ProtaGene
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White Paper

# Content and Degradation of Polysorbates in Biopharmaceutical Formulations

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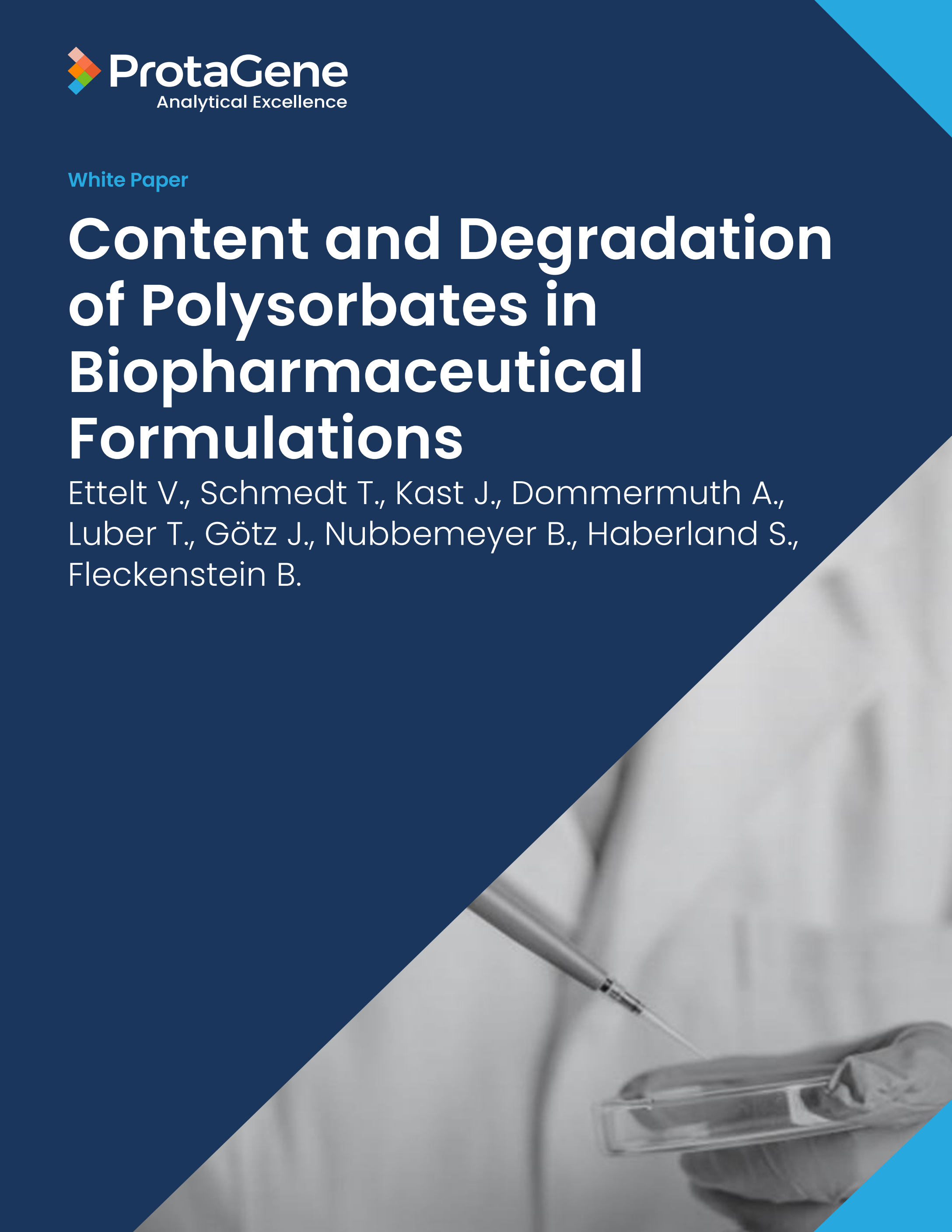


## Abstract

Polysorbates are a family of non-ionic surfactants widely applied in the development of biopharmaceuticals, as they play a critical role in stabilizing protein-based drug substances. Degradation of polysorbates therefore limits drug substance stability and must be carefully monitored. Overall, polysorbates are crucial for drug product quality, safety, and efficacy. Therefore, regulatory agencies require control of drug product polysorbate content and characterization of polysorbate degradation in case of increased product-related impurities.

Here, we present orthogonal analytical procedures for quantification of the total polysorbate content, determination of polysorbate identity, as well as characterization and quantification of polysorbate degradation products. Methods can be easily implemented in a pharmaceutical quality control laboratory.

## Introduction

Unlike most other surface-active substances, polysorbates are well tolerated by humans and animals upon consumption. As a result, polysorbates are widely used in the food and drug industries as emulsifiers and stabilizing agents, respectively.[1] For biopharmaceuticals in particular, polysorbates are included to prevent protein denaturation and aggregation. Being an integral part of the drug product formulation, polysorbates can have a profound influence on the drug substance. Moreover, being exposed to the same external factors that can affect the drug substance, they can also undergo changes that can in turn negatively impact the formulated biologic.[2]

The unifying chemical feature of polysorbates is a polyethoxylated sorbitol-derived core, which can be esterified with a variety of fatty acids. The two most common members of the family are polysorbate 20, a monolaurate, and polysorbate 80, a monooleate (see Figure 1).

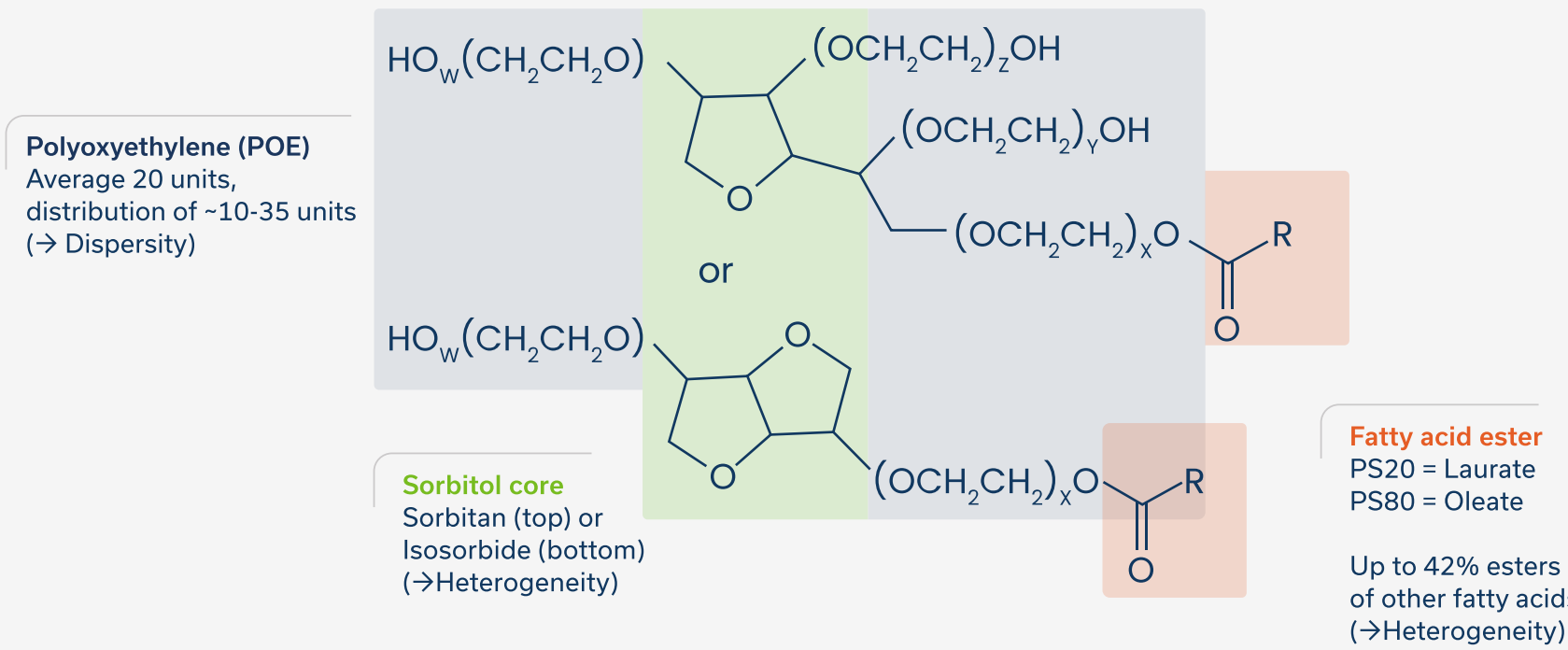



**Figure 1:** Chemical Structure and Building Blocks of Polysorbates.

Due to the nature of polymerization reactions, most polymeric species are heterogenous at the molecular level. This is also true for polysorbates. Nominally, they consist of 20 polyoxyethylene (POE) units that are attached to four hydroxyl groups of the sorbitan core. These 20 POEs only represent an average value, however. In practice, polysorbates are mixtures of species ranging from approximately 10 to 40 POE.[3] Unlike many other polymers, polysorbates have two additional aspects increasing their heterogeneity. In addition to the sorbitan core, an alternate isosorbide core can be formed which only carries two instead of four hydroxyl groups. This isosorbide core only carries half the number of POE units typically consisting of a range of 5-20 POE units with an average of 10.

