ANALYTICAL CHARACTERIZATION OF POLYSORBATE 80

Case Study

Sara Sequeira
November 2020
With 4 US FDA inspected facilities, Hovione is committed to deliver biopharma excipients with outstanding quality

As a solution seeking company, we embraced the challenging domain of polysorbates addressing the issue it presents in the formulation of biopharmaceuticals

**Case study:**

Hovione has conducted a mapping of commercial Polysorbate 80 and compared those with Hovione’s Biopharma grade
• Amphiphatic and nonionic surfactants

• Used in the formulations of biotherapeutic products

• Act as stabilizing agents against protein aggregation
Polysorbates present a variety of components such as partial esters of fatty acids, sorbitol and its anhydrides along with approximately 20 polymerized ethylene oxide units.
**CHALLENGES**

Polysorbates Degradation

- Oxidation resulting in hydroperoxide formation and side chain cleavage
- Hydrolysis and release of free fatty acids

**Oxidation**
- Fatty Acid Esters
- Aldehydes
- Ketones
- Acids
- Peroxides
- Alkanes

**Hydrolysis**
- Fatty Acids
ANALYTICAL SELECTION

- Compendial methods

Further characterization:
- Volatiles finger printing by GC-FID
- Polysorbate profile by HPLC-PDA
- Fatty acids screening by GC-FID
- Peroxide quantification by HPLC and UV/VIS using catalase
- Elemental impurities by ICP-MS
VOLATILE IMPURITIES
Degradation products:

**Alkyl aldehydes and Alkanes**
- Oxidative scission in the vicinity of the olefin

**Formates and esters**
- Peroxide scission in the propagation step of POE autoxidation

**Dicarboxylic Acids**
- Degradants of oleic acid
**Method 1: GC-Headspace**

![Graph showing GC chromatograms for Acetaldehyde, Ethanol, Acetone, Hexane, and Heptane for different suppliers.]

<table>
<thead>
<tr>
<th>Volatiles (ppm)</th>
<th>Acetaldehyde</th>
<th>Ethanol</th>
<th>Acetone</th>
<th>Hexane</th>
<th>Heptane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supplier A</td>
<td>&lt; LOQ</td>
<td>58</td>
<td>&lt; LOQ</td>
<td>&lt; LOQ</td>
<td>&lt; LOQ</td>
</tr>
<tr>
<td>Supplier B</td>
<td>11</td>
<td>ND</td>
<td>&lt; LOQ</td>
<td>&lt; LOQ</td>
<td>&lt; LOQ</td>
</tr>
<tr>
<td>Supplier C</td>
<td>19</td>
<td>&lt; LOQ</td>
<td>&lt; LOQ</td>
<td>&lt; LOQ</td>
<td>&lt; LOQ</td>
</tr>
<tr>
<td>Supplier D</td>
<td>&lt; LOQ</td>
<td>ND</td>
<td>&lt; LOQ</td>
<td>&lt; LOQ</td>
<td>&lt; LOQ</td>
</tr>
<tr>
<td>Hovione</td>
<td>&lt; LOQ</td>
<td>11</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

**Supplier to Supplier variability**

Adapted from EP Polysorbate monograph: Ethylene oxide and dioxane impurities.

**VOLATILES FINGER PRINTING BY GC**

**AT EUROPE 2020: Analytical characterization of Polysorbate 80**

Copyright Hovione © 2020
### Results

Formaldehyde (ppm)

- **Supplier A**: 8
- **Supplier B**: 6
- **Supplier C**: 2
- **Supplier D**: At LOQ level
- **Hovione**: < LOQ

**Method 2: GC-Headspace for Formaldehyde**

\[
\begin{align*}
\text{Formaldehyde} & \quad \text{Ethanol} \\
\text{CH}_2\text{O} + 2 \text{C}_2\text{H}_6\text{O} & \quad \text{Diethoxymethane} \\
\text{C}_5\text{H}_{12}\text{O}_2 + \text{H}_2\text{O}
\end{align*}
\]

Supplier to Supplier variability

Adapted from: "A Robust Static Headspace-GC-FID Method to Detect and Quantify Formaldehyde Impurity in Pharmaceutical Excipients" - 2018
PROFILE
PROFILE ASSESSMENT

Method: HPLC-PDA and UV

Adapted from: White paper (Agilent): Analysis of Tween 80 by high performance liquid chromatography with diode array detection (2018), 1-6

Eluting at 196 nm: PEG chains, Sorbitan, Sorbitol dehydration impurities esterified with one or more oleic acid chain

