QUALITY ASSESSMENT OF POLYSORBATES 80 AND 20 PHARMACEUTICAL RAW MATERIALS BY MEASURING FATTY ACIDS COMPOSITION USING HPLC WITH MASS DETECTION

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INTRODUCTION

Polysorbates are non-ionic surfactants widely used as excipients or inactive ingredients in food and pharmaceutical products[1,2]. The quality and purity of excipients are critical to the safety of the finished drug products and must be demonstrated using suitable and reliable test methods.

The U.S. Pharmacopoeia specifies a gas chromatography (GC) with flame ionization detector (FID) procedures for the polysorbate 80[3] and 20[4] based on the fatty acids composition by conversion of methylated acids to free fatty acids. Both procedures require hydrolysis and derivatization of the polysorbates to free fatty acids.

In this work, simple and fast HPLC-mass spectrometry (MS) methods were developed for the determination of fatty acids composition in the polysorbates 80 and 20 by direct analysis of the hydrolyzed samples. The new HPLC-MS methods offers fast quality assessment of the polysorbate 80 pharmaceutical raw materials 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 system wash)
Column	XBridge™ BEH C_{18}, 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 80

Time (min)	%A	%B	%C	Curve
Initial	60.0	40.0	0.0	6
1.00	60.0	40.0	0.0	6
14.00	20.0	80.0	0.0	6
14.10	0.0	50.0	50.0	6
16.00	0.0	50.0	50.0	6
16.10	60.0	40.0	0.0	6
20.00	60.0	40.0	0.0	6

Polysorbate 20

Time (min)	%A	%B	Curve
Initial	95.0	5.0	6
1.00	95.0	5.0	6
1.10	60.0	40.0	6
14.00	5.0	95.0	6
16.00	5.0	95.0	6
16.10	95.0	5.0	6
20.00	95.0	5.0	6

Wash solvents	Purge/sample wash: 60/40 water/acetontrile 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 hydrolyzed polysorbates samples were analyzed for fatty acids content using reverse-phase separation and mass detection. For quantitative analysis, fatty acids were measured using a single ion recording (SIR) mode. 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

Fatty acids specified by the USP for polysorbate 80 are shown Table 2. The developed HPLC-MS method successfully separated all the USP-specified fatty acids (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).

Acid	C:D *	Monoisotopic mass (Da)	Structure
Myristic	14:0	228.21	$CH_3(CH_2)_{11}CH_2COOH$
Palmitic	16:0	256.24	$CH_3(CH_2)_{13}CH_2COOH$
Palmitoleic	16:1	254.22	$CH_3(CH_2)_4CH{=}CH(CH_2)_7CH_2COOH$
Stearic	18:0	284.27	$CH_3(CH_2)_{15}CH_2COOH$
Oleic	18:1	282.26	$CH_3(CH_2)_6CH{=}CH_2$
Linoleic	18:2	280.24	$HO(CH_2)_3CH{=}CH_2$
Linolenic	18:3	278.22	$H_3C(CH_2)_5CH{=}CH_2$

Table 2. Fatty acids specified in the USP monograph for polysorbate 80[3].

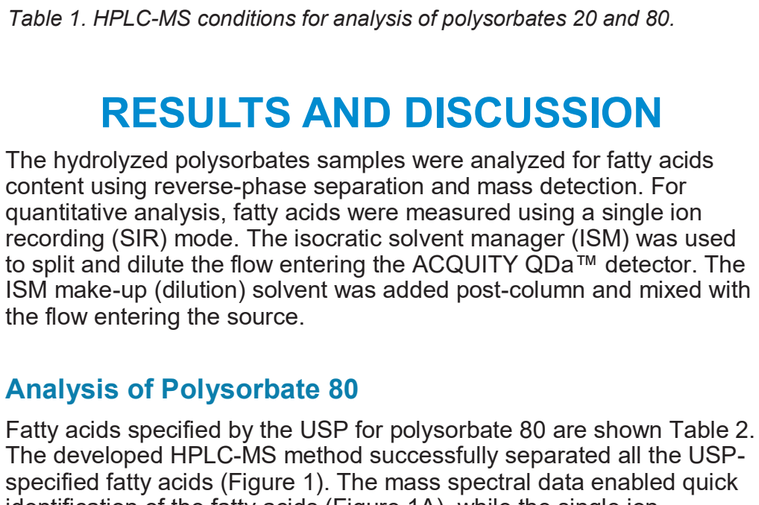


Figure 1. Chromatographic separation of the USP-specified fatty acids in polysorbate 80 by an ACQUITY Arc™ HPLC System with ACQUITY QDa Detector. Standard solution at 10 µg/mL. Total ion chromograph (TIC) with mass spectral data (A) and overlay of single ion recording (SIR) channels (B).