The second level of complexity is derived from esterification. Indeed, the fatty acid used for esterification is never pure, but contains varying amounts of other fatty acid species. For example, regulatory guidelines require polysorbate 80 to contain only 58% of the oleate species (see Table 1) whereas the remaining 42% may be comprised of a variety of other fatty acids. Consequently, monitoring product quality of polysorbates and quantification of degradation products is also defined by the regulatory agencies. The different Pharmacopeia (e.g., Ph.Eur., USP) specify limits of present peroxides, the fatty acid composition and different impurities like Ethylene Oxides (see Table 1, adapted from Martos, A. *et al.* [4]).

**Table 1:** Requirements regarding PS20 and PS80 quality according to European Pharmacopeia 8.5 and United States Pharmacopeial Convention from 2015

| | Ph. Eur | | USP | |
|---|---|---|---|---|
| | PS20 | PS80 | PS20 | PS80 |
| Peroxide Value (mEq/Kg) | ≤10 | ≤10 | ≤10 | ≤10 |
| Fatty Acid Composition (%) | | | | |
| Capric Acid | ≤10 | - | ≤10 | - |
| Caproic Acid | ≤1 | - | ≤1 | - |
| Caprylic Acid | ≤10 | - | ≤10 | - |
| Lauric Acid | 40-60 | - | 40-60 | - |
| Linoleic Acid | ≤3 | ≤18 | ≤3 | ≤18 |
| Linolenic Acid | - | ≤4 | - | ≤4 |
| Myristic Acid | 14-25 | ≤5 | 14-25 | ≤5 |
| Oleic Acid | ≤11 | ≥58 | ≤11 | ≥58 |
| Palmitic Acid | 7-15 | ≤16 | 7-15 | ≤16 |
| Palmitoleic Acid | - | ≤8 | - | ≤8 |
| Stearic Acid | ≤7 | ≤6 | ≤7 | ≤6 |
| Impurities | | | | |
| Ethylene Oxide (ppm) | ≤1 | ≤1 | ≤1 | ≤1 |

Moreover, more than one fatty acid chain can be attached to the terminal hydroxyl groups of the POE moieties, creating not only mono-, but also di-, tri- and tetraesters. If multiple fatty acids are attached to a single core, they are not necessarily of the same species, but reflect the underlying fatty acid composition, resulting in an even higher heterogeneity of the polysorbate. As a result, for a comprehensive characterization it is not only important to accurately determine the total polysorbate content but to also analyze polysorbate identity, heterogeneity, dispersity, and degradation. Several methods can be applied to analyze polysorbates (see Table 2).

Due to the widespread use of polysorbates in the formulation of biologics, their stability is critical for a high-quality product. External influences during production, formulation, storage, and handling can degrade polysorbates thereby affecting drug product stability.[5] Two main degradation pathways exist for polysorbates.[6] First, the hydrolysis of the ester functionality can compromise the surfactant function of polysorbates, which can result in increased aggregate formation.

Ester hydrolysis is thought to mainly be caused by residual enzymes, but can also be chemically induced.[7] Secondly, exposure to oxidative species can result in oxidation of unsaturated fatty acids. Their double bonds are particularly vulnerable and are readily converted into different oxidized species, including hydroxy-, peroxy- or keto forms. Reactions can even lead to cleavage of the fatty acid and formation of aldehydes and carbonic acids. In turn, these oxidative species can oxidize the drug substance and compromise drug product quality.[8,9] Monitoring polysorbate degradation and quantifying possible degradation products is therefore a key aspect of controlling product quality.

ProtaGene has established a plethora of analytical methods in order to support every aspect of polysorbate characterization, degradation, and quality control (see Figure 2).

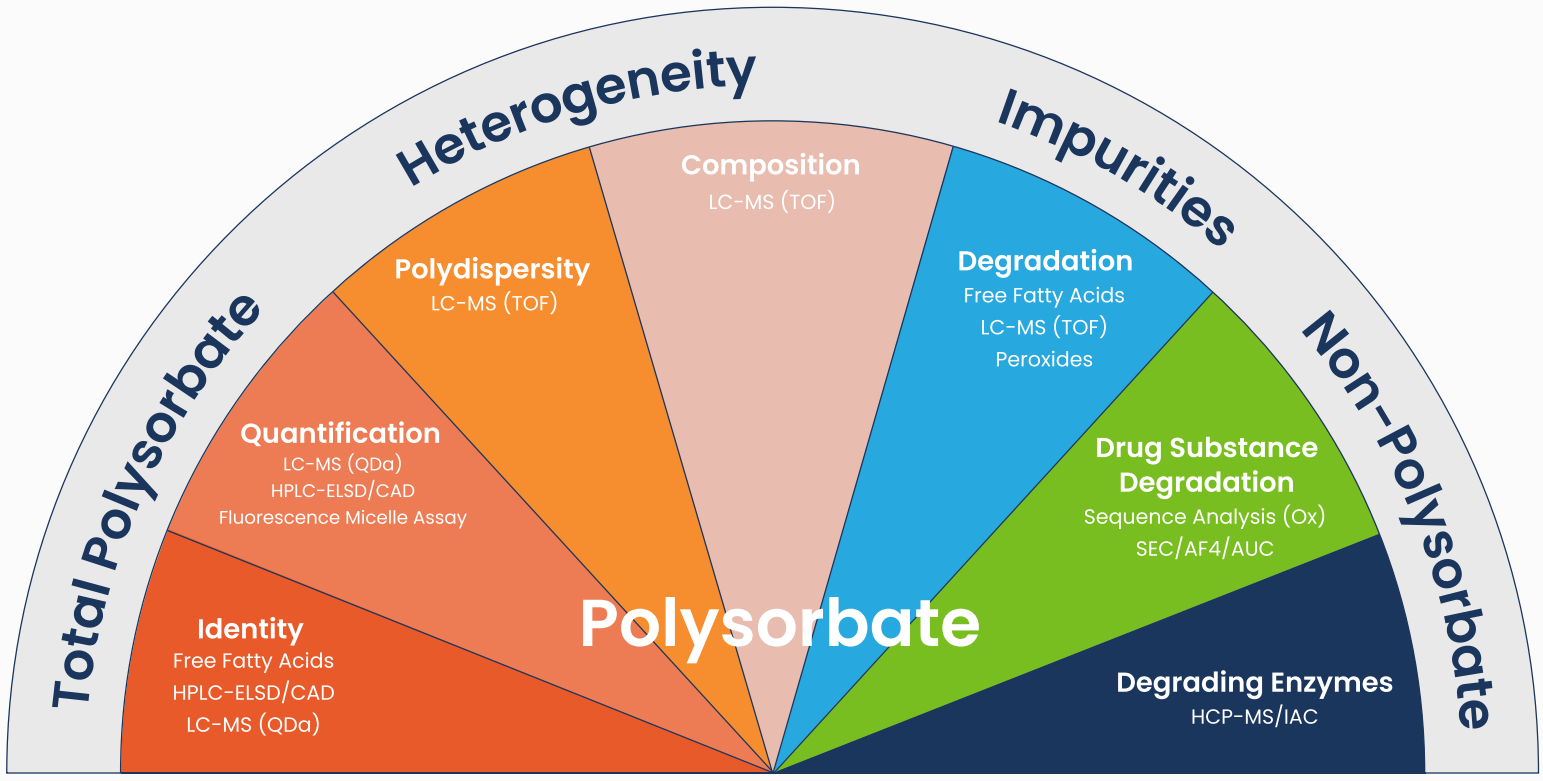



**Figure 2:** Overview of Analytical Methods for comprehensive Polysorbate analysis available at ProtaGene.

For the case study presented here, data has been obtained with the following methods and compared to each other (see Table 2). A defined set of polysorbate-containing samples was used.

**Table 2:** Overview of analytical methods used in this study.

| Analytical Method | Result | Quality Attribute |
|---|---|---|
| HPLC-CAD | Polysorbate content in g/L | Quantification |
| HPLC-QDa | Polysorbate content in g/L | Quantification, Identity |
| LC-MS | Polysorbate species relative content in % | Polydispersity, Composition, Degradation |
| Free Fatty Acid Analysis | Free Fatty Acid content in g/L | Identity, Degradation |
| Peroxide Assay | Peroxide content in mM | Degradation |
| Fluorescence Micelle Assay | Polysorbate content in g/L | Quantification |

## Results

A monoclonal antibody drug product containing 0.2 mg/mL PS80 was subjected to light stressing according to ICH Q2B recommendations ($1.2x10^6$ Lux*h and 200 $Wh/m^2$). As controls, the drug product spiked with 0.1 mg/mL PS80, Water for Injection, Water for injection spiked with 0.1 mg/mL PS20 and 0.1 mg/mL PS80 were prepared and underwent light stressing in parallel. Regular and light-stressed samples were analyzed for polysorbate content and characterization of degradation products.

