

ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF POLYSORBATE 80 IN ICE CREAM BY CHROMATOGRAPHIC RP-HPLC METHOD

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ABSTRACT: A simple, rapid and precise reverse phase high-performance liquid chromatographic method has been developed and validated for the estimation of Polysorbate 80 in ice cream. Polysorbate 80 was quantified as oleic acid released by alkaline hydrolysis (saponification) of its ester bonds, followed by hexane extraction and reconstitution in acetonitrile. Chromatography was carried out on a C18 column (4.6 × 150 mm, 5 µm) using a mixture of 0.1% phosphoric acid/formic acid in water and acetonitrile (45:55% v/v) as the mobile phase at a flow rate of 1.0 mL/min, and detection was carried out at 195 nm. The retention time of Polysorbate 80 (as oleic acid) was 3.2 min. The method produces linear responses in the concentration range of 50-150 µg/mL ($r = 0.9995$). The precision of the method, expressed as %RSD, was below 2.0%, the mean recovery ranged from 98.8% to 100.1%, and the LOD and LOQ were 0.57 µg/mL and 1.74 µg/mL respectively. The method was validated for system suitability, specificity, linearity, precision, accuracy, robustness, LOD and LOQ as per ICH Q2(R1) guidelines and was successfully applied to an ice cream sample, in which Polysorbate 80 was found to be 127 mg/kg, within the prescribed limit of 175 mg/kg.

Key Words: Polysorbate 80, Ice cream, RP-HPLC, Method Development, Validation, Emulsifier.

INTRODUCTION

All age groups like ice cream, a popular frozen delicacy. Its smooth texture, resistance to melting and uniform distribution of air cells depend heavily on the stabilizers and emulsifiers added during manufacture, which affect the texture of the product even though they comprise only a relatively minor fraction of its composition [1-4]. In dairy products, emulsifiers are typically limited to 0.2% on a weight basis, whereas polysorbate is allowed up to 0.1%. [5]. Chemically speaking, polysorbate 80, sometimes referred to as Tween 80, is polyoxymethylene sorbitan monooleate. This non-ionic surfactant is employed as an emulsifier in ice cream, where it helps the product resist melting, decreases overrun, and encourages fat instability [6,7]. The prescribed regulation of the recommended prescribed regulation is limited. Polyoxymethylene sorbitan monooleate, along with similar

sorbitan and isosorbide esters, is the main component of Polysorbate 80, which is a heterogeneous mixture rather than a single defined chemical. [8]. Concerns have also been raised about the effect of dietary emulsifiers, including Polysorbate 80, on immune function and intestinal inflammation [9,10]. Routine quantification of Polysorbate 80 in ice cream is therefore necessary to confirm that the level of the additive is within the prescribed limit. Polysorbate 80 has mostly been measured in pharmaceutical formulations and complex matrices using HPLC with evaporative light scattering detection (HPLC-ELSD), HPLC-ELSD combined with ESI-MS, LC-MS/MS, and GC-MS, according to a review of the literature [8,11-14]. Because Polysorbate 80 lacks a strong chromophore, direct UV detection is challenging, and the procedures above require mass spectrometers or specialized detectors. For food matrices like ice cream, very few

techniques utilizing a traditional HPLC-PDA system have been documented. Alkaline hydrolysis of Polysorbate 80 produces oleic acid from the monooleate ester, which gives a single sharp peak that may be monitored by a PDA detector. Using a standard C18 column and acetonitrile-water mobile phase, the suggested approach is quick, easy, accurate, and cost-effective. It also requires a modest sample size. In accordance with International Council for Harmonization (ICH) guideline Q2(R1) [15], this study details the development of a straightforward RP-HPLC technique for the quantification of Polysorbate 80 in ice cream and its validation parameters.

EXPERIMENTAL

Instruments Used

The analysis was performed on an RP-HPLC system (Agilent HPLC system and Shimadzu Prominence I-Series) fully equipped with an autosampler and a photodiode array (PDA) detector. Standard laboratory glassware (volumetric flasks, pipettes and reaction vials) was used for the preparation, dilution and handling of solutions.