Eluting at 236 nm: PEG chains, Sorbitan, Sorbitol dehydration impurities esterified with other esters (such as C18:2) depending on the purity of the oleic acid used

Adapted from: Characterization of polysorbate 80 with liquid chromatography-mass spectrometry and nuclear magnetic resonans specroscopy (2011)
Method: HPLC-PDA for Profile

Supplier to Supplier variability

Polysorbate 80 at 0.1% (w/v)

- Supplier C
- Supplier B
- Supplier A
- Supplier D
- Hovione

Max Plot

Adapted from: White paper (Agilent): Analysis of Tween 80 by high performance liquid chromatography with diode array detection (2018)
FATTY ACIDS
FATTY ACIDS SCREENING BY GC

Method: GC-FID

<table>
<thead>
<tr>
<th>Acid Methyl Ester</th>
<th>EP Reference A</th>
<th>EP Reference C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myristic (C14:0)</td>
<td>5%</td>
<td>4%</td>
</tr>
<tr>
<td>Palmitic (C16:0)</td>
<td>10%</td>
<td>10%</td>
</tr>
<tr>
<td>Palmitoleic (C16:1)</td>
<td>-</td>
<td>8%</td>
</tr>
<tr>
<td>Steric (C18:0)</td>
<td>16%</td>
<td>5%</td>
</tr>
<tr>
<td>Oleic (C18:1)</td>
<td>21%</td>
<td>55%</td>
</tr>
<tr>
<td>Linoleic (C18:2)</td>
<td>-</td>
<td>15%</td>
</tr>
<tr>
<td>Linolenic (C18:3)</td>
<td>-</td>
<td>3%</td>
</tr>
<tr>
<td>Arachidic (C20:0)</td>
<td>22%</td>
<td>-</td>
</tr>
<tr>
<td>Cis-11-Eicosenoic (C20:1)</td>
<td>6%</td>
<td>-</td>
</tr>
<tr>
<td>Behenic (C22:0)</td>
<td>12%</td>
<td>-</td>
</tr>
<tr>
<td>Lignoceric (C24:0)</td>
<td>9%</td>
<td>-</td>
</tr>
</tbody>
</table>

Adapted from EP Polysorbate monograph: Composition of fatty acids.
### Fatty Acids Screening by GC

<table>
<thead>
<tr>
<th>Acid Methyl Ester</th>
<th>Supplier A</th>
<th>Supplier B</th>
<th>Supplier C</th>
<th>Supplier D</th>
<th>Hovione</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capric (C10:0)</td>
<td>&lt; LOQ</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>&lt; LOQ</td>
</tr>
<tr>
<td>Lauric (C12:0)</td>
<td>&lt; LOQ</td>
<td>ND</td>
<td>1%</td>
<td>3%</td>
<td>&lt; LOQ</td>
</tr>
<tr>
<td>Myristic (C14:0)</td>
<td>&lt; LOQ</td>
<td>ND</td>
<td>&lt; LOQ</td>
<td>2%</td>
<td>&lt; LOQ</td>
</tr>
<tr>
<td>Palmitic (C16:0)</td>
<td>2%</td>
<td>14%</td>
<td>5%</td>
<td>6%</td>
<td>3%</td>
</tr>
<tr>
<td>Palmitoleic (C16:1)</td>
<td>ND</td>
<td>&lt; LOQ</td>
<td>&lt; LOQ</td>
<td>&lt; LOQ</td>
<td>ND</td>
</tr>
<tr>
<td>Steric (C18:0)</td>
<td>3%</td>
<td>4%</td>
<td>2%</td>
<td>2%</td>
<td>3%</td>
</tr>
<tr>
<td>Oleic (C18:1)</td>
<td>90%</td>
<td>70%</td>
<td>78%</td>
<td>76%</td>
<td>88%</td>
</tr>
<tr>
<td>Linoleic (C18:2)</td>
<td>&lt; LOQ</td>
<td>&lt; LOQ</td>
<td>ND</td>
<td>2%</td>
<td>&lt; LOQ</td>
</tr>
<tr>
<td>Linolenic (C18:3)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Arachidic (C20:0)</td>
<td>1%</td>
<td>1%</td>
<td>1%</td>
<td>ND</td>
<td>&lt; LOQ</td>
</tr>
<tr>
<td>c-11-Eicosenoic (C20:1)</td>
<td>&lt; LOQ</td>
<td>5%</td>
<td>2%</td>
<td>2%</td>
<td>2%</td>
</tr>
</tbody>
</table>

