DETERMINATION OF FATTY ACIDS COMPOSITION IN POLYSORBATES 80 AND 20 PHARMACEUTICAL RAW MATERIALS BY HPLC WITH MASS DETECTION

Waters™

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INTRODUCTION

Polysorbates are non-ionic surfactants used as excipients or inactive ingredients in food, pharmaceutical products, and cosmetics to stabilize emulsions, solubilize ingredients, and improve the overall formulation's stability and texture. The most common types are polysorbates 80 and 20.

For the determination of fatty acids composition in polysorbates 80 and 20, the U.S. Pharmacoeia recommends a gas chromatography (GC) with flame ionization detector (FID) procedures (USP–NF 2021 Issue 1). These procedures require hydrolysis and derivatization of polysorbates to convert methylated acids to free fatty acids.

In this work, new HPLC methods with mass detection were developed for the determination of free fatty acid composition in polysorbates 80 and 20 by direct analysis of hydrolyzed samples.

METHODS

Standard Solutions Preparation

Individual fatty acids stock standard solutions were prepared in ethanol at 1 mg/mL. Stock standard solutions were diluted with water/ethanol (50:50, v/v) to make two separate standard mixtures for analysis of polysorbates 20 and 80, respectively.

Sample Solutions Preparation

Polysorbates test samples were hydrolyzed with 1 M potassium hydroxide (KOH) in water to release fatty acids. The test samples prepared in 1 M KOH at 1.5 mg/mL were incubated for 6 hours at 40°C. Solutions were then cooled to room temperature, neutralized with equal volume of 1 M formic acid, and diluted with water/ethanol (50:50, v/v) to 0.1 mg/mL. All test sample solutions were filtered through GHP syringe filters prior analysis.

LC System	Arc™ HPLC System with column heater/cooler with passive pre-heater, ACQUITY™ QDa™ Detector, Isocratic solvent manager (ISM)
Mobile Phase	Solvent A: 10 mM Ammonium acetate in water Solvent B: Acetonitrile Solvent C: Isopropyl alcohol (used for seal wash)
Column	XBridge™ BEH™ C18 Column, 4.6 x 100 mm, 3.5 µm, at 60 °C
Flow Rate	2.0 mL/min
Injection Vol.	25.0 µL
Sample Temp.	10 °C

Gradient:

Polysorbate 80					Polysorbate 20				
Time (min)	%A	%B	%C	Curve	Time (min)	%A	%B	Curve	
Initial	60.0	40.0	0.0	6	Initial	95.0	5.0	6	
1.00	60.0	40.0	0.0	6	1.00	95.0	5.0	6	
14.00	20.0	80.0	0.0	6	1.10	60.0	40.0	6	
14.10	0.0	50.0	50.0	6	14.00	5.0	95.0	6	
16.00	0.0	50.0	50.0	6	16.00	5.0	95.0	6	
16.10	60.0	40.0	0.0	6	16.10	95.0	5.0	6	
20.00	60.0	40.0	0.0	6	20.00	95.0	5.0	6	

Wash solvents	Purge/sample wash: 60/40 water/acetonitrile Seal wash: 90:10 water/acetonitrile
MS Detection	Ionization mode: Electrospray negative (ESI-) MS Acquisition: range: 75 – 350 m/z, Single Ion Recording (SIR) for quantitation Probe temperature: 600°C Capillary Voltage: 0.5 kV Cone Voltage: 10 V
Isocratic solvent manager (ISM)	Makeup solvent: 50:50 water/acetonitrile with 1 mM ammonium acetate Flow rate: 0.2 mL/min, with 10:1 split and dilute ratio

Table 1. HPLC-MS conditions for analysis of polysorbates 20 and 80.

RESULTS AND DISCUSSION

The developed HPLC-MS methods were used to determine fatty acid composition in polysorbates 80 and 20 by direct analysis of hydrolyzed samples. The isocratic solvent manager (ISM) was used to split and dilute the flow entering the ACQUITY QDa Detector. The ISM make-up (dilution) solvent was added post-column and mixed with the flow entering the source.

