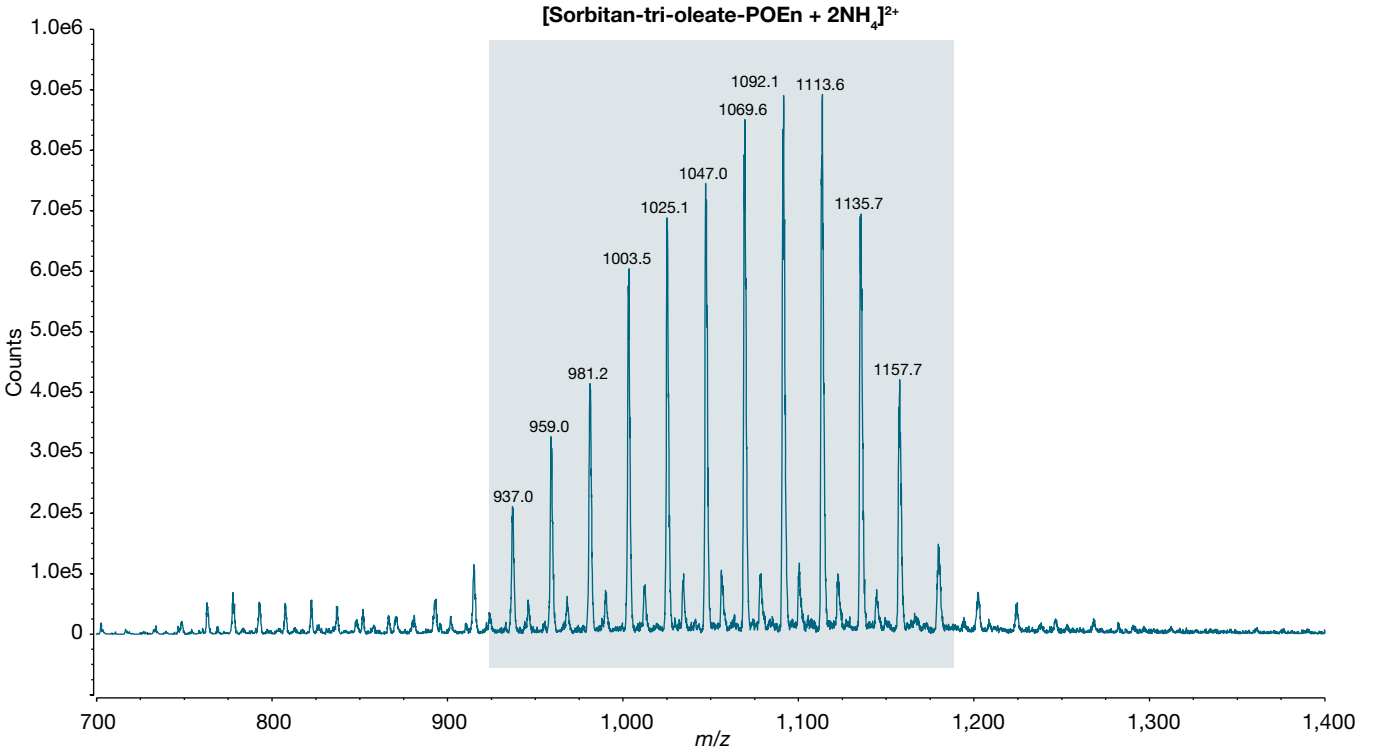

Figure 4F. Mass spectrum representing the averaged spectra across the retention time window 33.9–35.0 min. The signal ions are inferred as sorbitan-tri-oleate-POEn doubly ammoniated adducts. The detected species contain a number of oxyethylene units n between 20 and 31.