**Table 3:** Test panel

| Sample | HPLC-CAD | HPLC-QDa | LC-MS | Free Fatty Acid Analysis | Peroxide Assay | Fluorescence Micelle Assay |
|---|---|---|---|---|---|---|
| mAb | ✕ | | | ✕ | ✕ | ✕ |
| mAb, light stressed | ✕ | | | ✕ | ✕ | ✕ |
| mAb spiked with 0.1 mg/mL PS80 | ✕ | | | ✕ | ✕ | ✕ |
| mAb spiked with 0.1 mg/mL PS80, light stressed | ✕ | | | ✕ | ✕ | ✕ |
| Water or PBS | ✕ | ✕ | | ✕ | ✕ | ✕ |
| Water or PBS, light stressed | ✕ | ✕ | | ✕ | ✕ | ✕ |
| Water spiked with 0.1 mg/mL PS20 | ✕ | ✕ | | ✕ | ✕ | ✕ |
| Water spiked with PS20, light stressed | ✕ | ✕ | ✕ | ✕ | ✕ | ✕ |
| Water spiked with 0.1 mg/mL PS80 | ✕ | ✕ | ✕ | ✕ | ✕ | ✕ |
| Water spiked with 0.1 mg/mL PS80, light stressed | ✕ | ✕ | ✕ | ✕ | ✕ | ✕ |

# Evaluation of Polysorbates Content by HPLC-CAD

To perform total polysorbate quantification that can be applied as a QC method for routine testing, a platform method was established by applying mixed-mode chromatography on a HPLC system (MM-HPLC) with charged aerosol detection (CAD). In contrast to RP, MM-HPLC separation is not only based on one form of interaction between analytes and the stationary phase. The Oasis MAX column used allows separation based on a combination of reversed phase, anion-exchange, and dipole-dipole interactions.

A flowrate of 1 mL/min with a binary gradient of water with formic acid (eluent A) and an organic eluent with formic acid (eluent B), was applied. For elution, the amount of organic non-polar solvent was increased. With every analytical session, a calibration curve is performed using freshly prepared polysorbate calibration solutions starting at 0.05 g/L. Results of the quantification of Polysorbate 20 in different samples with and without light stress are summarized in Table 4. The impact of light stress on PS20 is graphically shown in Figure 3.

**Table 4:** Results of Polysorbate 20 quantification of regular and light stressed samples.

| Sample | PS20 Concentration [g/L] | | | Theoretical PS20 Concentration [g/L] | Recovery to Nominal Concentration [%] |
|---|---|---|---|---|---|
| | Mean | SD | CV [%] | | |
| Water | n.d. | n.d. | n.d. | 0.000 | n.a. |
| Water, light stressed | n.d. | n.d. | n.d. | 0.000 | n.a. |
| Water spiked with PS20 | 0.117 | 0.003 | 2.8 | 0.100 | 117.0 |
| Water spiked with PS20, light stressed | 0.110 | 0.001 | 1.3 | 0.100 | 110.0 |

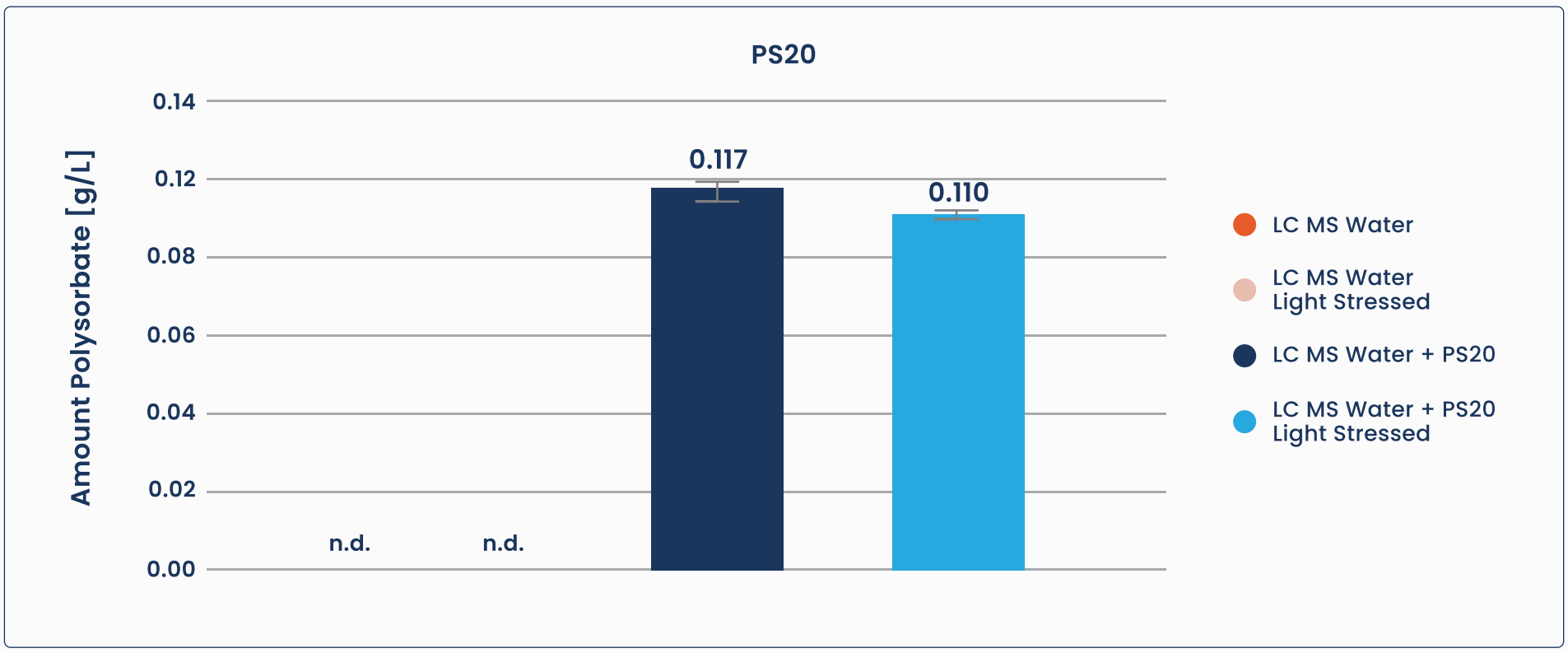



**Figure 3:** Impact of Light Stress on the PS20 content.

Results of the quantification of Polysorbate 80 in different samples subjected to and without light stress are summarized in Table 5. The impact of light stress on PS80 is shown in Figure 4.

**Table 5:** Results of Polysorbate 80 quantification of regular and light stressed samples.

| Sample | PS80 Concentration [g/L] | | | Theoretical PS80 Concentration [g/L] | Recovery to Nominal Concentration [%] |
|---|---|---|---|---|---|
| | Mean | SD | CV [%] | | |
| Water | n.d. | n.d. | n.d. | 0.000 | n.a. |
| Water, light stressed | n.d. | n.d. | n.d. | 0.000 | n.a. |
| Water spiked with PS80 | 0.103 | 0.001 | 1.0 | 0.100 | 103.0 |
| Water spiked with PS80, light stressed | 0.095 | 0.001 | 1.1 | 0.100 | 95.0 |
| mAb | 0.226 | 0.004 | 2.0 | 0.200 | 113.0 |
| mAb, light stressed | 0.217 | 0.002 | 0.8 | 0.200 | 108.5 |
| mAb spiked with PS80 | 0.317 | 0.020 | 6.4 | 0.300 | 105.7 |
| mAb spiked with PS80, light stressed | 0.301 | 0.001 | 0.4 | 0.300 | 100.3 |

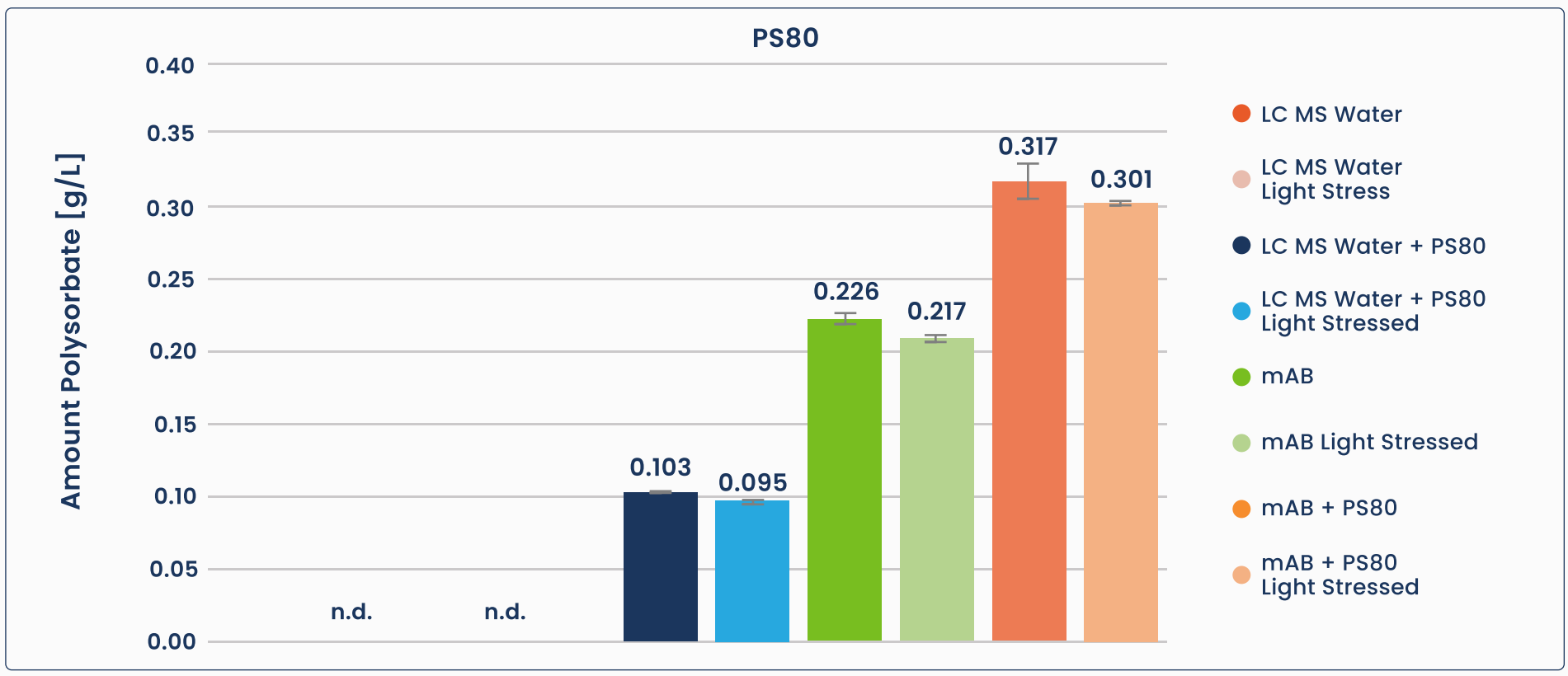



**Figure 4**: Impact of Light Stress on the PS80 content.

In summary, the total polysorbate content was determined with recoveries between 95% and 117% for the unstressed samples. A decrease of approximately 0.01 g/L (corresponding to 4% to 8%) in the total polysorbate content has been observed for the light-stressed materials with HPLC-CAD.