Analysis of Polysorbate 80

The developed HPLC-MS method successfully separated all the USP-specified fatty acids in polysorbate 80 (Figure 1). The mass spectral data enabled quick identification of the fatty acids (Figure 1A), while the single ion recording (SIR) was used for quantitative analysis (Figure 1B).
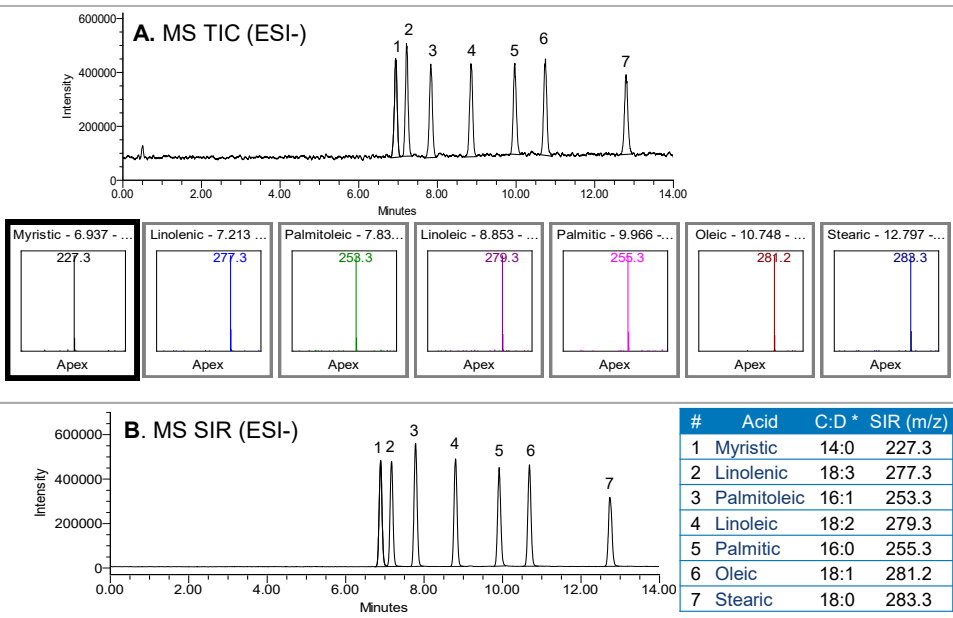

Figure 1. Chromatographic separation of the USP-specified fatty acids in polysorbate 80 by an ACQUITY Arc HPLC System with ACQUITY QDa Detector. Total ion chromogram (TIC) with mass spectral data (A) and single ion recording (SIR) channel (B). * C:D - carbon to carbon chain length: number of double bonds.

#	Acid	C:D *	SIR (m/z)
1	Myristic	14:0	227.3
2	Linolenic	18:3	277.3
3	Palmitoleic	16:1	253.3
4	Linoleic	18:2	279.3
5	Palmitic	16:0	255.3
6	Oleic	18:1	281.2
7	Stearic	18:0	283.3