**EP Spec.**

<table>
<thead>
<tr>
<th>C18:1</th>
<th>Supplier A</th>
<th>Supplier B</th>
<th>Supplier C</th>
<th>Supplier D</th>
<th>Hovione</th>
</tr>
</thead>
<tbody>
<tr>
<td>NL</td>
<td>&lt; 5%</td>
<td>&lt; 16%</td>
<td>&lt; 8%</td>
<td>&lt; 6%</td>
<td>&lt; 16%</td>
</tr>
<tr>
<td>NL</td>
<td>&lt; 18%</td>
<td>&lt; 58%</td>
<td>&lt; 4%</td>
<td>ND</td>
<td>&lt; 58%</td>
</tr>
</tbody>
</table>

**Supplier to Supplier variability**

- **ND**: Not detected
- **NL**: Not listed

---

AT EUROPE 2020: Analytical characterization of Polysorbate 80

Copyright Hovione © 2020
Peroxides

\[
\text{Phenylphosphine} + \text{RO}_{2}H \rightarrow \text{Phenylphosphoryl} + \text{ROH}
\]
Method 1: UV/VIS

Hydroperoxides Content by UV/VIS

Fox Assay II Methodology

Total hydroperoxides content

- Reduction of Hydroperoxides by Fe (II) under acid conditions

\[ \text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{HO}^- \]

\[ \text{Fe}^{2+} + \text{ROOH} \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{RO}^- \]

- The resulting ferric ion forms a strong complex with xylenol orange

- The complex has maximum absorbance at 560 nm

\[ \text{Abs} = 0.0523x + 0.0493 \]

\[ R^2 = 0.9993 \]

Adapted from: "Method for the simultaneous determination of free/protein malondialdehyde and lipid/protein hydroperoxides", Free Radical Biology and Medicine 2013

Adapted from: "Hydroperoxide Assay with the Ferric–Xylenol Orange Complex", School of Biological Sciences, Macquarie University, Sydney 2109, Australia, 1998

Adapted from: https://www.edinst.com/blog/the-lambert-law/
**HYDROPEROXIDES CONTENT BY UV/VIS**

**Method 1: UV/VIS**

**Catalase Methodology**

Alkoxy peroxides content

- Catalase reacts with the free peroxide but not with the alkoxy peroxide

\[
\text{ROOH} + 2 \text{H}_2\text{O}_2 \xrightarrow{\text{Catalase}} \text{ROOH} + 2\text{H}_2\text{O} + \text{O}_2
\]

- The quenched sample is then analyzed by the Fox Assay II

Free peroxides content

- The difference between the total hydroperoxides and the alkoxy peroxides gives the amount of free peroxide
Hydroperoxides Content by UV/VIS

<table>
<thead>
<tr>
<th>Supplier</th>
<th>Total Hydroperoxides (ppm)</th>
<th>ROOH (ppm)</th>
<th>ROOH (%)</th>
<th>H₂O₂ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supplier A</td>
<td>68</td>
<td>33</td>
<td>49</td>
<td>51</td>
</tr>
<tr>
<td>Supplier B</td>
<td>120</td>
<td>46</td>
<td>38</td>
<td>62</td>
</tr>
<tr>
<td>Supplier C</td>
<td>148</td>
<td>65</td>
<td>44</td>
<td>56</td>
</tr>
<tr>
<td>Supplier D</td>
<td>412</td>
<td>172</td>
<td>42</td>
<td>58</td>
</tr>
<tr>
<td>Hovione</td>
<td>64</td>
<td>36</td>
<td>44</td>
<td>56</td>
</tr>
</tbody>
</table>

- Our findings suggest that nearly 60% of the peroxides present in Polysorbate 80 are H₂O₂
**Method 2: HPLC-PDA**

- **Triphenylphosphine** (TPP) reacts rapidly and quantitatively with $\text{H}_2\text{O}_2$ and $\text{ROOH}$ to yield 1 mole of Triphenylphosphine oxide (TPPO) for each mole of peroxides reacted.

- Polysorbates are added to a standard solution of 0.1 mg/mL TPP in methanol. The sample is then allowed to react for about **15 min** prior to HPLC analysis.