Various ISM makeup solvents were screened to enhance the MS signal, while ensuring acceptable repeatability of the replicate injections (Figure 2). The ammonium acetate/acetonitrile makeup solvent provided highest signal for fatty acids and lowest % RSD of peak areas (n = 5), hence it was selected for final method.

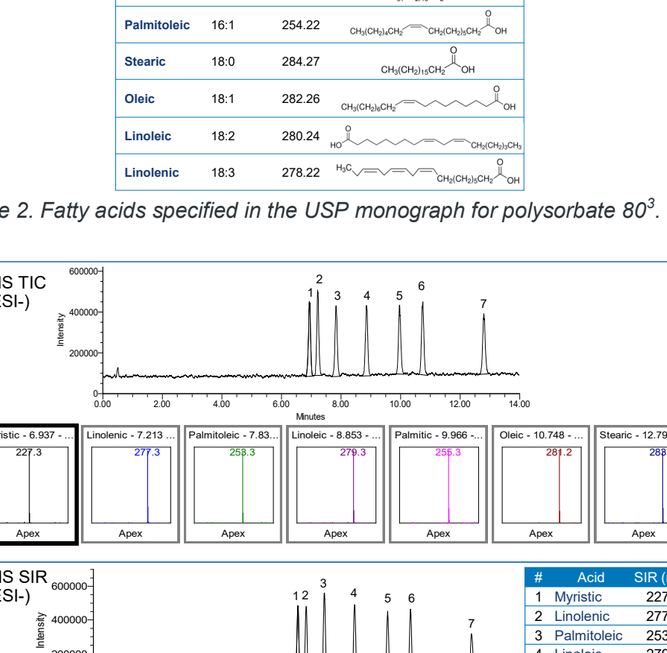


Figure 2. ISM makeup solvents screening. A: acetonitrile/water (50:50 v/v), B: methanol/water (50:50 v/v), C: 1 mM ammonium acetate in acetonitrile/water (50:50 v/v). MS SIR data for 20 µg/mL fatty acid standard. Ammonium acetate/acetonitrile provided best signal and lowest %RSD.

Different reaction media were investigated during the study to ensure complete extraction of all fatty acids from the test samples (Figure 3). Hydrolysis with base released most fatty acids. Therefore, all samples were hydrolyzed with 1 M KOH for 6 hours at 40°C, neutralized with 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 3. Hydrolysis study of polysorbate 80 in different reaction media to release fatty acids. ACQUITY Arc System with an ACQUITY QDa Detector, MS SIR data. Hydrolysis with base released most fatty acids.

Analysis of the polysorbate 80 samples revealed 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 4). It was concluded that the unknown peaks were positional isomers of the linoleic and oleic acids.

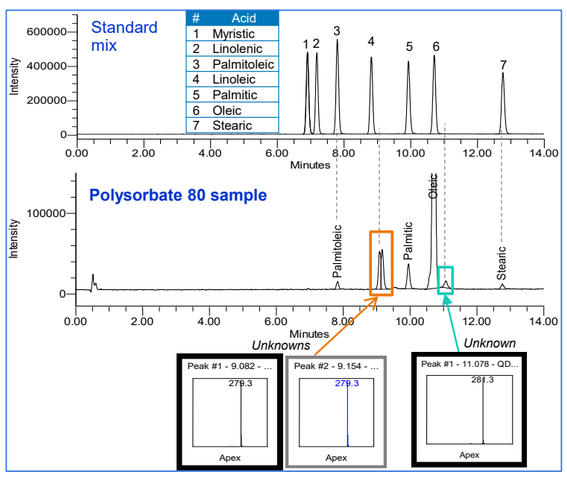


Figure 4. Polysorbate 80 analysis for free fatty acids revealed presence of unknowns peaks with m/z of 279.2 and 281.3.

