Complexity of the Analytical Characterization of Polysorbate

Case Studies for Degradation Profiling

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The perspectives and opinions in this talk are those of the presenter.

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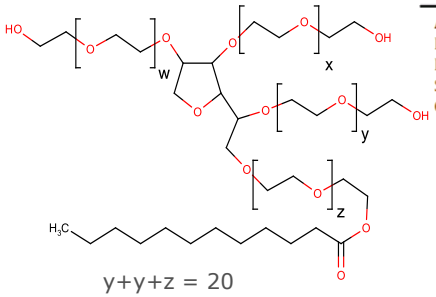
Functional role of Polysorbates (PS) in biopharmaceutical formulations

- Polysorbate 20 and 80 are the most common non-ionic surfactants in biopharmaceutical products
- PS have a high surface activity at low concentration and are used as excipient to:
  - Prevent protein adsorption
  - Protect protein against interfacial stress, surface-induced aggregation and particle formation
- Concentration range in protein therapeutics from 0.001 to 0.1% (w/v)
  - $[PS]_{optimum}$ is experimentally determined by stability studies and stress-studies
  - Depends on the stabilization mechanism (competition/ protein-PS interaction)
  - Formation of side/degradation products (e.g., PS related particles)

➢ **Analytical assessment of polysorbate composition, purity and stability are important during drug product development to ensure sufficient stabilization while minimizing negative side-effects of PS**

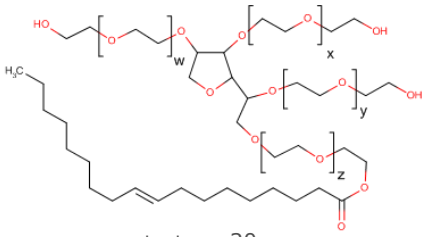
Compendial Grade PS

Idealized structure of PS20



y+y+z = 20

Idealized structure of PS80



y+y+z = 20

Requirements of Different Pharmacopoeias Regarding PS20 and PS80 for Injection

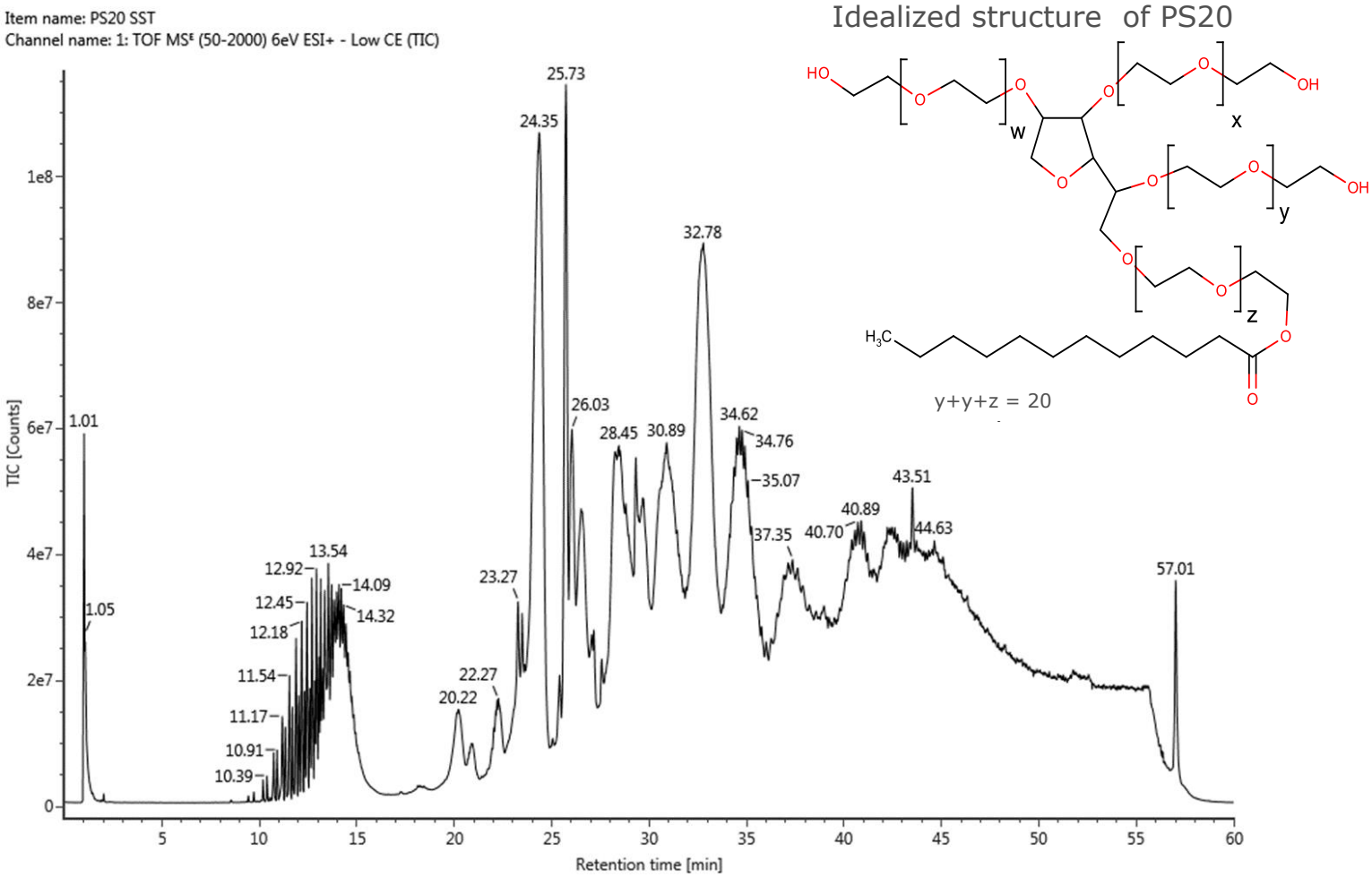
Tests	Ph. Eur.		USP		JP		ChP	
	PS20	PS80	PS20	PS80	PS20	PS80	PS20	PS80
Acid value (mg KOH/g)	≤2	≤2	≤2	≤2	≤4	≤2	≤2	≤1
Hydroxyl value (mg KOH/$g_{acetylated}$)	96-108	65-80	96-108	65-80	–	65-80	96-108	65-80
Peroxide value (mEq/Kg)	≤10	≤10	≤10	≤10	–	≤10	≤10	≤3
Saponification value (mg KOH/g)	40-50	45-55	40-50	45-55	43-55	45-55	40-50	45-55
Composition of FA (%)								
Capric acid	≤10	–	≤10	–	–	–	≤10	–
Caproic acid	≤1	–	≤1	–	–	–	≤1	–
Caprylic acid	≤10	–	≤10	–	–	–	≤10	–
Lauric acid	40-60	–	40-60	–	–	–	40-60	–
Linoleic acid	≤3	≤18	≤3	≤18	–	≤18	≤3	≤0.5
Linolenic acid	–	≤4	–	≤4	–	≤4	–	≤0.5
Myristic acid	14-25	≤5	14-25	≤5	–	≤5	14-25	≤0.5
Oleic acid	≤11	≥58	≤11	≥58	–	≥58	≤11	≥98
Palmitic acid	7-15	≤16	7-15	≤16	–	≤16	7-15	≤0.5
Palmitoleic acid	–	≤8	–	≤8	–	≤8	–	≤0.5
Stearic acid	≤7	≤6	≤7	≤6	–	≤6	≤7	≤0.5
Ethylene oxide (ppm)	≤1	≤1	≤1	≤1	–	≤1	≤1	≤1
Dioxane (ppm)	≤10	≤10	≤10	≤10	–	≤10	≤10	≤10
Heavy metal (ppm)	≤10	≤10	≤10	≤10	–	≤20	≤10	≤10
Water (%)	≤3	≤3	≤3	≤3	≤3	≤3	≤3	≤0.5
Total ash (%)	≤0.25	≤0.25	≤0.25	≤0.25	≤1	≤0.25	≤0.25	≤0.1
pH	–	–	–	–	–	–	4-7.5	5-7.5
Relative density (kg/m^3)	~1.1	~1.1	–	1.06-1.09	–	~1.1	1.09-1.12	1.06-1.09
Viscosity (mPa·s, 25°C)	~400	~400	–	–	–	~400	250-400	350-450
Iodine value	–	–	–	–	–	–	–	18-24
Arsenic content (ppm)	–	–	–	–	–	–	≤2	≤2
Ethylene glycol (%)	–	–	–	–	–	–	≤0.01	≤0.01
Diethylene glycol (%)	–	–	–	–	–	–	≤0.01	≤0.01
Triethylene glycol (%)	–	–	–	–	–	–	–	≤0.01
Endotoxin (EU)	–	–	–	–	–	–	–	0.012