# Evaluation of Polysorbate Content by HPLC-QDa

In order to quantify as well as selectively distinguish polysorbate 20 from polysorbate 80, a quantification method was developed applying mixed-mode chromatography (MM-HPLC) on a UPLC System equipped with a single quadrupole detection (QDa) for mass detection (QDa, 500-1100 m/z). The Acclaim™ Surfactant column used allows separation based on a combination of reversed phase, anion-exchange, and dipole-dipole interactions. A flowrate of 0.8 mL/min with a tetrameric gradient of water (eluent A), isopropanol (eluent B), 10 mM ammonium formiate (eluent C), and acetonitrile (eluent D) was applied. For elution, the amount of organic non-polar solvent was increased.

The amounts of polysorbate 20 and polysorbate 80 determined in different samples with and without light stress are summarized in Figure 5. The impact of light stress on Polysorbate 20 is shown in Figure 5a.

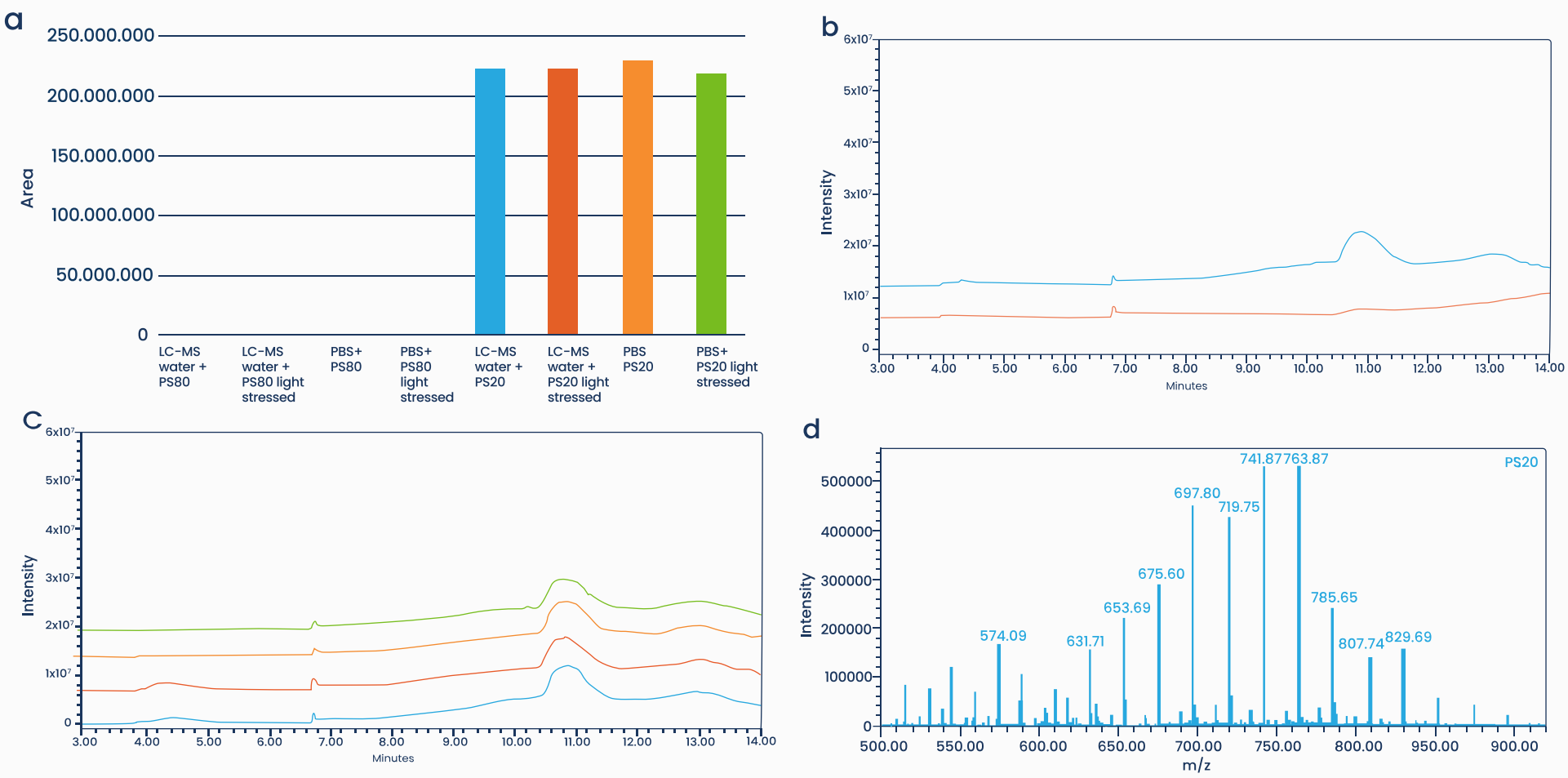



**Figure 5:** PS20 MM-HPLC quantification method. a: Peak area within the total ion current (TIC), b: overlay of TIC traces from unstressed samples of LC water + PS20 (blue), LC water + PS80 (orange) and LC water (black), c: overlay of TIC traces of PS20 within LC water (unstressed: blue, stressed: red) or PBS (unstressed: orange, stressed: green), d: MS spectra of PS20 with LC water unstressed, the $[M+2H]^{2+}$ polymer distribution is shown with a characteristic peak distance of 22 m/z.

In summary, changes in the amount of polysorbate 20 due to light stress are quantifiable in water and PBS buffer (Figure 5a). A decrease of 2% to 4% was observed for the light-stressed samples. For the determination of polysorbate 20, no difference was detected in the presence of salts compared to salt-free conditions, both under non-stressed and light-stressed conditions (Figure 5c). The identification of polysorbate 20 was performed based on the MS fingerprint (Figure 5d).

Since no signals were detected for polysorbate 80 in the total ion currents (TIC) and the MS spectra, the method is specific for polysorbate 20 quantification (Figure 5b). The method can also be adapted for specific quantification of polysorbate 80. Using a calibration curve, a quantification of polysorbate is possible.

# Evaluation of Polysorbates by LC-MS Analysis

For a more comprehensive characterization of polysorbates, LC-MS allows the direct analysis of individual polysorbates. This allows the direct comparison of samples regarding polydispersity, esterification and degradation.

During LC-MS analysis the different polysorbates were separated on an UPLC instrument equipped with a C18 column. A flowrate of 0.3 mL/min with a quaternary gradient of water (eluent A), isopropanol (eluent B), acetonitrile (eluent C), and 2% formic acid and 0.064% ammonium formiate in water (eluent D) was applied. Here ammonium formiate was added to improve MS detection. This method allows the separation and detection of non-esterified species, mono-, di-, tri- and tetra-esters elute at increasingly less polar conditions, resulting in separate elution time windows for each polysorbate component. The annotation of the peaks was based on MS identification of the different polysorbates (Figure 6 and Figure 7).

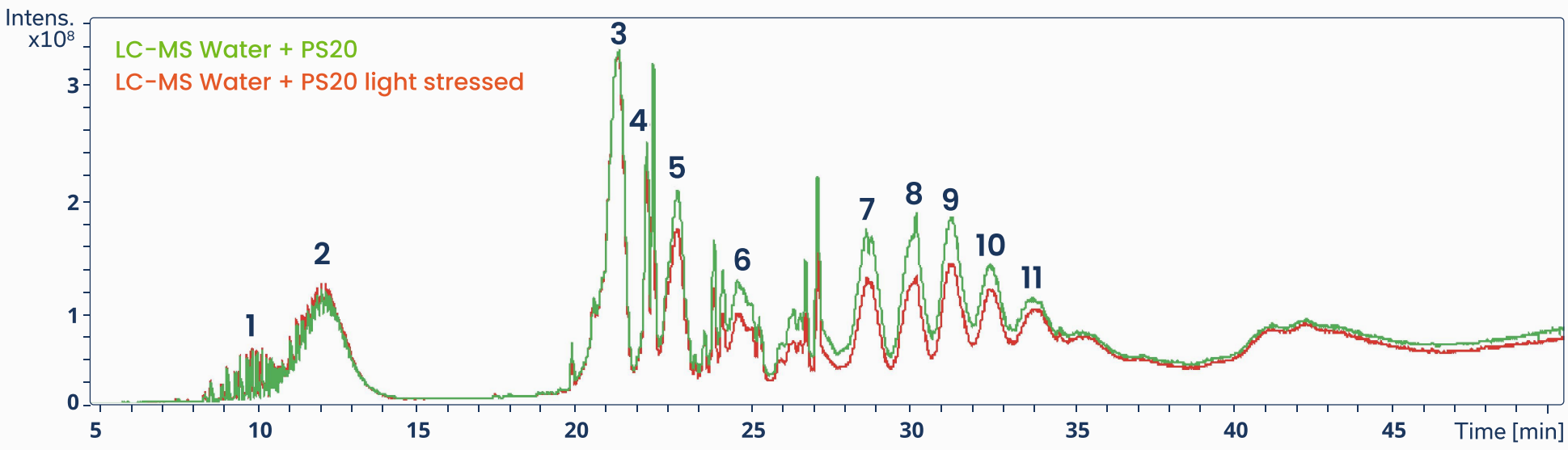



**Figure 6:** Total Ion chromatogram of PS20 with and without Light Stress.

1: non-esterified Isosorbide; 2: non-esterified Sorbitan; 3: Sorbitan mono-Laureate; 4: Isosorbide mono-Laureate; 5: Sorbitan mono-Myristinate; 6: Sorbitan mono-Palmitate; 7: Sorbitan di-Laureate; 8: Sorbitan mono-Laureate mono-Myristinate; 9: Sorbitan mono-Laureate mono-Palmiate; 10: Sorbitan mono-Laureate mono-Stearate; 11: Sorbitan tri-Laureate