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 coupled to a UPLC system. For UPLC separation, the HPLC conditions were scaled to a 1.7 µm particle size column with 2.1 x 150 mm dimension.

The unknown peak with *m/z* 279 was identified as a mixture of conjugated linoleic acid isomers (Δ 9, 11; Δ 10, 12). (Figure 5). Mass accuracy was found to be -0.5 and 0.4 mDa (Figure 5C), respectively.

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 6). These compounds were identified as cis-vaccenic and elaidic acids, with mass accuracy of 0.7 mDa and 0.8 mDa, respectively (Figure 6C).

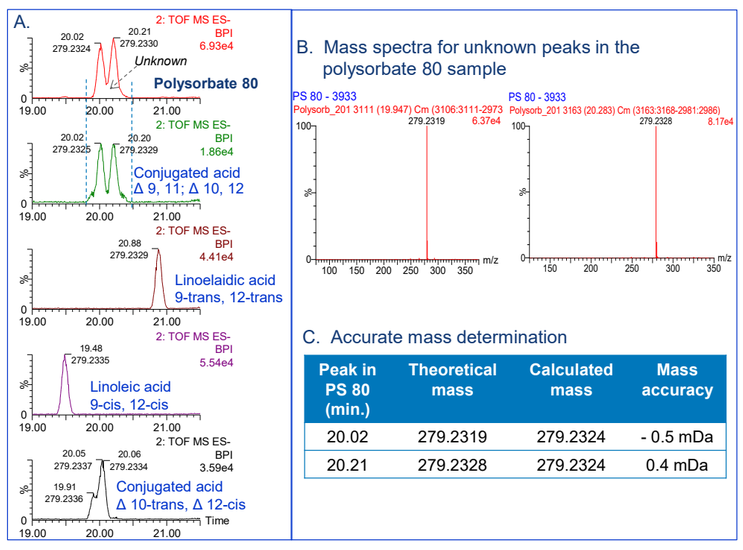


Figure 5. Identity verification of a peak with m/z 279 using Xevo™ G2-XS QTof. Isomers of linoleic acid (A), mass spectral data (B) and accurate mass determination (C). The unknown peak was identified as a mixture of conjugated linoleic acid isomers (Δ 9, 11; Δ 10, 12).

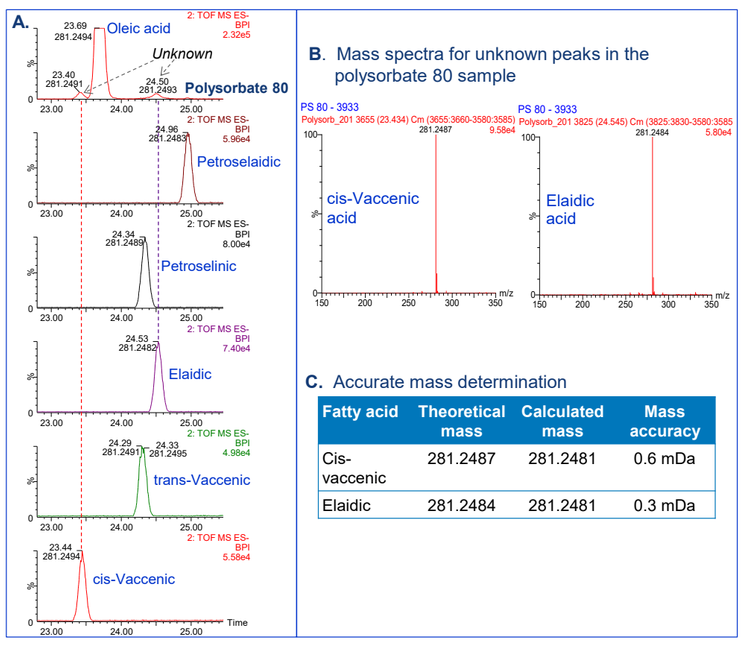


Figure 6. Identity verification of a peak with m/z 281 using Xevo G2-XS QTof . Isomers of oleic acid (A), mass spectral data (B) and accurate mass determination (C). Peaks were identified as cis-vaccenic and elaidic acids.

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 3). 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 3. Determination of fatty acids composition (% acid) in the polysorbate 80 batches using an ACQUITY Arc System with ACQUITY QDa Mass Detector, MS SIR. NMT: not more than, NLT: not less than. * USP monograph for polysorbate 80[3].*

Analysis of Polysorbate 20

Fatty acids specified by the USP for polysorbate 20 are shown Table 4. All the USP-specified fatty acid were successfully using the new developed HPLC-MS (Figure 7).