Ph. Eur.—European Pharmacopoeia 8.5 and 8.0 versions for PS20 and PS80, respectively. USP—United States Pharmacopoeial Convention from 2014 and 2015 for PS20 and PS80, respectively. JP—Japanese Pharmacopoeia, 17th edition, for PS80. JP has no monograph specific for PS20. The information shown here corresponds to PS20 mention under General Test 9.41 (Reagents, Test solutions). ChP—Chinese Pharmacopoeia, 2015. –Indicates value not reported in the corresponding pharmacopoeia.

Martos et al. (2017), J Pharm Sci, 106:1722-1735

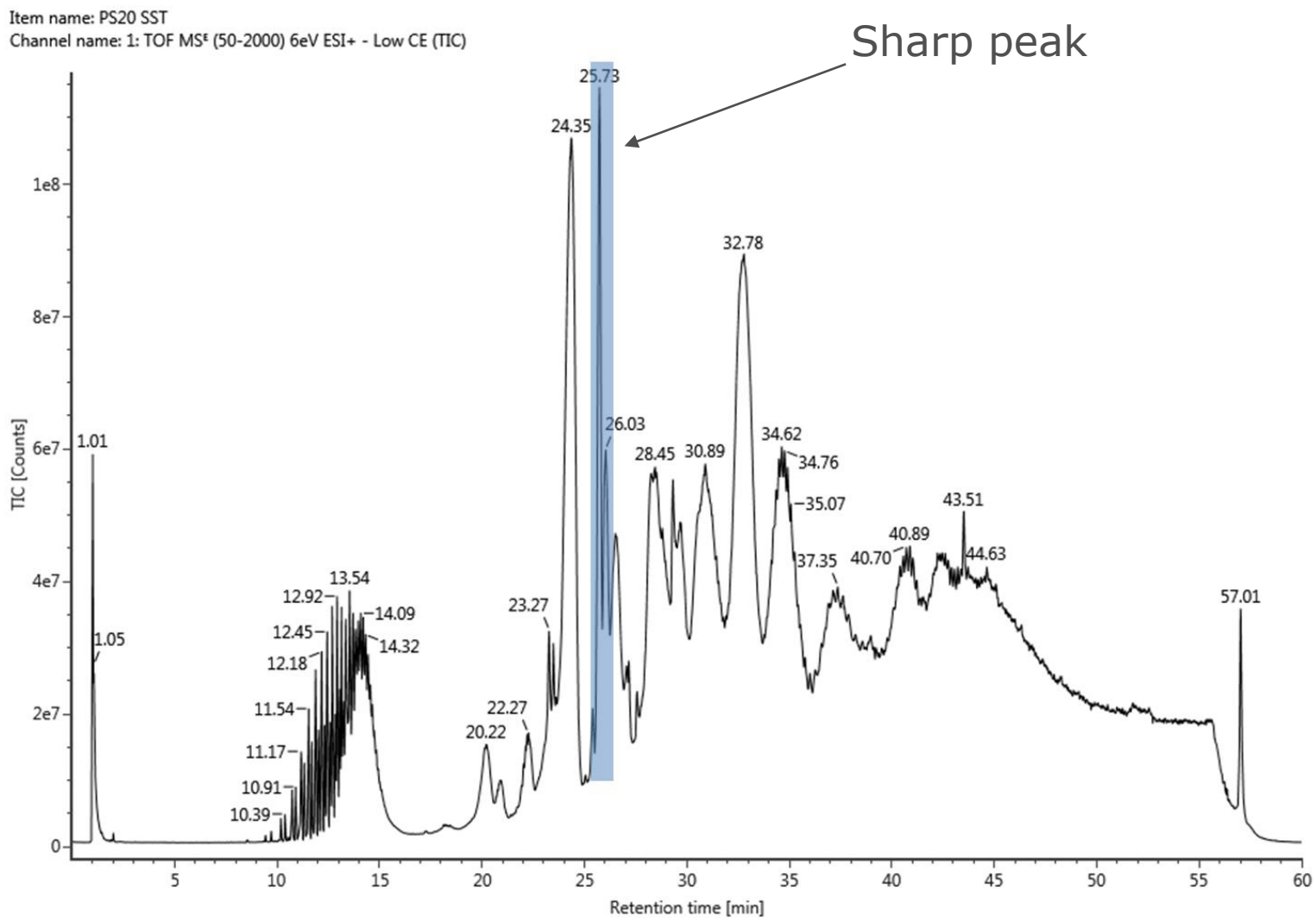
- **EP, USP and JP have harmonized requirements for PS80, which is composed of multiple fatty acid (FA) esters (PS20: JP is not harmonized)**
- **China enforces compliance to the ChP 2015 and requires "all oleic acid (≥98.0%)" Grade PS80**