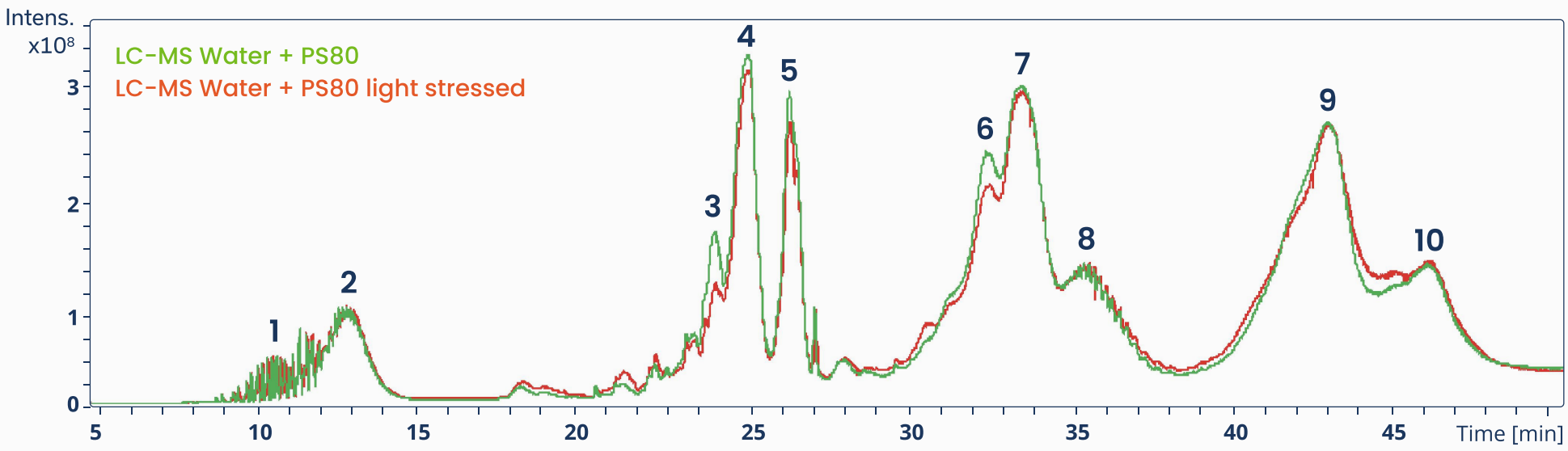



**Figure 7:** Total Ion chromatogram of PS80 with and without Light Stress.

1: non-esterified Isosorbide; 2: non-esterified Sorbitan; 3: Sorbitan mono-Linoleate; 4: Sorbitan mono-Oleate; 5: Isosorbide mono-Oleate; 6: Sorbitan monooleat mono-Linolate; 7: Sorbitan di-Oleate; 8: Isosorbide di-Oleate; 9: Sorbitan tri-Oleate; 10: Sorbitan tetra-Oleate

In summary, the species are separated based on the fatty acid(s) and the type of core, thus generating discernable chromatographic peaks for individual polysorbate subspecies. For some peaks, additional separation based on the number of POE units can be observed, but baseline separation is no longer achieved. As each POE unit results in a distinct mass increase of 44 Da, the POE composition is easily determined by mass spectrometry. The sum of all ions detected at any given time, or total ion current (TIC), also provides a convenient quantitative readout. As an example, the two core forms of the main species of polysorbate 20 and 80, respectively, are compared for PS20 (Figure 8 and Figure 9). For PS20 the most abundant POE form of the monolaurate is 24 and 11 for the sorbitan and isosorbide cores, respectively. For PS80, the most abundant POE species of the monooleate is 28 for the sorbitan and 12 for the isosorbide core.

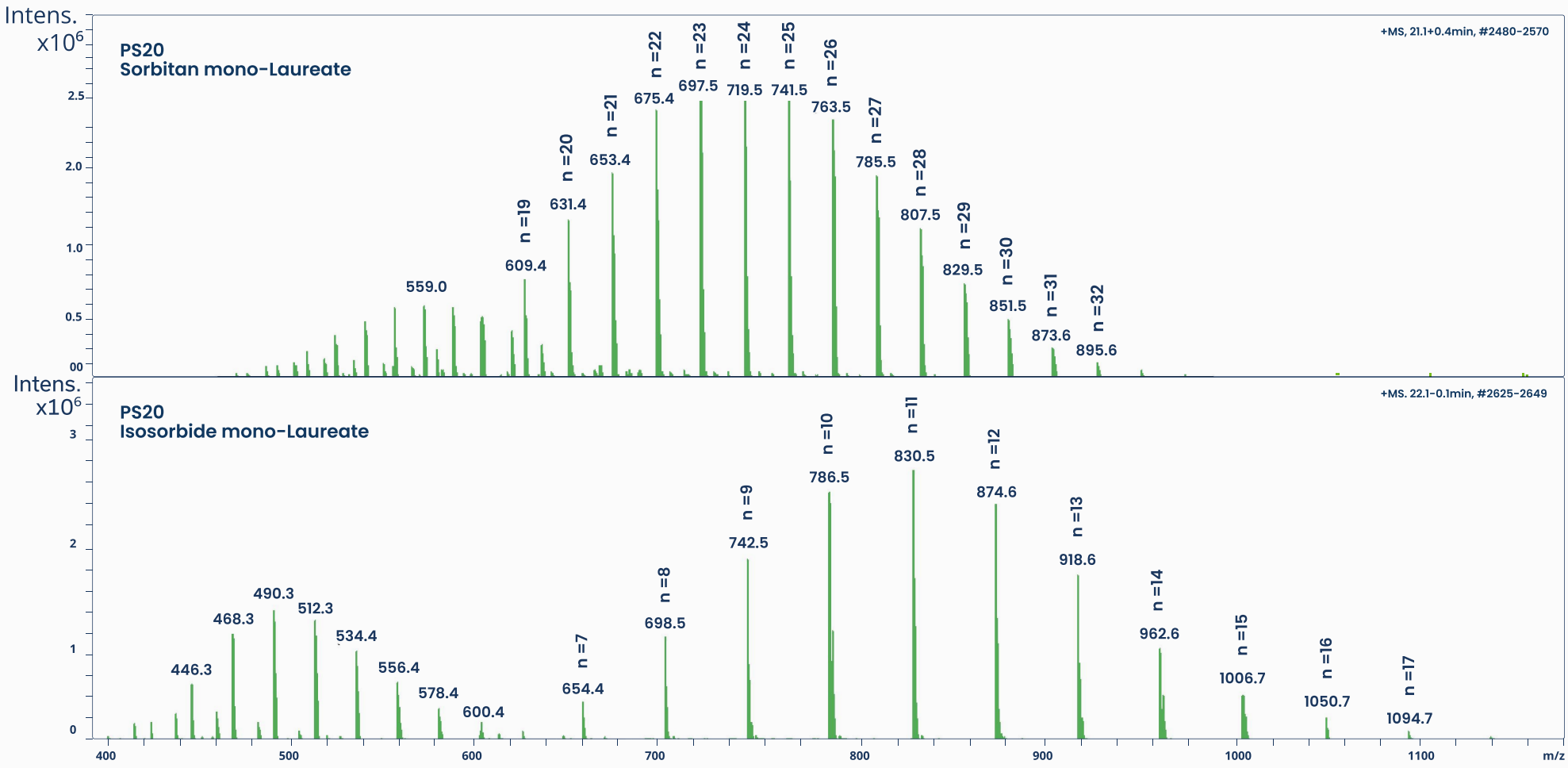



**Figure 8:** Determination of Polyoxyethylene (POE) Distribution of Sorbitan and Isosorbide Esters for PS20.

The detailed analysis also allows to determine differences in the degree of esterification and the variability regarding the attached fatty acids in PS80 compared to PS20 (Figure 9).