Chemicals Used

The ice cream sample was collected for the analysis. Polysorbate 80 reference standard (Supelco) was used. Potassium hydroxide (KOH), ethanol, oleic acid, dilute hydrochloric acid (HCl), hexane, acetonitrile, distilled water and 0.1% phosphoric acid or formic acid were used. All chemicals and solvents were of appropriate analytical/HPLC grade to ensure reliable and reproducible chromatographic results.

HPLC METHOD DEVELOPMENT:

Preparation of Sample Solution:

Polysorbate 80 in the sample is hydrolyzed to yield oleic acid, which provides a clean single peak on a PDA detector. Accurately weigh 5.0 g of melted ice cream into a reaction vial and add 5 mL of 1 M potassium hydroxide in ethanol/water. Heat at 70 °C for 1 hour to hydrolyze the ester bonds and release the oleic acid. Cool and neutralize the solution with dilute hydrochloric acid. Extract the fatty acids by adding 10 mL of hexane, vortex, and recover the top organic layer. Evaporate the hexane under nitrogen gas and reconstitute the dry residue in 2 mL of acetonitrile. Filter through a 0.22 µm PTFE filter.

Preparation of Standard Solution:

Accurately weigh 50 mg of Polysorbate 80 reference standard, dilute to 10 mL with water and subject the solution to the same hydrolysis and extraction procedure as the sample. A standard curve was prepared from the hydrolyzed standard.

Procedure:

Inject the samples by changing the chromatographic conditions and record the chromatograms, note the conditions of proper peak elution for performing validation parameters as per ICH guidelines.

Mobile Phase Optimization:

Initially the mobile phase tried was water: acetonitrile with 254 nm detection, then 0.1% phosphoric acid/formic acid in water with acetonitrile in different proportions (20:80 and 30:70) at 200 nm. Finally, the mobile phase was optimized to 0.1% phosphoric acid/formic acid (A) and acetonitrile (B) in the proportion 45:55 v/v (Table-1).

Optimization of Column:

A C18 reversed-phase column (4.6 × 150 mm, 5 µm) was used, which gave a sharp, symmetrical peak with acceptable retention at 1 mL/min flow.

Diluent Preparation:

Water in acetonitrile was used as the diluent.

METHOD VALIDATION PARAMETERS

System Suitability

Prepare the standard solution of Polysorbate 80 as described above and inject it five times into the HPLC system. Record the chromatograms and evaluate the retention time, tailing factor, theoretical plate count and the %RSD of the peak area. The tailing factor should be less than 2, the theoretical plate count more than 2000, and the %RSD of the peak area not more than 2.0.

Specificity

Other extraneous components should not interact with the analyte and should be well resolved from them. Specificity was determined by comparing the chromatograms of the Polysorbate 80 standard and the ice cream sample solution for interfering peaks at the retention time of the analyte.

Assay:

Inject three replicate injections of the standard and the sample solutions and calculate the amount of Polysorbate 80 using the formula:

$$\% \text{ Assay} = (\text{Sample area} / \text{Standard area}) \times (\text{Standard concentration} / \text{Sample concentration}) \times 100$$

Linearity:

Prepare a primary stock solution of Polysorbate 80. Pipette 0.50, 0.75, 1.00, 1.25 and 1.50 mL of the primary stock solution into separate 10 mL volumetric flasks and dilute to volume with diluent to obtain the 50%, 75%, 100%, 125% and 150% levels (50, 75, 100, 125 and 150 µg/mL). Inject each level into the chromatographic system and measure the peak area. Plot a graph of peak area versus concentration (concentration on X-axis and peak area on Y-axis) and calculate the correlation coefficient.

Precision

Precision was studied as intraday (repeatability) and inter day (intermediate) precision. The 100% standard solution was injected four times on the same day and on a different day under the same conditions, and the %RSD of the peak areas and the amount found was calculated.

Accuracy:

To achieve high dosing accuracy with micro-volumes, a 1.0 mg/mL spiking solution was prepared by dissolving 10.0 mg of Polysorbate 80 reference standard in 10 mL of diluent. The sample was spiked at 50%, 100% and 150% levels (50, 100 and 150 µg/mL), each in triplicate, and the recovery was calculated. Acceptance criterion: the % recovery for each level should be between 98.0 and 102.0%.