Complexity of Multi-compendial PS

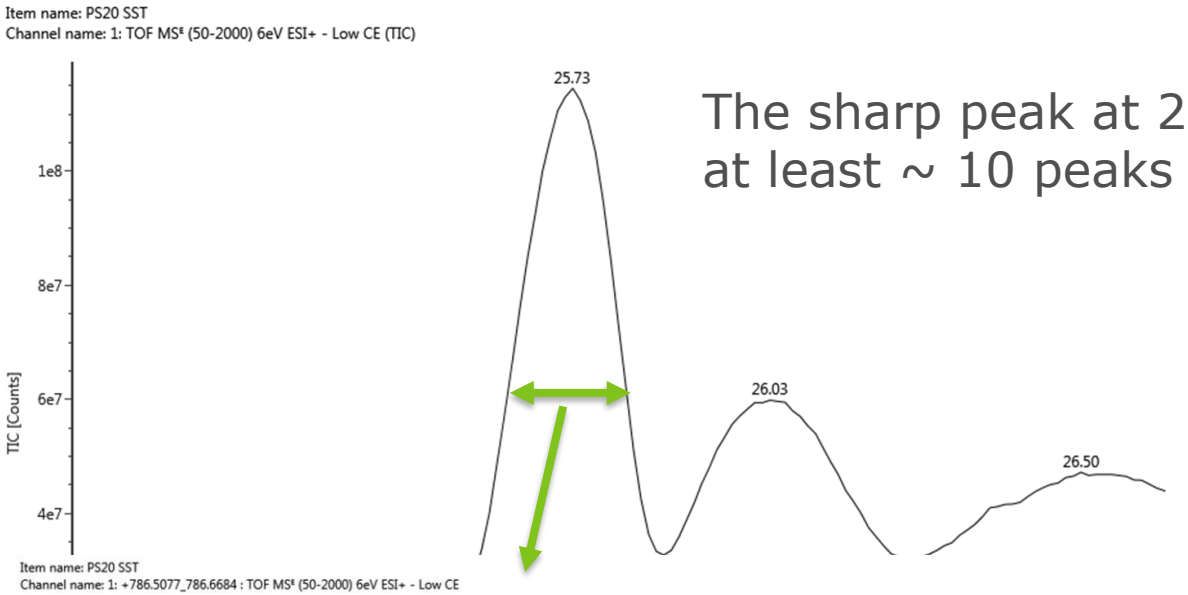


- **Optimized UPLC-MS separation of PS20 shows complex chromatogram with approximately 4000 peaks based on MS spectra** (parent (adduct) ions are counted as one peak)

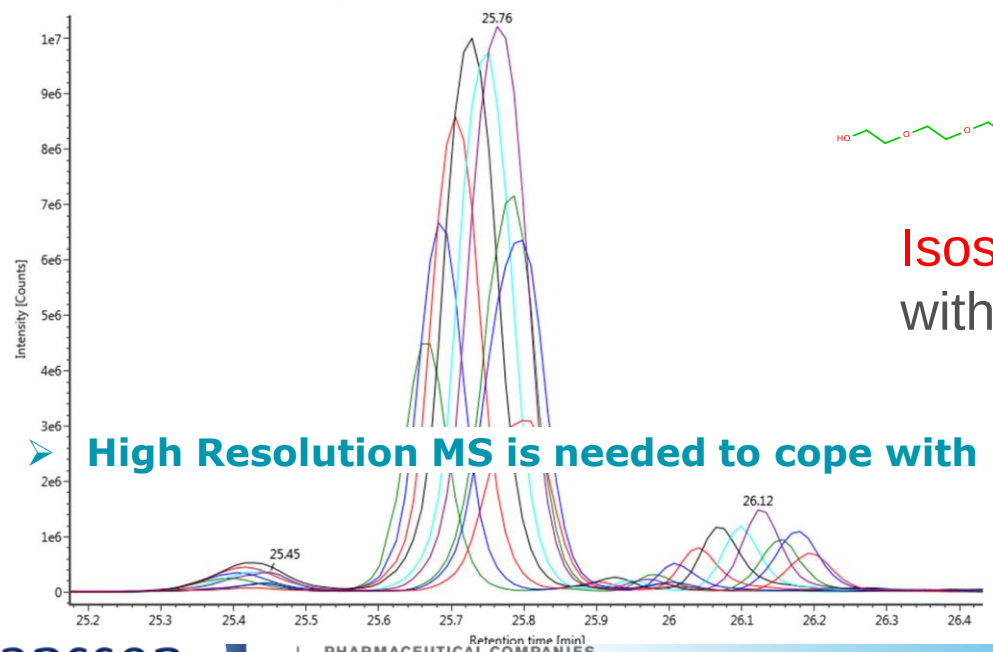
Complexity of Multi-compendial PS

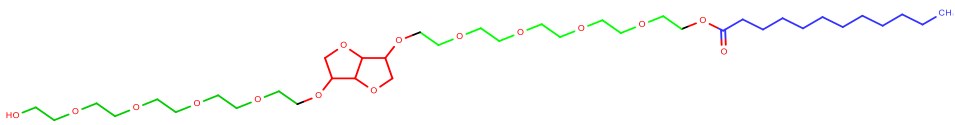


Complexity of Multi-compendial PS



The sharp peak at 25.7 min is composed by at least ~ 10 peaks based on mass data





Isosorbitane-PEGx10-mono-laurate
with different PEG-ylation levels

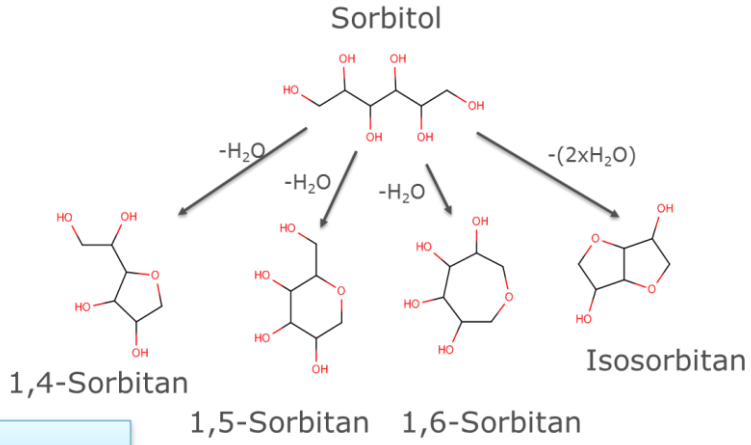
➢ **High Resolution MS is needed to cope with heterogeneous composition of PS**

Degradation increases complexity

Native Polysorbate

Potential isomeric forms of sorbitan core

Various poly(oxyethylene) chains (PEGs)
Different fatty acids (FA) esterified
Mono, di-, tri-, tetra-esters



Impurities/byproducts

Peroxides
Free FAs, PEGs..