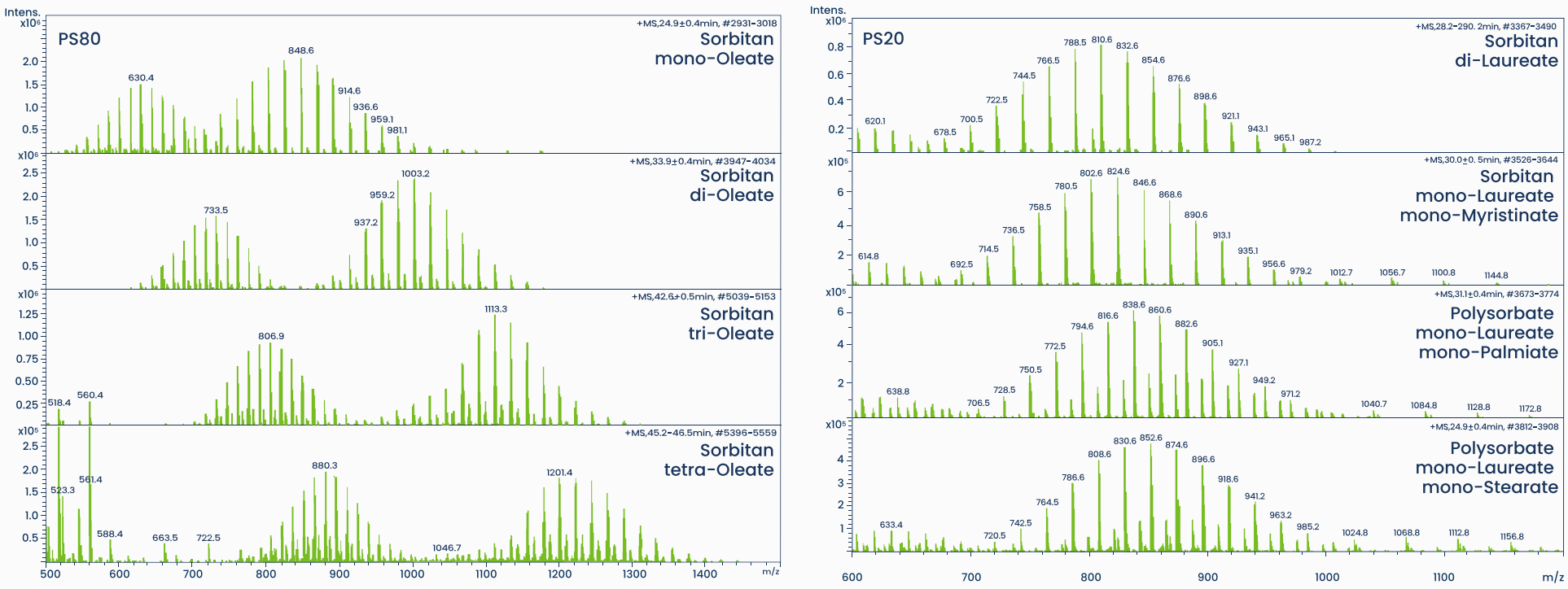



**Figure 9:** Determination of Polyesterification in PS80 (left) and Determination of Fatty Acid Heterogeneity in PS20 (right).

The data obtained during LC-MS analysis additionally allows the determination of degradation products in different polysorbate samples. Here, the two main degradation products were analyzed, de-esterification and oxidation of unsaturated fatty acids caused by exposure to VIS and UV light. For polysorbate 20, a decrease in the signal intensity of the di- and tri-ester species and a concomitant increase in the non-esterified species was observed (Figure 10).

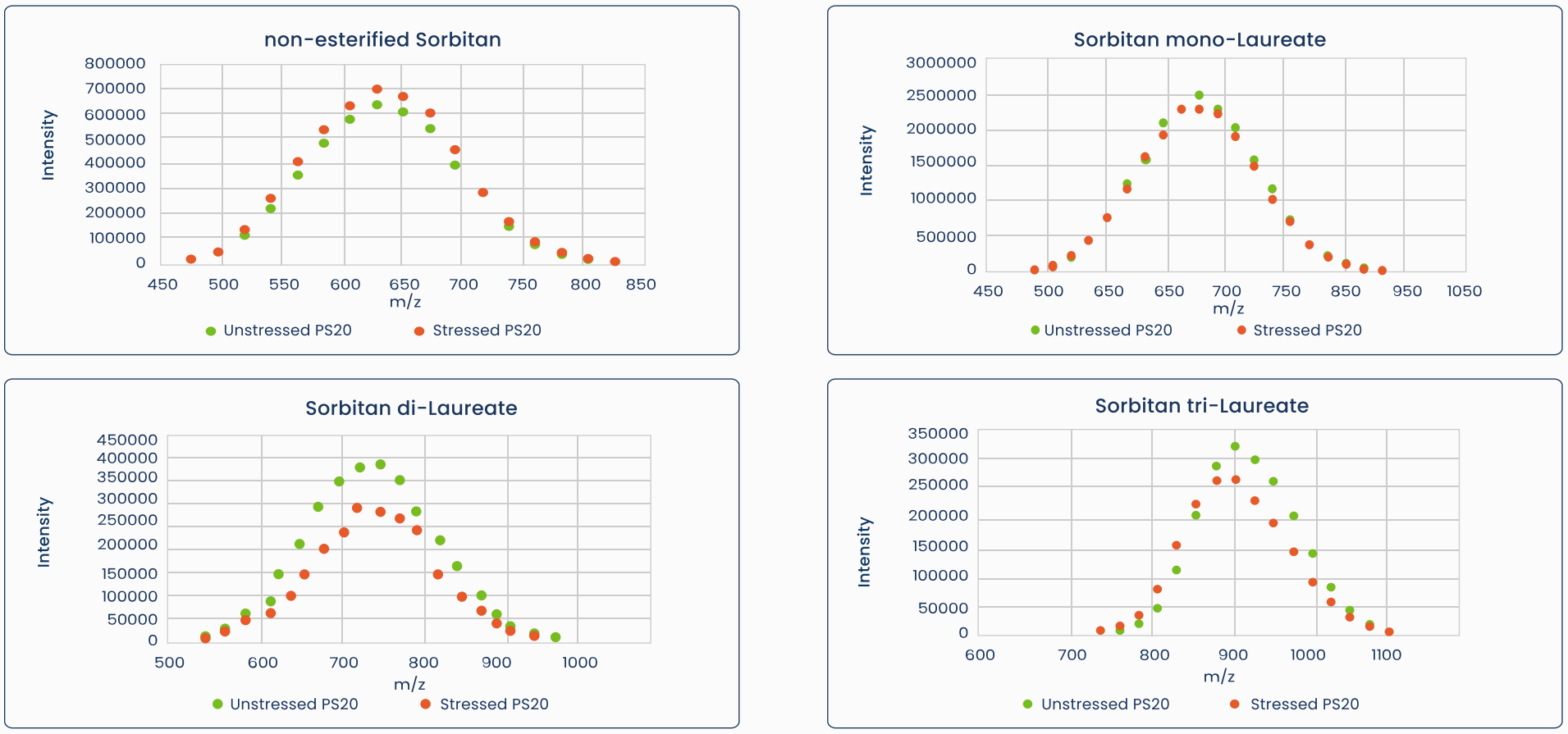



**Figure 10:** Hydrolysis of Sorbitan Polyesters in PS20. Y-axis not aligned.

For polysorbate 80, a decrease in the abundance of species containing unsaturated fatty acids was observed. The decrease was notably stronger for doubly unsaturated linolate species than the singly unsaturated oleate esters (Figure 11). Species containing saturated fatty acids were unchanged (data not shown). In parallel, the abundance of oxidation species notably increased (Table 5).

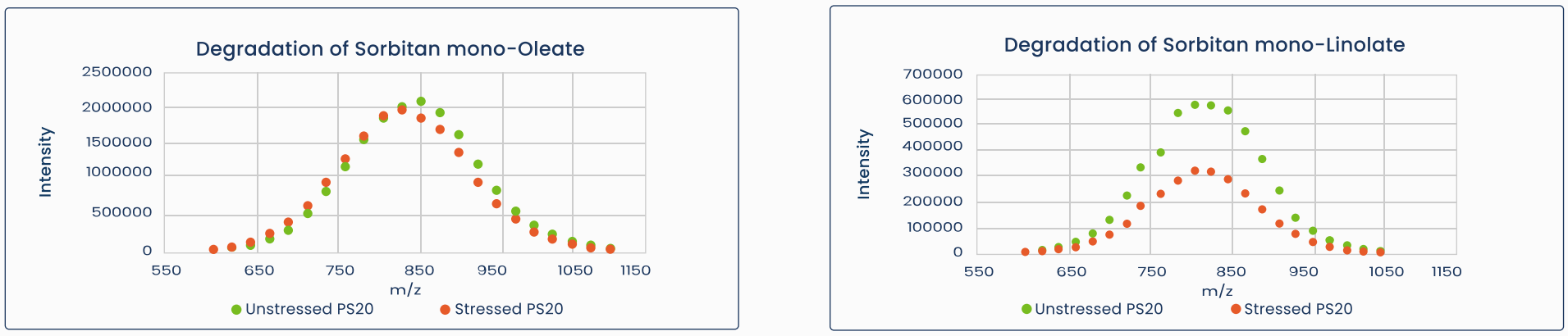



**Figure 11:** Degradation of Sorbitan esters containing unsaturated Fatty Acids in PS80. Y-axis not aligned.

**Table 6:** Results of oxidation products in unstressed and light-stressed PS80 containing samples.

| Species | LC-MS water + PS80 [%] | LC-MS water + PS80 Light stressed [%] |
|---|---|---|
| Oleate | 98.30 | 95.56 |
| Hydroxy Oleate | 0.26 | 0.59 |
| Peroxy Oleate | 1.12 | 2.98 |
| Keto Oleate | 0.19 | 0.50 |
| 9-oxo-nonanoate | 0.12 | 0.38 |

# Evaluation of Degradation by Detection of Free Fatty Acids

The Free Fatty Acid (FFA) assay discerns and quantifies individual free fatty acids (FFAs). FFAs are isolated from polysorbate-containing samples by solid phase extraction and then separated and quantified by RP-UPLC-FLR after fluorescent labeling with 1-Pyrenyldiazomethan. Chromatographic separation was achieved with a gradient of water and acetonitrile. Quantification of individual FFAs is achieved through normalization using an internal standard and based on a calibration curve in the range of 0.05 ng/µL – 8 ng/µL for up to eight different FFAs in parallel.