Different reaction media were investigated during the study to ensure complete extraction of all fatty acids from the test samples (Figure 2). Hydrolysis with base released most fatty acids. Therefore, all samples were hydrolyzed with 1 M KOH for 6 hours at 40°C, neutralized with equal concentration of formic acid, and diluted with water/ethanol (50:50, v/v) to 0.1 mg/mL. Previously published studies also used base hydrolysis[5].
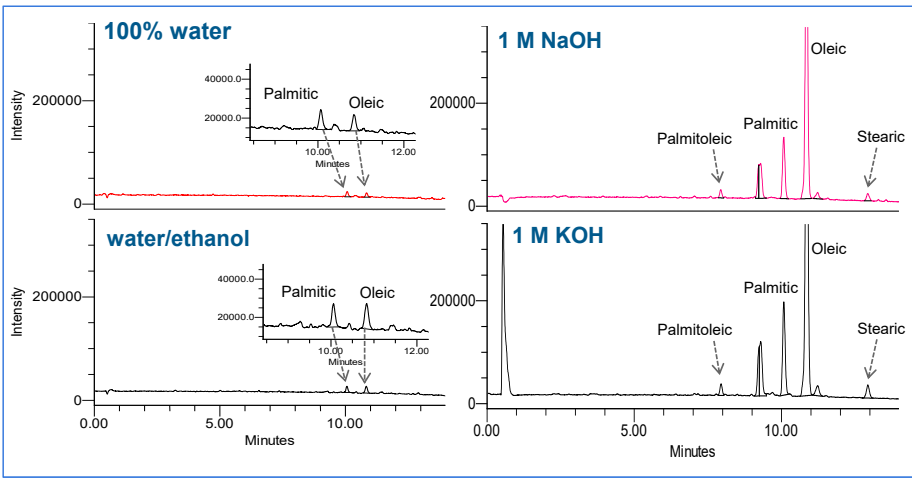

Figure 2. Hydrolysis study of polysorbate 80 in different reaction media to release fatty acids. Arc HPLC System with an ACQUITY QDa Detector, MS SIR data. Hydrolysis with base released most fatty acids.

Analysis of the polysorbate 80 samples revealed the presence of unknown peaks around 9 and 11 minutes with the same m/z values as the linoleic (18:2) and oleic (18:1) acids of 279.2 and 281.3, respectively (Figure 3). It was concluded that the unknown peaks were positional isomers of the linoleic and oleic acids.
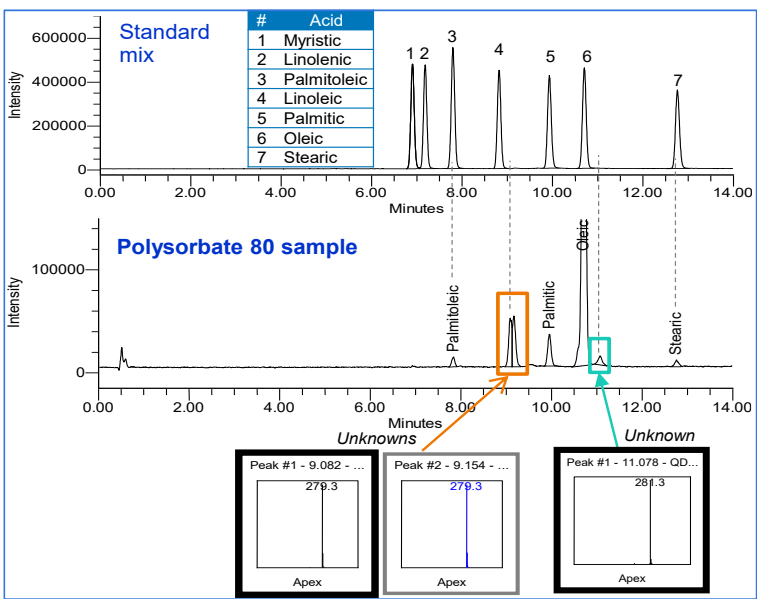

Figure 3. Polysorbate 80 analysis for free fatty acids revealed presence of unknowns peaks with m/z of 279.2 and 281.3.