Oxidation

Free FAs, free PEGs..
Peroxides
Oxidized FAs, PEGs (intermediates)
Short chain organic acids, aldehydes, ketones

Hydrolysis (chemical, enzymatic)

Mono-, di, tri-esters
Free FAs (FFAs)
Free PEGs

PS degradation leads to
Loss of PS surfactant functionality
and loss of solubilization capacity

Protein aggregation/precipitation
Protein oxidation

Generation of hydrophobic PS degradants (e.g., FFAs)

→ Formation of particles composed of PS degradants

Challenges for analytical characterization of PS

- Polysorbate heterogeneity

  → complex mixture, markers for degradation pathway are difficult to identify (needle in haystack)

  →(reactive) degradants: transient nature and difficult to isolate

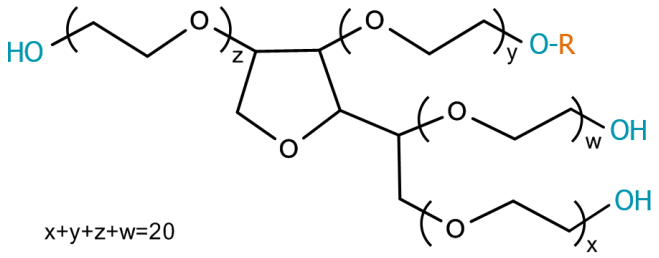
  → no universal PS standard available as reference (e.g. for calibration curves)

- Lack of chromophores

  → derivatization or universal detectors (CAD, ELSD, MS) required

- Interference of protein and excipients with the analytical methods

  → sample work-up required (in particular protein removal)



Analytical methods for PS in biopharmaceutical formulations

- PS content (validated method with acceptance criteria, R/S)

  Fluorescent micelle assay (e.g. NPN assay)

  Liquid Chromatography (LC) with charged aerosol detector (CAD) or evaporative light scattering detector (ELSD)

- PS subclass analysis (characterization method, fast, quantitative)

  HPLC-CAD or –ELSD incl. sample preparation for selective isolation of PS

- PS degradants in protein-formulations (characterization method, MS for identification)

  UPLC- MS (positive and/or negative mode) incl. sample preparation for selective isolation of PS; FA specific methods

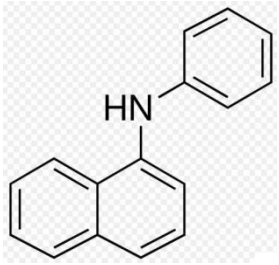
- Other methods

  Particle characterization methods to determine composition/nature

  Peroxide assays, ICP-MS, excipient related methods

PS content in biopharmaceutical formulations

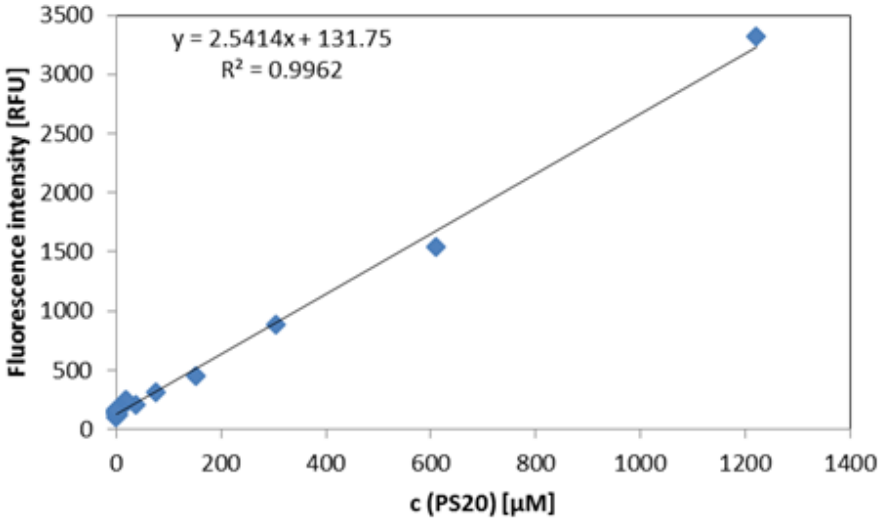
Method based on the fluorescent dye N-phenyl-1-napthylamine (NPN)



NPN

- Fluorescence assay can be used to determine PS content in aqueous solutions
- No sample preparation (just dilution), automated method (use of well plates)
- Good linearity

[PS] ≥ CMC: NPN in hydrophobic micellar interior -> high quantum yield



Caveats of the NPN assay:

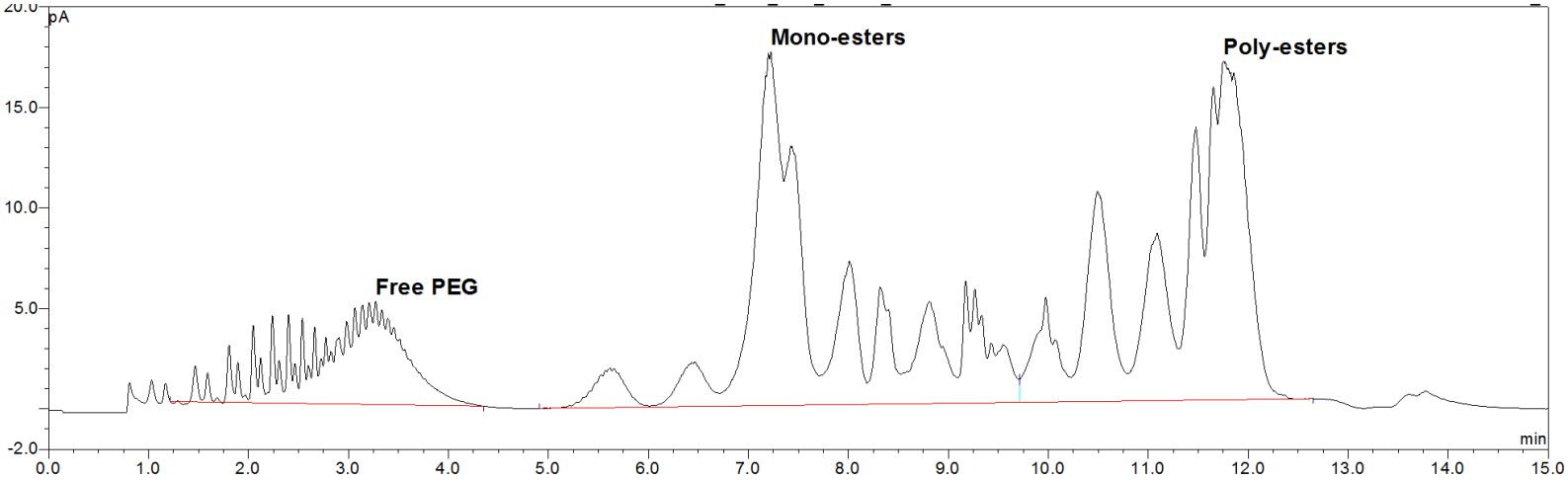
NPN interacts with hydrophobic compounds

→ interference by certain proteins and silicone oil

➢ **NPN assay is not suitable to quantify PS in each biopharmaceutical formulation**

PS content and PS class determinations in biopharmaceutical formulations

Fast LC-CAD method to monitor major components of PS



PS can be determined in aqueous solutions (without sample preparation)