Limit of Detection (LOD) and Limit of Quantitation (LOQ):

A series of dilute Polysorbate 80 solutions below the working level (1.0 µg/mL down to 0.05 µg/mL) was injected along with a blank extraction sample. LOD was taken as the concentration giving a signal-to-noise (S/N) ratio between 2:1 and 3:1, and LOQ as the lowest concentration giving an S/N ratio of 10:1.

Robustness:

The analysis was performed under small deliberate changes in the chromatographic conditions to find the variability of the test results. The flow rate was varied to 0.9 and 1.1 mL/min instead of 1.0 mL/min, the mobile phase organic composition was

varied by ±2% acetonitrile, and the temperature was varied to 65 °C and 75 °C, the remaining conditions being the same.

RESULTS AND DISCUSSION

Development of Analytical Method:

The chromatographic conditions were optimized by the trial-and-error method (Table-1). In Trial 1 (water: acetonitrile, 254 nm) the peak was tailing due to lack of ionization control and the response was weak. In Trials 2 and 3 (acid in water: acetonitrile, 20:80 and 30:70, at 200 nm) the peak was sharp but the retention time was too long and the mobile phase was not perfectly balanced. The optimized conditions consisted of 0.1% phosphoric acid/formic acid in water and acetonitrile in the ratio of 45:55 v/v as the mobile phase at a flow rate of 1 mL/min, with detection at 195 nm, an injection volume of 20 µL and a run time of 15 minutes (Table-2). Polysorbate 80 (as oleic acid) eluted at about 3.2 min with a sharp, symmetrical peak (Fig-1 and Fig-2).

Table-1: Method Development Trials

Trial	Mobile phase	λ (nm)	Observation
1	Water: ACN	254	Peak tailing; weak response
2	Acid in water: ACN (20:80)	200	Sharp peak; RT too long
3	Acid in water: ACN (30:70)	200	Sharp peak; RT too long; phase not balanced
Optimized	Acid in water: ACN (45:55)	195	Sharp peak; RT 3.2 min

Table-2: Optimized Chromatographic Conditions

Parameter	Condition
Column	C18 reversed phase (4.6 × 150 mm, 5 µm)
Mobile phase	0.1% Phosphoric acid/formic acid in water (A): Acetonitrile (B), 45:55 v/v
Flow rate	1.0 mL/min
Detector	PDA at 195 nm
Injection volume	20 µL
Run time	15 min
Retention time	3.2 min

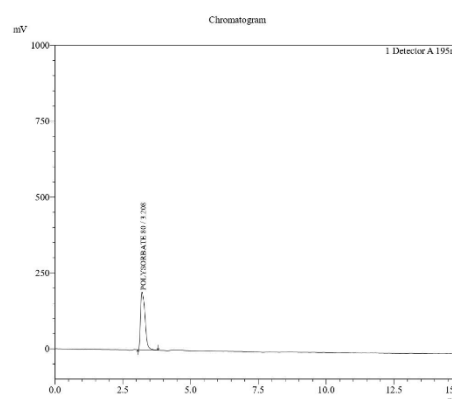


Fig-1: Chromatogram of Polysorbate 80 Standard (as Oleic acid)

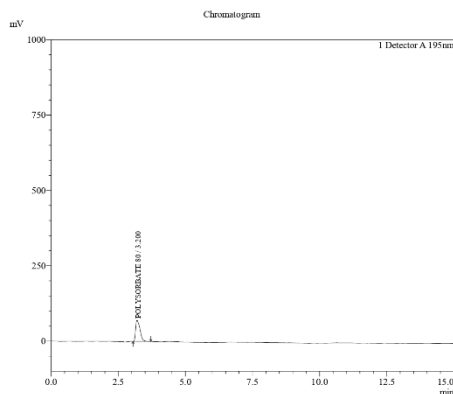


Fig-2: Chromatogram of Polysorbate 80 in Ice Cream Sample

Method Validation

Once the chromatographic and the experimental conditions were established, the method was validated by the determination of the following parameters: system suitability, specificity, linearity, precision, accuracy, robustness, limit of detection (LOD) and limit of quantitation (LOQ) as per ICH Q2(R1) guidelines.