The unknown peaks identity was verified via retention times and analysis of isomers standards (purchased from Nu-Chek Prep. Inc.) using a Xevo™ G2-XS QTof Mass Spectrometer (MS) coupled to an ACQUITY™ UPLC™ I-Class System. For the separation, the HPLC conditions were scaled to a 1.7 µm particle size column with 2.1 x 150 mm dimension.
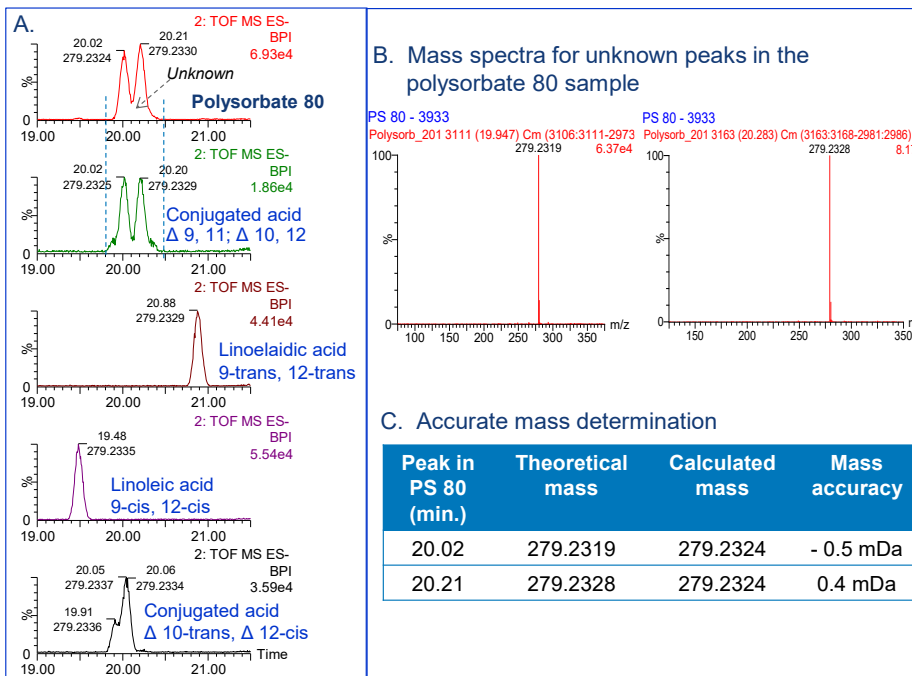

Figure 4. Identity verification of a peak with m/z 279 using Xevo G2-XS QTof MS. Isomers of linoleic acid (A), mass spectral data (B) and accurate mass determination (C). The unknown peak was identified as a mixture of conjugated linoleic acid isomers (Δ 9, 11; Δ 10, 12).

For peak with m/z 281, the analysis showed presence of two positional isomers of oleic acid, eluting before and after the oleic peak (Figure 5). These compounds were identified as cis-vaccenic and elaidic acids.
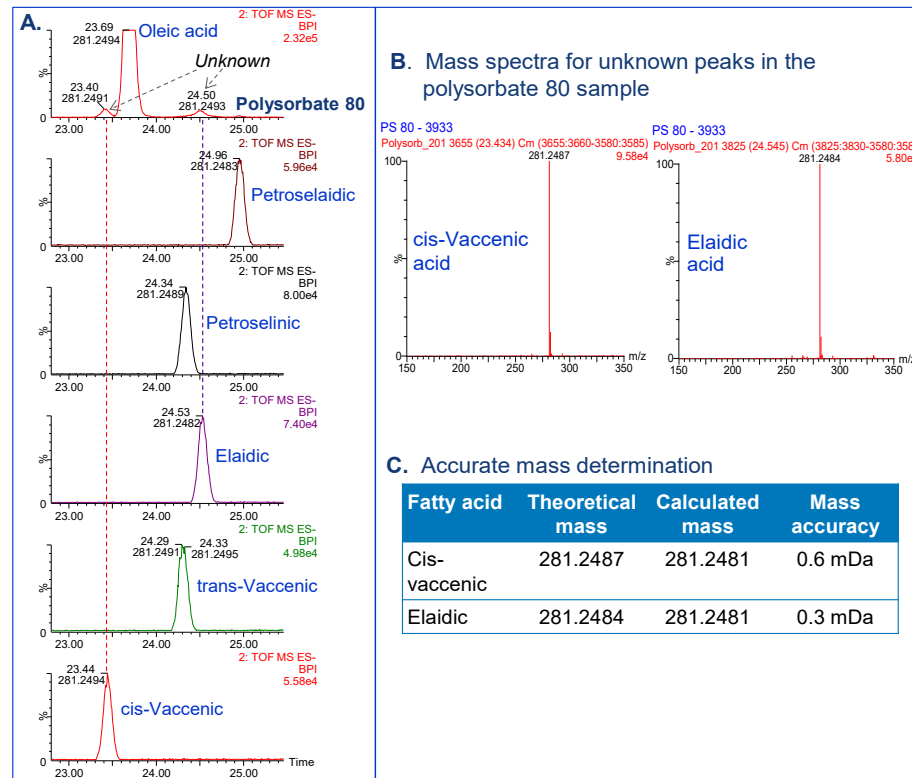