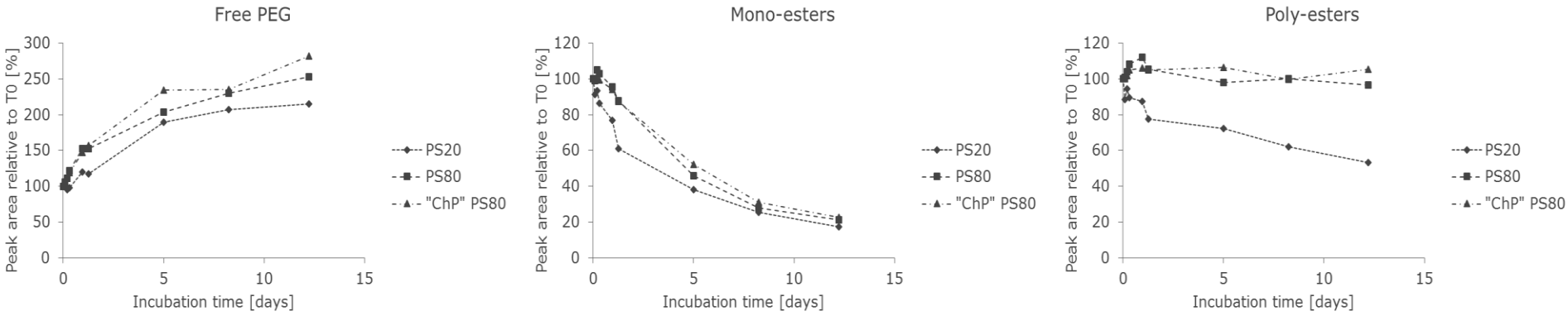
Protein needs to be removed e.g., by off-line SPE for biopharmaceutical formulations

Short cycle times of 15 min

Range: 60 – 1000 µg/mL (~0.006 – 0.1% w/v) PS

Enzymatic degradation studies

LC-CAD method to monitor esterase (0.005 U/mL) induced degradation of 0.04% PS formulated at pH 6



Mono-ester species degrade faster than Poly-esters

Free PEG fractions increase concomitantly

Part of poly-esters degrades in PS20 but not in PS80 (no change)

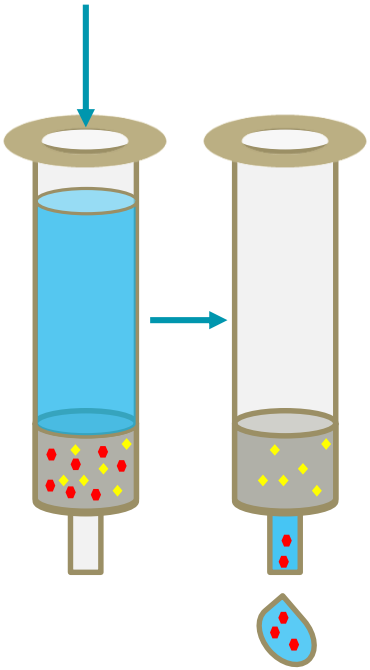
No significant difference between Chinese and multicompendial grade PS80

- **LC-CAD with high sample throughput enabled monitoring of PS degradation at multiple time points to get insight into kinetics**

Characterization of PS related molecules and degradants in biopharmaceutical formulations

Off-line SPE

Isolate PS constituents from Protein/other excipients based on hydrophobicity



UPLC-HRMS method

Positive Mode:

Sensitive detection of PS related molecules

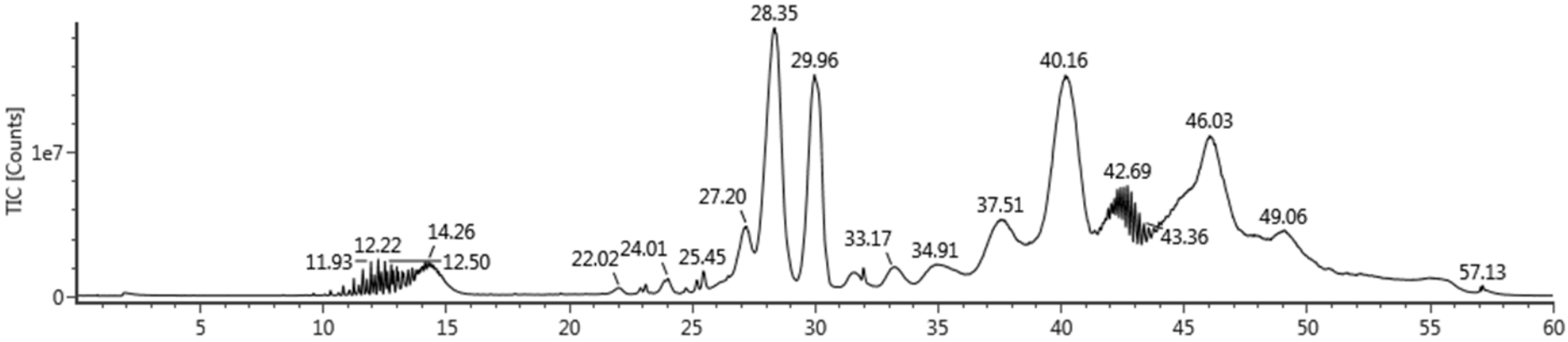
PS degradation pattern of esters

Negative Mode:

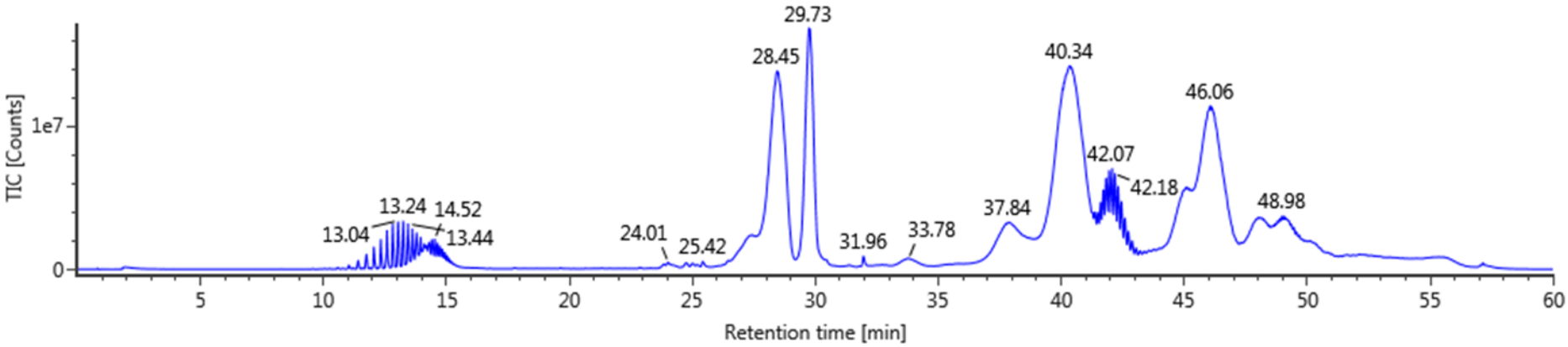
Sensitive detection of FFAs and related oxidized degradants

Example for multicompendial and Chinese grade PS80 by UPLC-HRMS (pos mode)