Results of the FFA content in Polysorbate 20 containing samples with and without light stress are summarized in Table 6. The impact of light stress on the FFA content is shown in Figure 12.

**Table 7:** Results of FFA quantification in PS20 containing samples with and without light stress.

| Sample | Water | | Water, light stressed | | Water spiked with PS20 | | Water spiked with PS20, light stressed | |
|---|---|---|---|---|---|---|---|---|
| FFA | Amount [ng/L] | | | | | | | |
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| Caprylic acid | 0.037 | 0.001 | 0.039 | 0.003 | 0.039 | 0.003 | 0.044 | 0.001 |
| Capric acid | 0.058 | 0.005 | 0.057 | 0.005 | 0.056 | 0.003 | 0.058 | 0.004 |
| Lauric acid | 0.041 | 0.002 | 0.043 | 0.003 | 0.188 | 0.018 | 0.277 | 0.024 |
| Myristic acid | n.a. | n.a. | 0.039 | 0.001 | 0.081 | 0.008 | 0.065 | 0.003 |
| Linoleic acid | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. |
| Palmitic acid | 0.094 | 0.003 | 0.049 | 0.035 | 0.079 | 0.045 | 0.052 | 0.049 |
| Oleic acid | n.a. | n.a. | 0.050 | 0.002 | 0.071 | 0.006 | 0.056 | 0.003 |
| Stearic acid | 0.069 | 0.034 | 0.079 | 0.011 | 0.088 | 0.027 | 0.074 | 0.028 |

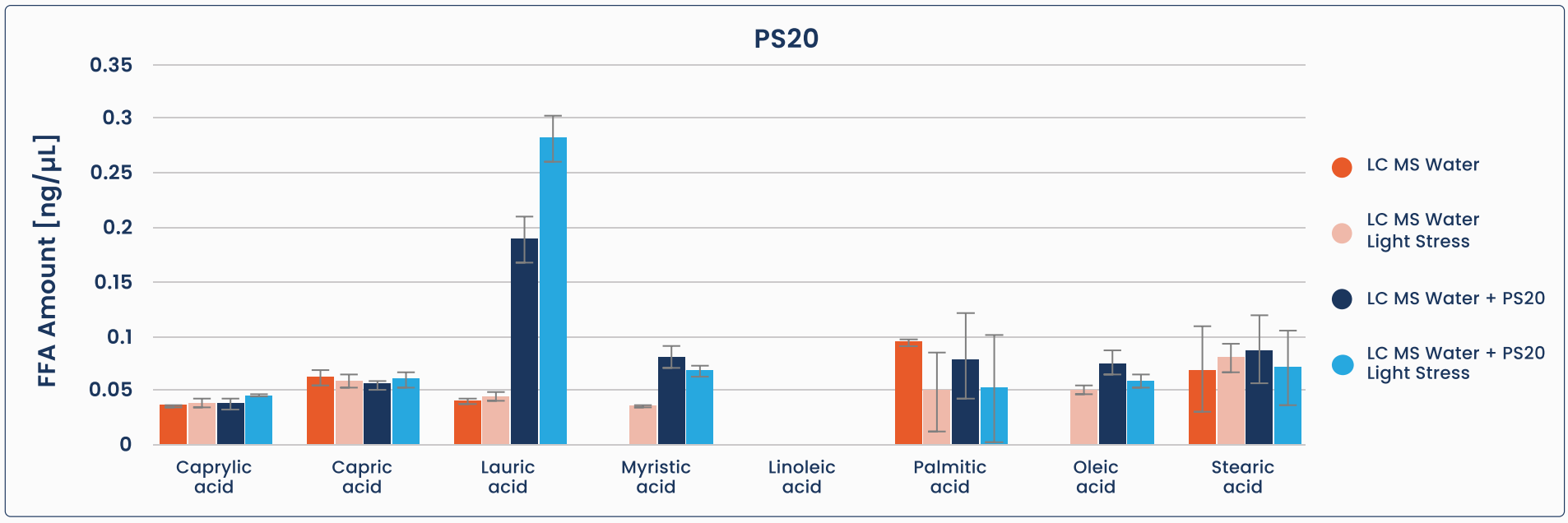



**Figure 12:** Impact of Light Stress on the FFA content in PS20 containing samples.

Results of the FFA content in Polysorbate 80 containing samples with and without light stress are summarized in Table 8 and Table 9. The impact of light stress on PS80 is shown in Figure 13.

**Table 8:** Results of FFA quantification in PS80 containing water samples with and without Light Stress.

| Sample | Water | | Water, light stressed | | mAB spiked with PS80 | | Water spiked with PS80, light stressed | |
|---|---|---|---|---|---|---|---|---|
| FFA | Amount [ng/L] | | | | | | | |
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| Caprylic acid | 0.037 | 0.001 | 0.039 | 0.003 | 0.041 | 0.003 | 0.045 | 0.001 |
| Capric acid | 0.058 | 0.005 | 0.057 | 0.005 | 0.055 | 0.004 | 0.054 | 0.001 |
| Lauric acid | 0.041 | 0.002 | 0.043 | 0.003 | 0.04 | 0.001 | 0.048 | 0.001 |
| Myristic acid | n.a. | n.a. | 0.039 | 0.001 | n.a. | n.a. | 0.045 | 0.011 |
| Linoleic acid | n.a. | n.a. | n.a. | n.a. | 0.068 | 0.006 | n.a. | n.a. |
| Palmitic acid | 0.094 | 0.003 | 0.049 | 0.035 | 0.075 | 0.049 | 0.053 | 0.038 |
| Oleic acid | n.a. | n.a. | 0.050 | 0.002 | 0.278 | 0.041 | 0.112 | 0.011 |
| Stearic acid | 0.069 | 0.034 | 0.079 | 0.011 | 0.087 | 0.018 | 0.096 | 0.036 |

**Table 9:** Results of FFA quantification in PS80 containing samples with and without Light Stress.

| Sample | Water | | Water, light stressed | | mAB spiked with PS80 | | Water spiked with PS80, light stressed | |
|---|---|---|---|---|---|---|---|---|
| FFA | Amount [ng/L] | | | | | | | |
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| Caprylic acid | 0.039 | 0.004 | 0.046 | 0.001 | 0.040 | 0.005 | 0.050 | 0.003 |
| Capric acid | 0.059 | 0.001 | 0.055 | 0.001 | 0.061 | 0.001 | 0.056 | 0.001 |
| Lauric acid | 0.049 | 0.001 | 0.040 | 0.001 | 0.052 | 0.001 | 0.039 | 0.001 |
| Myristic acid | 0.054 | 0.008 | 0.037 | 0.002 | 0.037 | 0.002 | n.a. | n.a. |
| Linoleic acid | 0.086 | 0.004 | n.a. | n.a. | 0.133 | 0.003 | n.a. | n.a. |
| Palmitic acid | 0.067 | 0.051 | 0.076 | 0.003 | 0.126 | 0.029 | 0.077 | 0.049 |
| Oleic acid | 0.358 | 0.010 | 0.117 | 0.005 | 0.669 | 0.022 | 0.163 | 0.006 |
| Stearic acid | 0.084 | 0.036 | 0.104 | 0.020 | 0.114 | 0.016 | 0.101 | 0.030 |