Figure 5. Identity verification of a peak with m/z 281 using Xevo G2-XS QTof MS. Isomers of oleic acid (A), mass spectral data (B) and accurate mass determination (C). Peaks were identified as cis-vaccenic and elaidic acids.

Composition of the fatty acids in the polysorbate 80 batches was determined by comparing peak area of each fatty acid to the total area of all fatty acids found in the chromatographic injection. Calculations performed following the USP monograph[3]. In this case, the calculations included the USP-specified fatty acids found in the test samples and isomers of linoleic and oleic acids detected by the new HPLC-MS method, all performed using Empower™ Software (Table 2). The USP-specified fatty acids found in all batches were within the USP criteria limits.

Acid Name	% Acid Batch 1	% Acid Batch 2	% Acid Batch 3	USP Criteria *
Myristic	0.1	0.5	ND	NMT 5.0%
Linolenic	ND	ND	ND	NMT 4.0%
Palmitoleic	1.2	1.1	1.0	NMT 8.0%
Linoleic	0.2	ND	ND	NMT 18.0%
Conjugated Δ 9, 11; Δ 10, 12	11.5	12.2	11.6	N/A
Palmitic	11.4	4.2	4.3	NMT 16.0%
Cis-vaccenic	1.1	ND	ND	N/A
Oleic	70.6	79.2	79.8	NLT 58.0%
Elaidic	1.9	1.3	2.0	N/A
Stearic	2.0	1.7	1.1	NMT 6.0%

Table 2. Fatty acids composition (% acid) in the polysorbate 80 batches. MS SIR. NMT: not more than, NLT: not less than. * Criteria according to USP monograph for polysorbate 80[3].

Analysis of Polysorbate 20

The developed HPLC-MS successfully separated all fatty acids specified by the USP for polysorbate 20 (Figure 6).
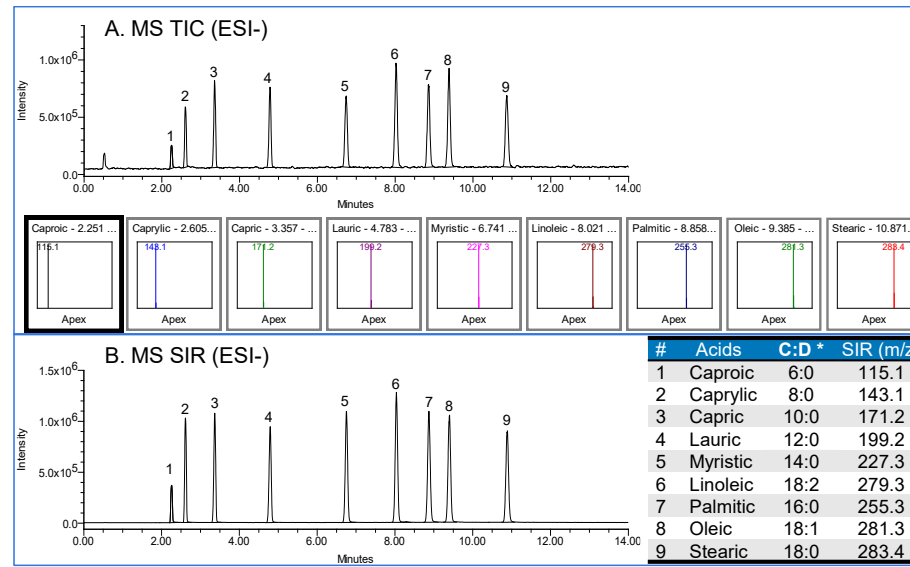