**Challenge**

- TPP undergoes **slow air oxidation**, gradually oxidizing to TPPO even in the absence of peroxides.
Challenge

- Low peroxide content is difficult to quantify accurately due to the residual TPPO
- Method suitability for low peroxide samples should be assessed
- Qualitative method: fast and easy to set up
- Fast peroxide screening
ELEMENTAL IMPURITIES
### ELEMENTAL IMPURITIES BY ICP-MS

#### Method: ICP-MS

![Diagram of elemental impurities in drug product](image)

<table>
<thead>
<tr>
<th>Elemental Impurities</th>
<th>Specifications (parenteral) ppm</th>
<th>Supplier A ppm</th>
<th>Supplier B ppm</th>
<th>Supplier C ppm</th>
<th>Hovione ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd</td>
<td>≤ 0.2</td>
<td>&lt; LOQ (0.148)</td>
<td>&lt; LOQ (0.148)</td>
<td>&lt; LOQ (0.148)</td>
<td>&lt; LOQ (0.148)</td>
</tr>
<tr>
<td>Pb</td>
<td>≤ 0.5</td>
<td>&lt; LOQ (0.071)</td>
<td>&lt; LOQ (0.071)</td>
<td>&lt; LOQ (0.071)</td>
<td>&lt; LOQ (0.071)</td>
</tr>
<tr>
<td>As</td>
<td>≤ 1.5</td>
<td>&lt; LOQ (0.137)</td>
<td>&lt; LOQ (0.137)</td>
<td>&lt; LOQ (0.137)</td>
<td>&lt; LOQ (0.137)</td>
</tr>
<tr>
<td>Hg</td>
<td>≤ 0.3</td>
<td>0.093</td>
<td>0.096</td>
<td>0.094</td>
<td>0.094</td>
</tr>
<tr>
<td>Co</td>
<td>≤ 0.5</td>
<td>&lt; LOQ (0.006)</td>
<td>&lt; LOQ (0.006)</td>
<td>&lt; LOQ (0.006)</td>
<td>&lt; LOQ (0.006)</td>
</tr>
<tr>
<td>V</td>
<td>≤ 1</td>
<td>&lt; LOQ (0.060)</td>
<td>&lt; LOQ (0.060)</td>
<td>&lt; LOQ (0.060)</td>
<td>&lt; LOQ (0.060)</td>
</tr>
<tr>
<td>Ni</td>
<td>≤ 2</td>
<td>&lt; LOQ (0.174)</td>
<td>&lt; LOQ (0.174)</td>
<td>&lt; LOQ (0.174)</td>
<td>&lt; LOQ (0.174)</td>
</tr>
<tr>
<td>Li</td>
<td>≤ 25</td>
<td>&lt; LOQ (2.359)</td>
<td>&lt; LOQ (2.359)</td>
<td>&lt; LOQ (2.359)</td>
<td>&lt; LOQ (2.359)</td>
</tr>
<tr>
<td>Sb</td>
<td>≤ 9</td>
<td>&lt; LOQ (0.456)</td>
<td>&lt; LOQ (0.456)</td>
<td>&lt; LOQ (0.456)</td>
<td>&lt; LOQ (0.456)</td>
</tr>
<tr>
<td>Cu</td>
<td>≤ 30</td>
<td>&lt; LOQ (2.701)</td>
<td>&lt; LOQ (2.701)</td>
<td>&lt; LOQ (2.701)</td>
<td>&lt; LOQ (2.701)</td>
</tr>
<tr>
<td>Mo</td>
<td>≤ 150</td>
<td>&lt; LOQ (8.618)</td>
<td>&lt; LOQ (8.618)</td>
<td>&lt; LOQ (8.618)</td>
<td>&lt; LOQ (8.618)</td>
</tr>
</tbody>
</table>

**Method:**
- ICP-MS

**Specifications:**
- Parenteral

**Adapted from:**
- [ICH guideline Q3D](https://www.ich.org/page/quality-guidelines)
STABILITY DATA

**Acetaldehyde**
- Hovione
- Supplier D
- Supplier A
- Supplier C

**Formaldehyde**
- Hovione
- Supplier D
- Supplier A
- Supplier C

**Peroxides**
- Hovione
- Supplier D
- Supplier A
- Supplier C

<table>
<thead>
<tr>
<th></th>
<th>T0</th>
<th>T1W</th>
<th>T1M</th>
<th>T2M</th>
<th>T3M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hovione</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supplier D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supplier A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supplier C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
CONCLUSIONS

Volatile s by GC-FID: Degradation Products
• Understanding product stability, purification and identify degradation agents

Profile by HPLC-PDA: Variability indicator
• Stability studies throughout samples and supply chain fingerprinting

Peroxides by HPLC and UV/VIS: Peroxide determination
• Critical attribute in product quality and stability, since it is the major responsible for oxidative degradation of Polysorbate containing formulations

Fatty acids by GC-FID: Fatty acids screening
• Supply chain fingerprinting

Elemental impurities by ICP-MS: Trace metals
• Compliance with ICH guidelines
Thank you for your attention

ssequeira@hovione.com

Sara Sequeira, MSc
Analytical Scientist