Fatty acid	C:D *	Monoisotopic mass (Da)	Structure
Caproic	6:0	116.08	$CH_3(CH_2)_3CH_2COOH$
Caprylic	8:0	144.11	$CH_3(CH_2)_6CH_2COOH$
Capric	10:0	172.14	$CH_3(CH_2)_8CH_2COOH$
Lauric	12:0	200.11	$CH_3(CH_2)_{10}COOH$
Myristic	14:0	228.21	$CH_3(CH_2)_{11}CH_2COOH$
Palmitic	16:0	256.24	$CH_3(CH_2)_{13}CH_2COOH$
Stearic	18:0	284.27	$CH_3(CH_2)_{15}CH_2COOH$
Oleic	18:1	282.26	$CH_3(CH_2)_6CH{=}CH_2$
Linoleic	18:2	280.24	$HO(CH_2)_3CH{=}CH_2$

Table 4. Fatty acids specified in the USP monograph for polysorbate 20[4].

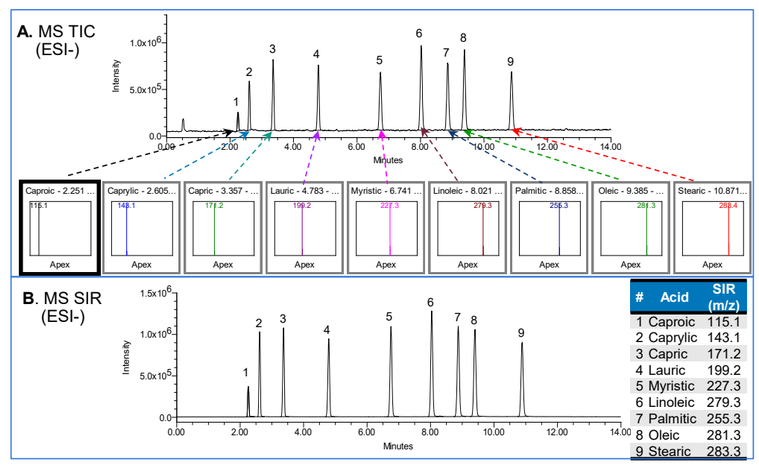
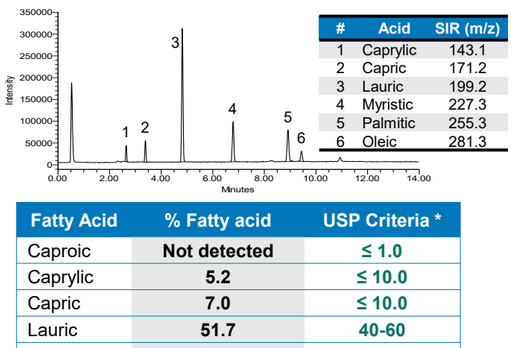


Figure 7. Chromatographic separation of the USP-specified fatty acids in polysorbate 20 using an Arc HPLC with ACQUITY QDa Detector. Standard solution at 20 µg/mL. A: total ion chromograph (TIC), B: overlay of single ion recording (SIR) channels.

The hydrolyzed Polysorbate 20 samples were analyzed for the fatty acids content following calculations in the USP monograph[4].
The percent (%) of each fatty acid was calculated using Empower Software by comparing peak area of each fatty acid to the total area of all fatty acids in the chromatographic injections. The results for composition of fatty acids in the polysorbate 20 sample solutions met the USP criteria (Figure 8).



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 8. Determination of fatty acids composition (average of n = 6) in the polysorbate 20 samples using an ACQUITY Arc System with ACQUITY QDa Mass Detector, MS SIR. . * Criteria according to USP monograph for polysorbate 20[4].*

CONCLUSION

- **The developed HPLC-MS method offers fast quality assessment of the polysorbates 80 and 20 pharmaceutical raw materials by measuring fatty acids composition in hydrolyzed samples**
  - **Direct injection of hydrolyzed samples eliminates the need for a complex sample pretreatment procedure required for analysis by GC.**
  - **Easy and accurate identification of fatty acids by mass detection using mass spectral data from 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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