Figure 6. Chromatographic separation of the USP-specified fatty acids in polysorbate 20 using an Arc HPLC System with ACQUITY QDa Detector. Standard solution at 20 µg/mL. A: total ion chromogram (TIC), B: overlay of single ion recording (SIR) channels.

#	Acids	C:D *	SIR (m/z)
1	Caproic	6:0	115.1
2	Caprylic	8:0	143.1
3	Capric	10:0	171.2
4	Lauric	12:0	199.2
5	Myristic	14:0	227.3
6	Linoleic	18:2	279.3
7	Palmitic	16:0	255.3
8	Oleic	18:1	281.3
9	Stearic	18:0	283.4

Composition of fatty acids calculated using Empower Software following the USP monograph for polysorbate 20[4]. Results met the USP criteria (Figure 7).

#	Acid	SIR (m/z)
1	Caprylic	143.1
2	Capric	171.2
3	Lauric	199.2
4	Myristic	227.3
5	Palmitic	255.3
6	Oleic	281.3

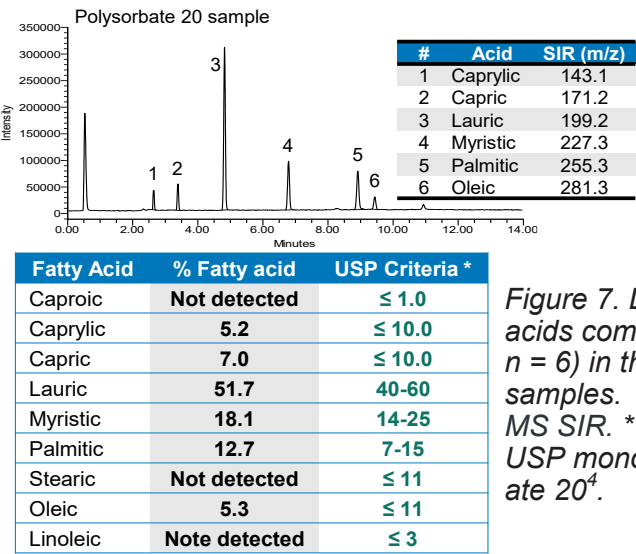| Fatty Acid	% Fatty acid	USP Criteria *
Caproic	Not detected	≤ 1.0
Caprylic	5.2	≤ 10.0
Capric	7.0	≤ 10.0
Lauric	51.7	40-60
Myristic	18.1	14-25
Palmitic	12.7	7-15
Stearic	Not detected	≤ 11
Oleic	5.3	≤ 11
Linoleic	Note detected	≤ 3

Figure 7. Determination of fatty acids composition (average of n = 6) in the polysorbate 20 samples. MS SIR. * Criteria according to USP monograph for polysorbate 20[4].

CONCLUSION

- The developed HPLC-MS methods offers fast quality assessment of the polysorbates 80 and 20 by measuring fatty acids composition.
  - Direct injection of hydrolyzed samples.
  - Easy and accurate identification of fatty acids by mass detection using an ACQUITY QDa Detector.
  - Integrated with a compliant-ready Empower Software, suitable for routine QC testing.
- HPLC-MS method separates additional fatty acids not listed in the GC-FID procedure for polysorbate 80 recommended by the USP (USP–NF 2021 Issue 1).
- The QTof mass spectrometer enables accurate identity verification of unknown peaks.

References

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