PS80 multicompendial



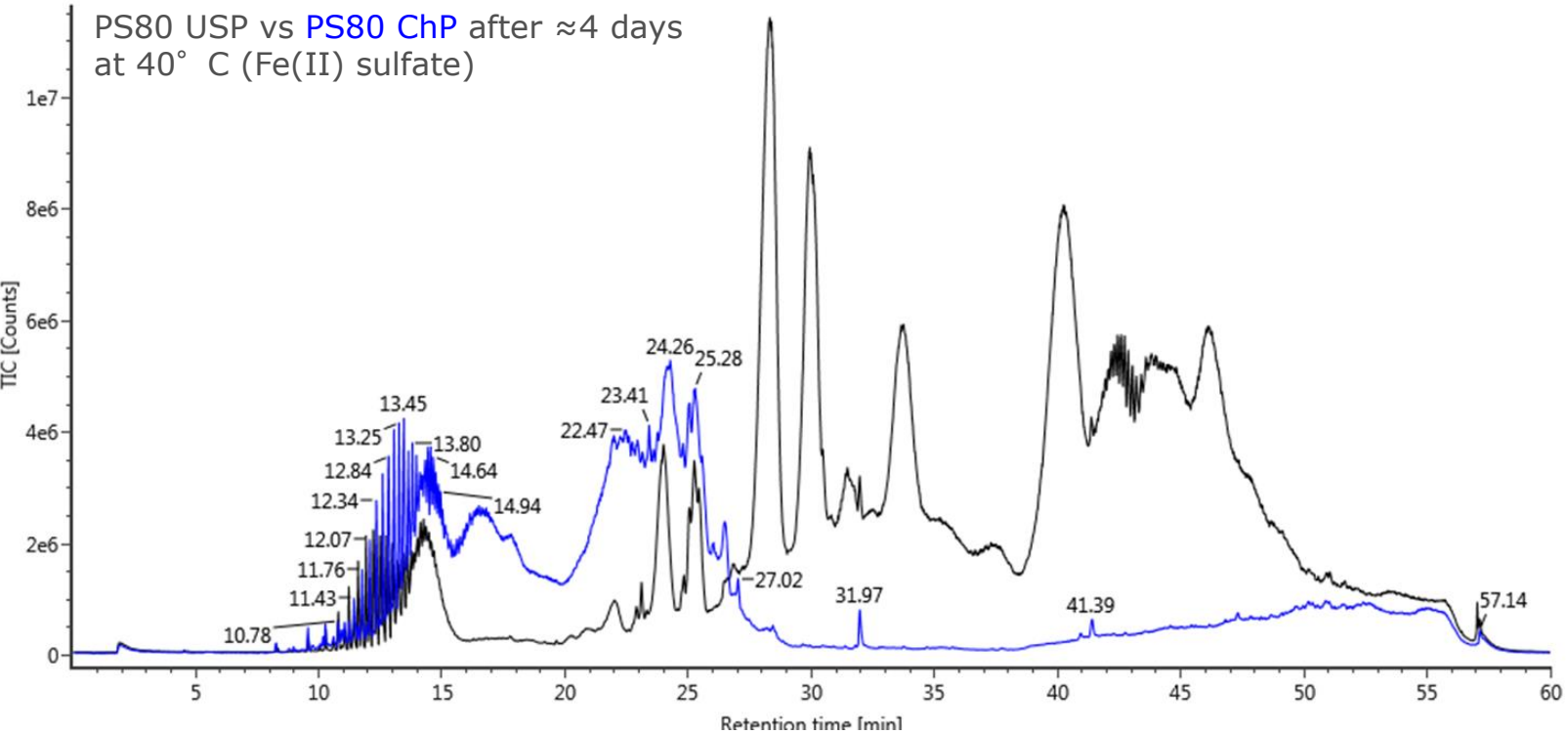
PS80 ChP



- **Differences in overall pattern (base peak chromatogram) is less pronounced as would be expected based on the ChP requirement for oleic acid only grade (≥98%)**

Oxidative degradation studies ($Fe(II)SO_4$)

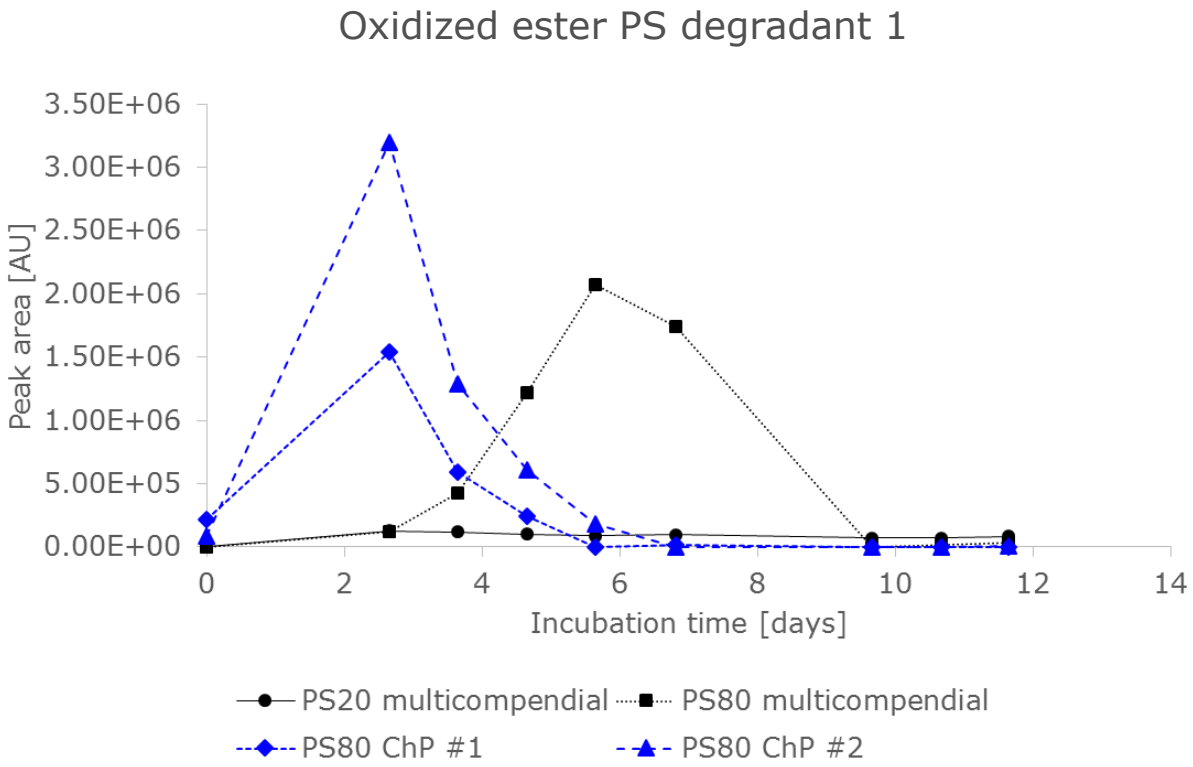
UPLC-HRMS (pos mode) method to monitor oxidative degradation of 0.04% PS formulated at pH 6