System Suitability:

The standard solution was injected five times and the system suitability parameters were evaluated. The %RSD of the peak area of five replicate injections was 0.54%, the tailing factor ranged from 1.18 to 1.28 and the theoretical plate count from 2284 to 2325, all within the acceptance limits (Table-3).

Table-3: Results of System Suitability for Polysorbate 80

S.No.	RT (min)	Area ($\mu V \cdot sec$)	USP Plate Count	USP Tailing
1	3.208	788312	2306	1.25
2	3.210	795986	2284	1.28
3	3.200	785886	2318	1.18
4	3.202	786720	2284	1.20
5	3.200	785828	2325	1.18
Mean	3.204	788546.4		
Std. Dev.		4278.02		
% RSD		0.54		

Specificity:

According to the ICH publications, specificity is the capacity to definitively evaluate the analyte in the presence of elements such contaminants, degradation products, and matrix components that would be expected to be present. The method is particular for measuring Polysorbate 80 in ice cream, as

evidenced by the chromatogram of the ice cream sample, which displayed a single peak at the same retention period as the standard (3.20 min) with no interference from any other peak.

Linearity:

Linearity was studied over the range of 50-150 $\mu g/mL$ (50% to 150% levels). The plot of concentration (x) versus peak area (y) was a straight line (Fig-3), with slope (m) = 7976, intercept (c) = -11816 and correlation coefficient (r) = 0.9995 ($r^2 = 0.999$).

Table-4: Linearity Data of Polysorbate 80

Concentration Level (%)	Concentration ($\mu g/mL$)	Peak Area
50	50	382000
75	75	597002
100	100	786924
125	125	971000
150	150	1192003
r^2		0.999

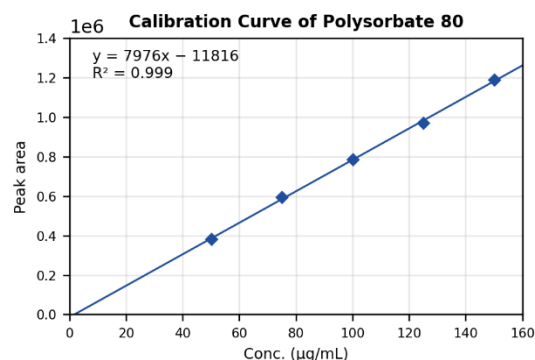


Fig-3: Calibration Curve of Polysorbate 80

Validation Criteria: The response linearity is verified if the correlation coefficient is 0.99 or greater. Conclusion: The correlation coefficient (r) of 0.9995 meets the validation criteria.

Precision:

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. The %RSD of the peak area and of the amount found was 0.2% for both intraday and inter day precision (Table-5 and Table-6), well within the limit of NMT 2.0%, indicating the repeatability of the method.

Table-5: Results of Intraday Precision for Polysorbate 80

S.No.	Peak Area	Amount Found (ppm)
1	786924	127.12
2	785112	126.83
3	788440	126.99
4	786119	127.49
5	786112	127.44
6	785113	126.82
% RSD	0.2	0.2

Table-6: Results of Inter Day Precision for Polysorbate 80

S.No.	Peak Area	Amount Found (ppm)
1	791200	127.81
2	793450	128.18
3	789110	127.48
4	792600	127.68
5	792701	127.64
6	788110	127.72
% RSD	0.2	0.2

Accuracy:

Accuracy at different concentrations (50%, 100% and 150%) was prepared in triplicate and the % recovery was calculated (Table-7). The % mean recovery was found to be 99.37%, 98.78% and 100.12% at the three levels, which is within the acceptance criterion of 98.0-102.0% and indicates the accuracy of the method.