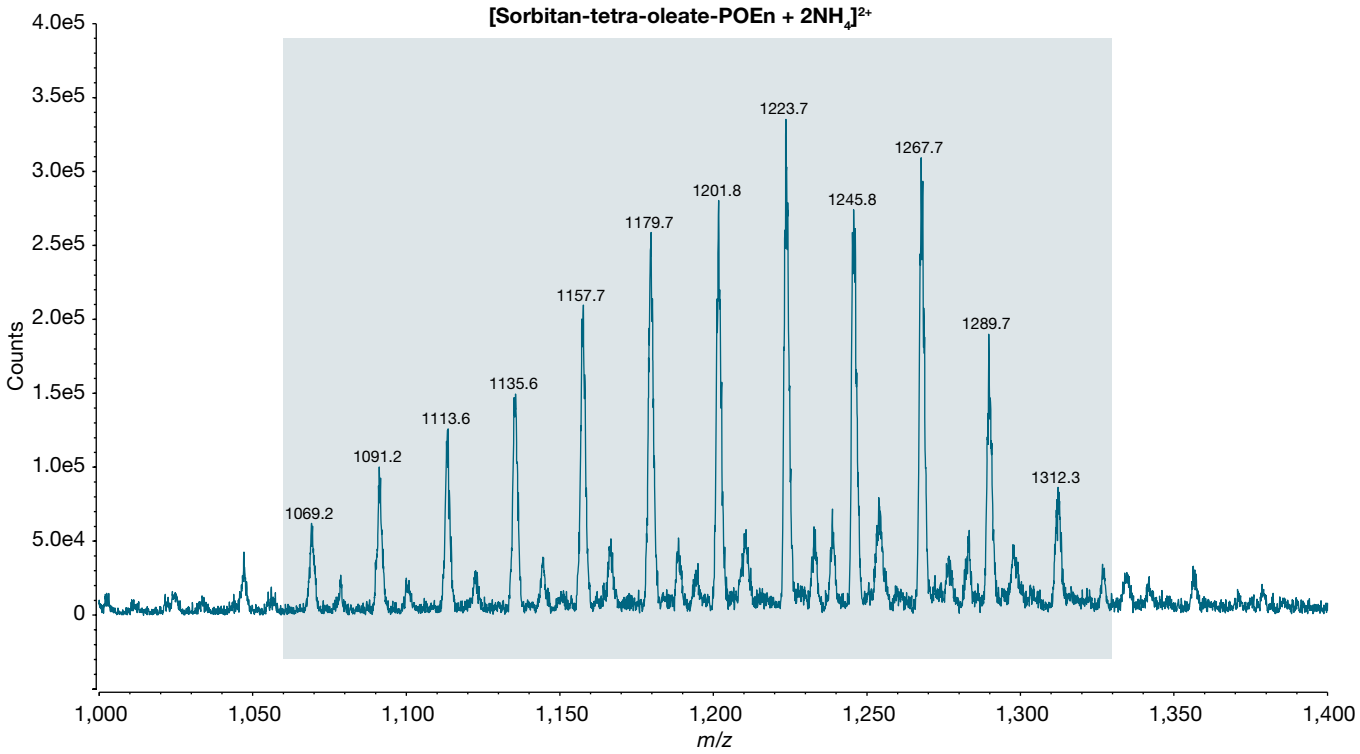

Figure 4G. Mass spectrum representing the averaged spectra across the retention time window 36.9–37.7 minutes. The signal ions are inferred as sorbitan-tetra-oleate-POEn doubly ammoniated adducts. The detected species contain a number of oxyethylene units n between 20 and 31.

The sum of the area of all peaks measured in inverse gradient varies with the sample (Table 6). Since the detector response is independent of the species, and the concentration of PS used for the test is the same, this observation points to different purities of the PS standards. When comparing relative areas of the peak groups for inverse gradient analysis (Table 6), it is observed that SA1 and SA2 contain the least amount of non-ester species (Group 1). Interestingly, these samples are those with the highest main peaks in Group 2 and Group 3. Combining this insight with the information obtained by the mass detection, we can now conclude that those peaks contain the POE sorbitan mono-esters and POE sorbitan di-esters. Thus, for the samples analyzed in this work, it appears that the amount of the polyols of Group 1 inversely correlates with the amount of oleic acid esters of sorbitan. On the other hand, the correlation with isosorbide esters is not evident.

Table 6. Overview of the relative areas of the peak groups (standard and inverse gradient) and sum of all peak areas (inverse gradient). CAD detection: 1 mg/mL samples, and ISQ EM detection: 0.5 mg/mL. The data is the average of two injections.

Gradient type	Peak group	Relative area (%)				
		SA1 (with MS detection)	SA1	SA2	SA3	SB4
Standard	G1	7.89	1.58	1.66	2.31	2.16
	G2	26.04	20.09	20.46	26.04	21.34
	G3	36.50	38.77	38.60	36.58	39.84
	G4	29.58	39.59	39.30	29.59	36.67
Inverse	G1	n.a.	8.72	8.40	11.35	10.89
	G2		33.69	34.09	34.95	33.87
	G3		35.09	34.90	33.98	34.80
	G4		22.51	22.62	19.73	20.45
			Area (pA × min)			
	Sum area all groups		10.23	10.44	9.71	9.43

As shown in Table 7 and Figure 5, peak group area measured for the sample concentration range between 0.5 and 2.5 mg/mL showed good linearity, for both standard and inverse gradient analyses. This observation, proves that the assessment of the groups' relative abundance is consistent across the investigated concentration range. The sum of the area of the four groups also correlates linearly with the amount of injected sample (Table 7). Utilizing the separation of groups based on the level of esterification, quantitation can aid in monitoring trends in ester and polyol population. This is useful for stability studies of PS-based drug formulations.