- **Chinese grade PS80 shows after 4 days exposure complete loss of polyester and monoester fractions**
- **Chinese grade PS80 also with higher levels of oxidative degradants compared to multi-compendial PS80**

Oxidative degradation studies ($Fe(II)SO_4$)

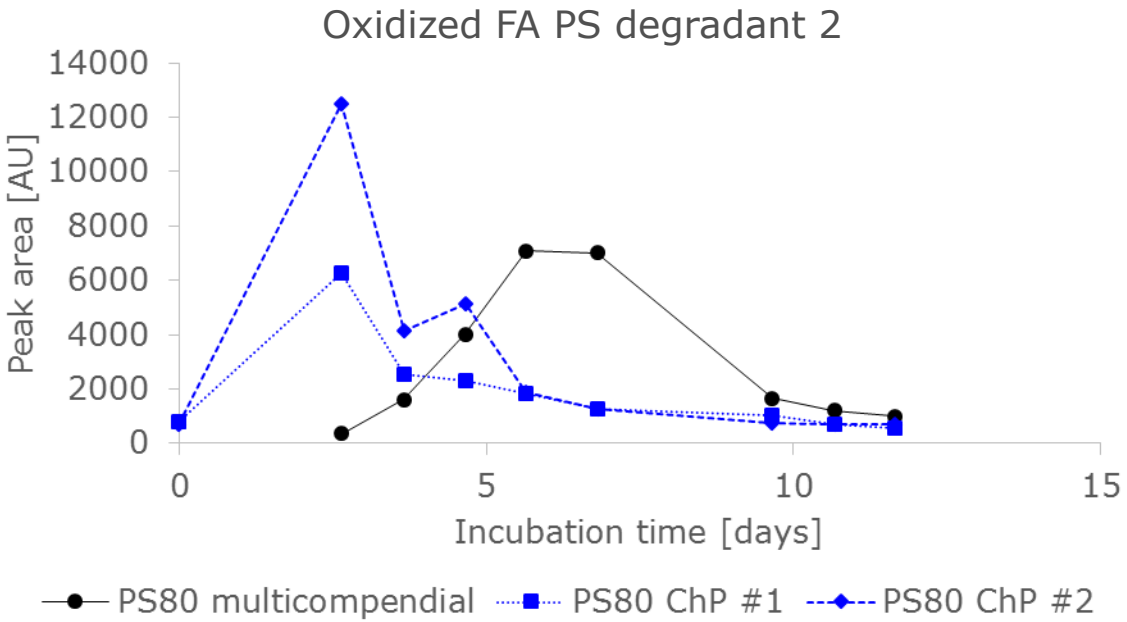
UPLC-HRMS (pos mode) method to monitor oxidative degradation of 0.04% PS formulated at pH 6 at 25° C



- **Chinese grade PS80 show a shorter lag-phase for the formation and higher levels of oxidized degradants compared to multi-compendial grade**
- **PS20 shows lower levels compared to PS80 for intermittent oxidized ester PS degradant**

Oxidative degradation studies ($Fe(II)SO_4$)

UPLC-HRMS (neg mode) method to monitor oxidative degradation of 0.04% PS formulated at pH 6 at 25° C

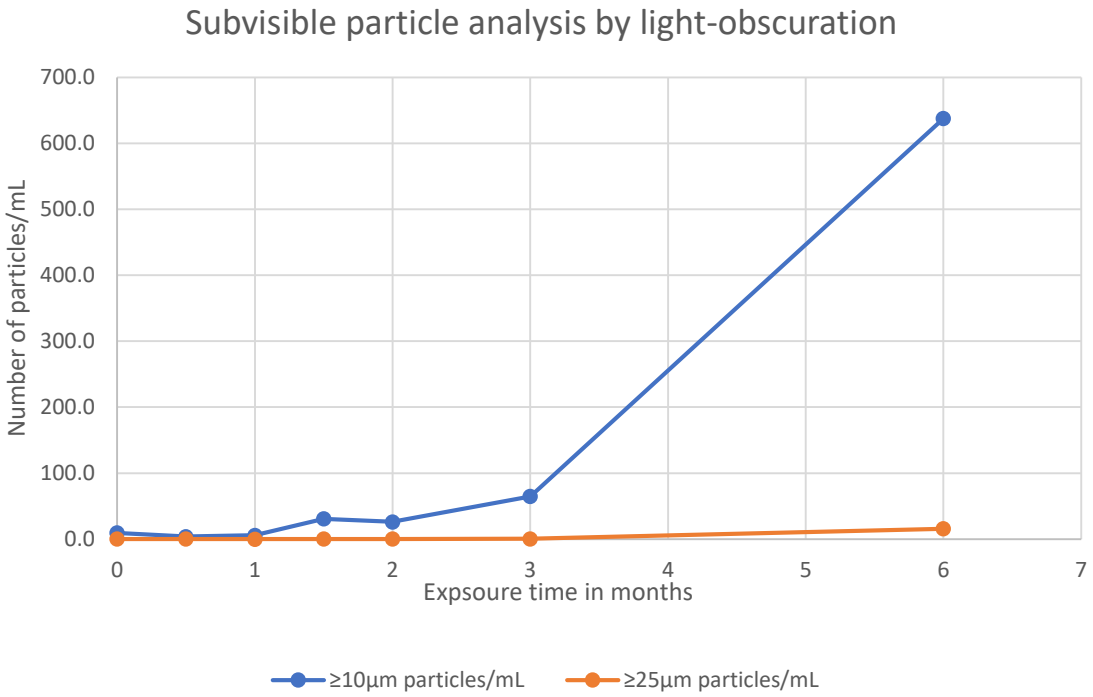


Koch et al. (2018), manuscript under preparation".

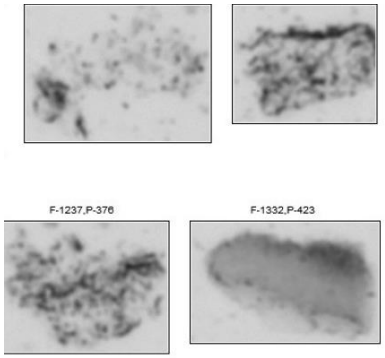
- **Some oxidized products are intermittent, others do not decompose during monitored time period**
- **Free ("soluble") oleic acid was not detected at high levels (either only present in oxidized forms or as FA in mixed micelles or as emulsion at 25° C)**