Table-7: The Accuracy Results for Polysorbate 80

% Level	Amount Spiked (µg/mL)	Amount Recovered (µg/mL)	% Recovery	Mean % Recovery
50%	50	49.74	99.48	99.37
	50	49.50	99.00	
	50	49.82	99.64	
100%	100	98.62	98.62	98.78
	100	98.72	98.72	
	100	99.01	99.01	
150%	150	150.70	100.46	100.12
	150	149.91	99.94	
	150	149.94	99.96	

Limit of Detection:

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. The LOD corresponds to an S/N ratio of about 3:1 ($LOD = 3.3 \times \sigma/S$, where σ is the standard deviation of the response and S is the slope of the calibration curve). Result: $LOD = 0.57 \mu\text{g/mL}$.

Quantitation Limit:

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be

quantitatively determined. The LOQ corresponds to an S/N ratio of 10:1 ($LOQ = 10 \times \sigma/S$). Result: $LOQ = 1.74 \mu\text{g/mL}$.

Robustness

The robustness was performed for the flow rate variation (0.9 and 1.1 mL/min), the mobile phase organic composition variation ($\pm 2\%$ acetonitrile) and the temperature variation (65 °C and 75 °C) for Polysorbate 80. There was no significant change in the chromatographic parameters, and the %RSD was 0.81, which is within the limit of NMT 2.0%, proving that the method is robust (Table-8).

Table-8: Robustness Conditions Evaluated

Parameter varied	Condition
Flow minus	0.9 mL/min
Flow plus	1.1 mL/min
Mobile phase minus	-2% ACN
Mobile phase plus	+2% ACN
Temperature minus	65 °C
Temperature plus	75 °C
Overall % RSD	0.81

Table-9: Summary of Validation Parameters

Parameter	Polysorbate 80	Limit
Linearity range	50-150 µg/mL	-
Correlation coefficient (r)	0.9995	NLT 0.99
Regression equation	$y = 7976x - 11816$	-
Specificity	Specific	No interference
System precision % RSD	0.54	NMT 2.0%
Method precision % RSD	0.2	NMT 2.0%
Accuracy (% recovery)	98.78-100.12	98.0-102.0%
LOD	0.57 µg/mL	S/N ~3:1
LOQ	1.74 µg/mL	S/N 10:1
Robustness % RSD	0.81	NMT 2.0%

Quantification of Polysorbate 80 in Ice Cream:

Polysorbate 80 acts as an emulsifier in ice cream formulation and should be quantified within the prescribed limit. The ice cream sample was analyzed by the developed method (Table-10). The mean standard and sample peak areas were 2195593 and 786924 respectively, and the assay was found to be 0.0127% (127 mg/kg), which is within the safe acceptance limit of not more than 175 mg/kg.

Table-10: Quantification of Polysorbate 80 in Ice Cream

S.No.	Standard Area	Sample Area
1	2195986	786720
2	2195312	787128
3	2195482	-
Mean	2195593	786924
Assay	0.0127% (127 mg/kg)	
Limit	NMT 175 mg/kg	

SUMMARY

The analytical method was created. The injection volume was set at 20 µL, and detection at 195 nm produced an excellent response for oleic acid released from Polysorbate 80. The flow rate was set at 1 mL/min due to the adequate retention duration and good peak shape of the C18 column (4.6 × 150 mm, 5 µm). The mobile phase 0.1% phosphoric acid/formic acid in water and acetonitrile (45:55) was fixed due to the sharp symmetrical peak and short retention time of 3.2 min. Saponification at 70 °C for 1 hour followed by hexane extraction was used to release and isolate the oleic acid from the ice cream matrix. The method was linear over the range of 50-150 µg/mL. The percent recovery was found to be 98.78-100.12%. Both system and method precision were well within range, and the method passed the robustness test with %RSD well below 2.0.

CONCLUSION

In the present investigation, a simple, precise and accurate RP-HPLC method was developed for the quantitative estimation of Polysorbate 80 in ice cream. The method uses a common C18 column and an acetonitrile-aqueous acid mobile phase with a short run and retention time, which makes it economical. The %RSD values were within 2 and the method was found to be precise, accurate, linear and robust as per ICH Q2(R1). The method was applied to an ice cream sample, in which the Polysorbate 80 content (127 mg/kg) was within the prescribed limit of 175 mg/kg. The method can be used for the routine determination of Polysorbate 80 in ice cream.

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