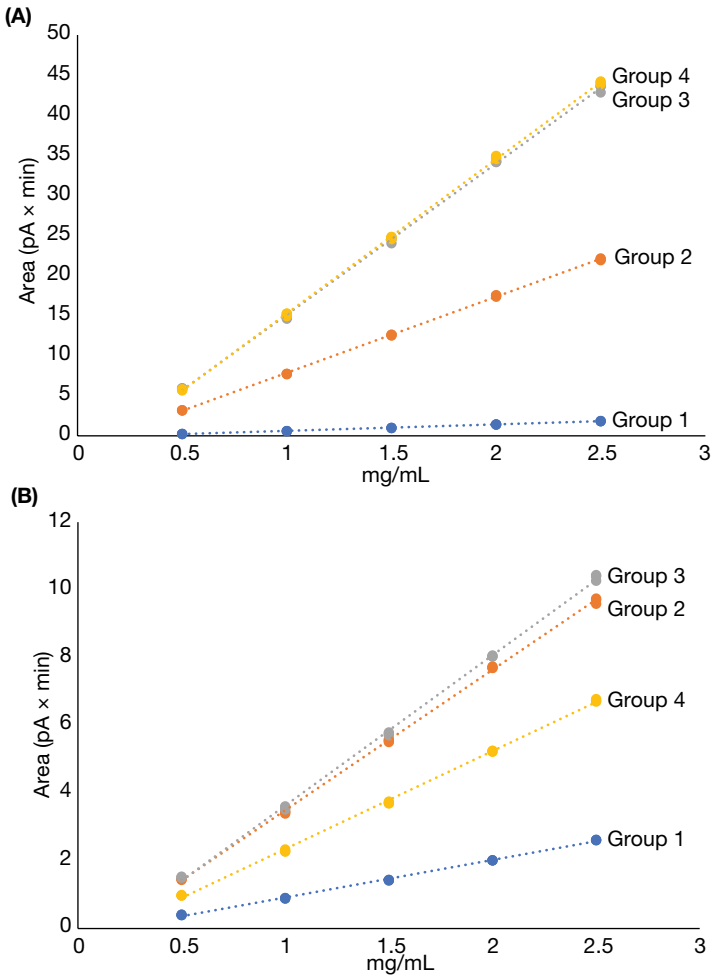

Figure 5. Group area measured at different PS 80 concentration in SA1 (0.5–2.5 mg/mL with 3 injections for each level). Standard gradient (A) and inverse gradient (B).

Table 7. Results of linear regression for the plots in Figure 5. (0.5–2.5 mg/mL with 3 injections for each level)

		Rel. Std. Dev. (%)	Coeff. of Det.	C0 (Intercept)	C1 (Slope)
Standard gradient	Group 1	2.30	0.9985	-0.1895	0.8083
	Group 2	0.94	0.9997	-1.6252	9.4848
	Group 3	1.30	0.9995	-3.7318	18.8525
	Group 4	0.82	0.9998	-3.9703	19.2355
	All groups	0.92	0.9998	-9.5167	48.3811
Inverse gradient	Group 1	2.40	0.9982	-0.1907	1.1080
	Group 2	1.16	0.9996	-0.6553	4.1464
	Group 3	1.66	0.9992	-0.8064	4.4371
	Group 4	1.77	0.9991	-0.5396	2.8892
	All groups	1.36	0.9994	-2.1920	12.5809

Conclusion

- An HPLC-CAD method for fingerprinting of PS 80 raw material capable of resolving the components based on the degree of esterification was developed.
- The method enables monitoring of different productions, thereby providing a simple, albeit reliable, tool to ensure the consistency of PS 80 raw materials and contribute to consistent drug formulation production.
- Thanks to the uniform response, CAD with inverse gradient provides the real mass balance between species with different degrees of esterification.
- Confirmation of the identity of the main components of PS 80, namely POE, sorbitan POE, isosorbide POE, isosorbide and sorbitan POE esters of oleic acid, is easily achieved by LC-MS with the ISQ EM mass detector.
- The total ion chromatogram based on Full MS scans is a viable alternative to CAD for PS 80 fingerprinting.

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