Characterization of insoluble fraction of PS

Biopharmaceutical product stressed at 40° C



Examples of MFI images

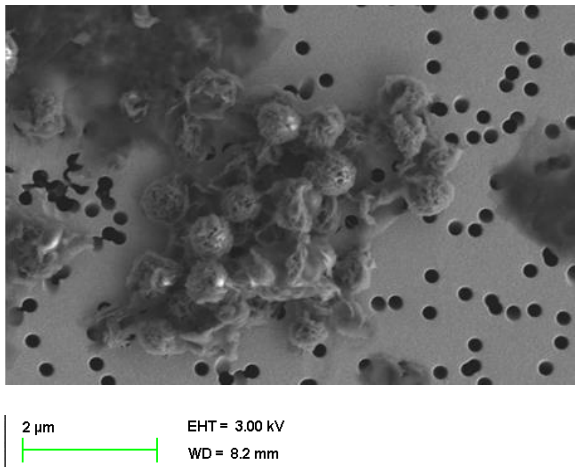


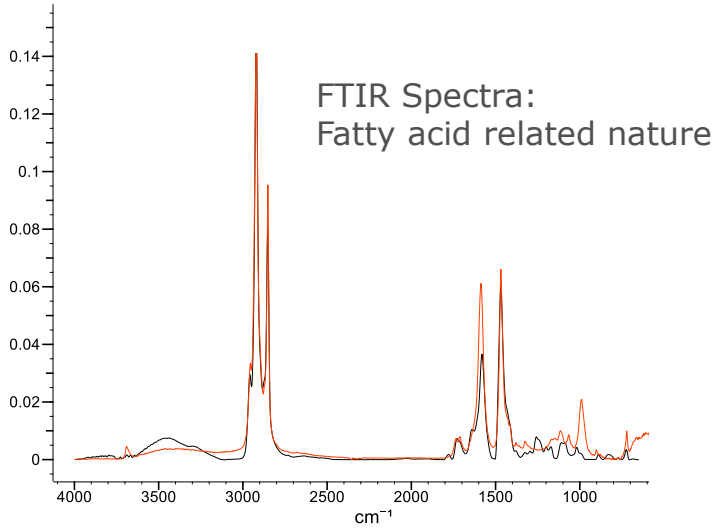
- **Increase in subvisible particles after lag-time**
- **Smaller (≥10µm) particles increase first, then larger (≥25µm) particles**
- **Morphology of PS related particles is variable and not always easily distinguishable from proteinaceous particles**

Characterization of PS related (subvisible) particles

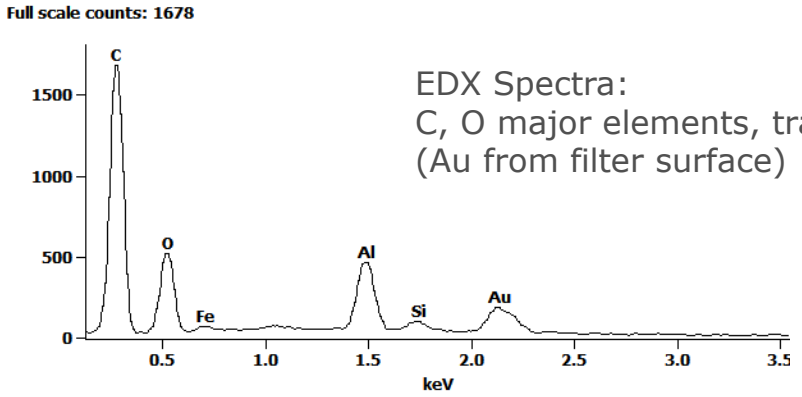
Isolation of particles on filter for advanced characterization of their nature and composition by FTIR- microscopy and SEM-EDX

SEM image on filter surface



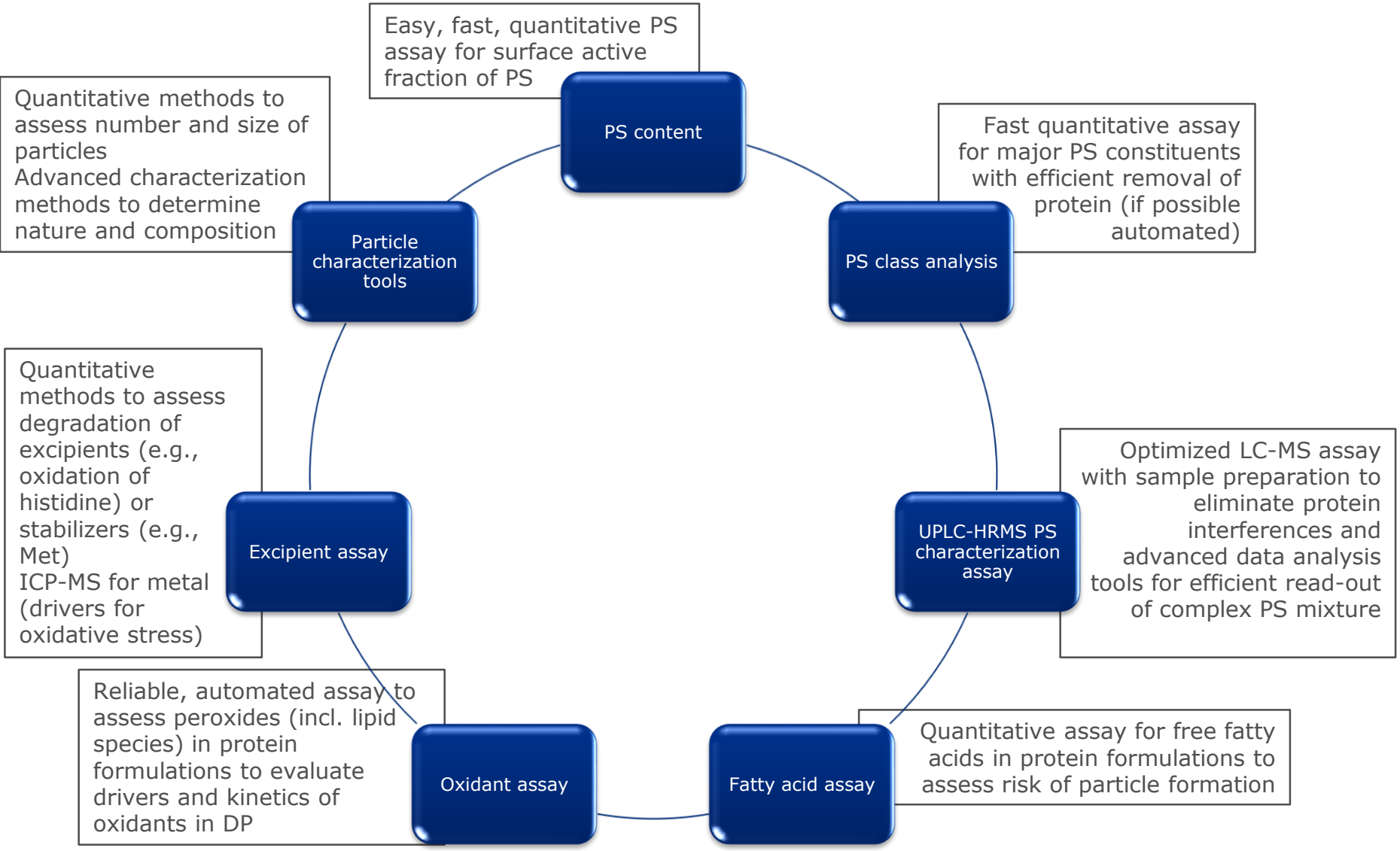


FTIR Spectra:
Fatty acid related nature



EDX Spectra:
C, O major elements, trace metals
(Au from filter surface)

PS Analytical Toolbox with Complementary Assays



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