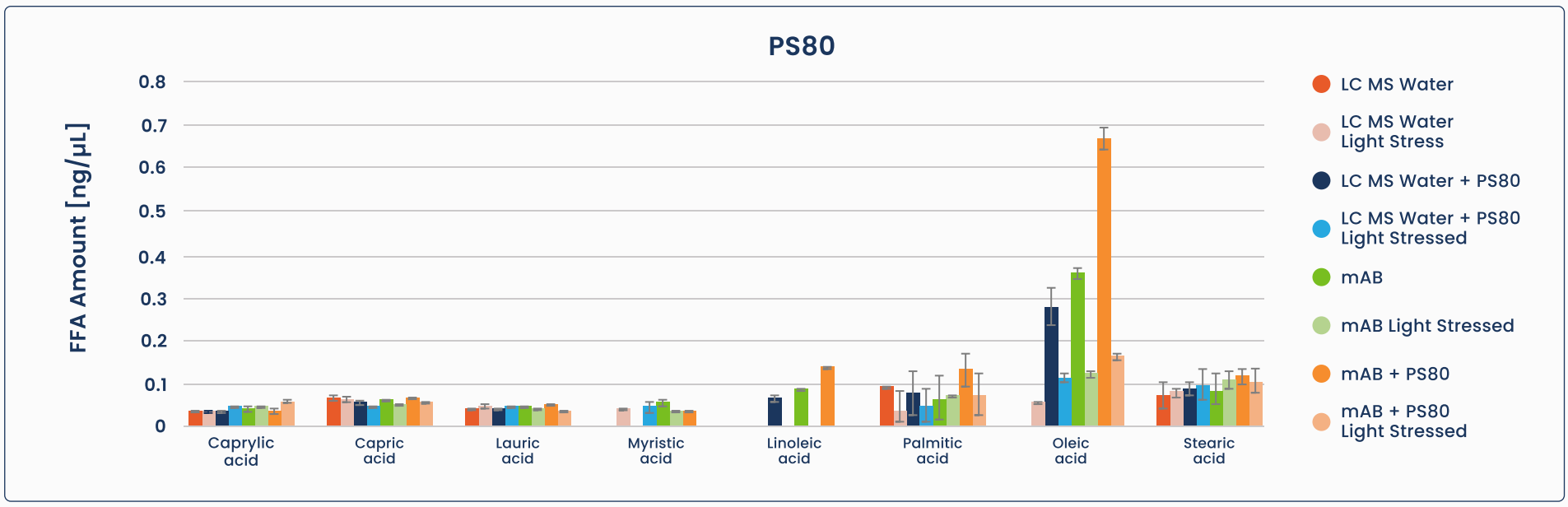



**Figure 13:** Impact of Light Stress on the FFA content in PS80 containing samples.

In summary, as expected for PS20 containing samples, lauric acid was detected as the major FFA, whereas for PS80 containing samples, the major FFA was found to be Oleic acid. Light stress led to an increase of lauric acid in PS20 containing samples, presumably due to de-esterification of polysorbate.

In contrast, for PS80 containing samples, a decrease of Oleic acid and Linoleic acid was observed, presumably due to light stress-induced oxidation and breakdown of the unsaturated fatty acids (such as oleic acid and Linoleic acid), therefore decreasing the amount of those FFAs.

# Evaluation of Degradation by Detection of Peroxides

The Peroxide assay quantifies the total content of peroxide species present in a test solution. The detection is based on the oxidation of 10-acetyl-3, 7-dihydroxyphenoxazine to resorufin in the presence of $H_2O_2$ and Horseradish Peroxidase. Quantification is based on a calibration curve from 75 pM to 1 µM $H_2O_2$. The samples were analyzed without further preparation in a 96-well plate reader format using excitation at 530 ± 12.5 nm and detecting emission at 590 ± 17.5 nm.

Analysis of the peroxide content showed significant differences between unstressed and light-stressed samples (Figure 14). Peroxides are detected in light-stressed LC-MS water, but that does not account for the greater peroxide content in LC-MS water samples spiked with PS20. For the stressed mAb samples, the increase in peroxides compared to unstressed mAb was about 40-fold. Notably, a significantly higher increase in peroxide species was observed for light-stressed mAb samples (containing PS80) compared to the PS-spiked water samples (Figure 15).

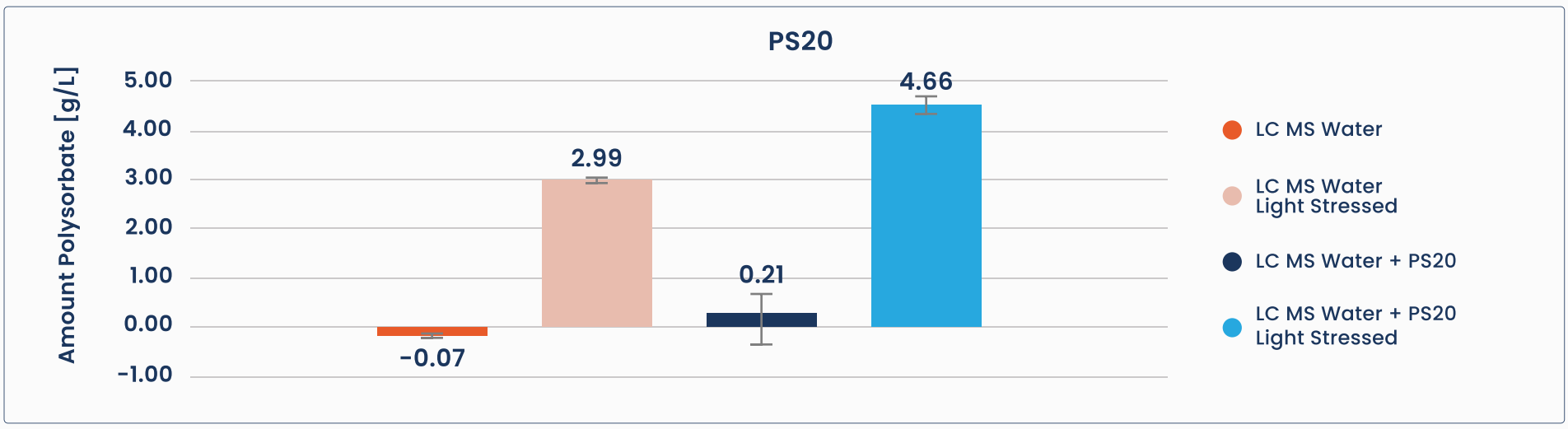



**Figure 14:** Impact of Light Stress on the Peroxide content in PS20 containing samples.

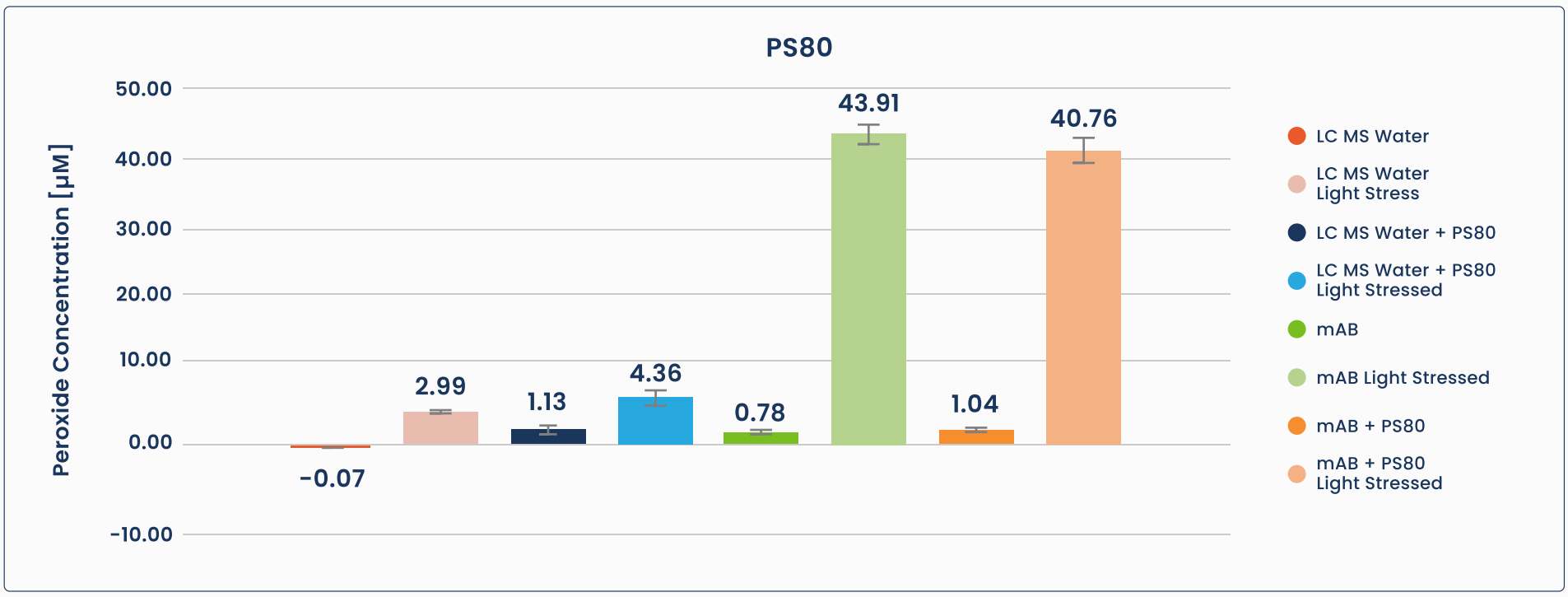
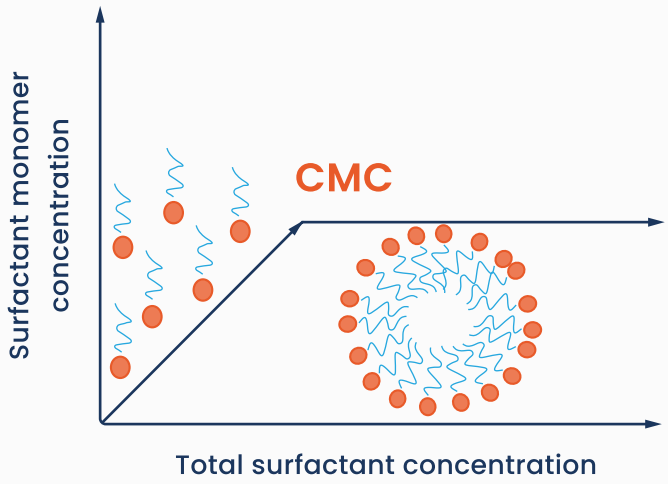



**Figure 15:** Impact of Light Stress on the Peroxide content in PS80 containing samples.

In summary, as expected low levels of peroxides were detected in unstressed samples. After light-stressing, peroxide levels increased for all samples. Interestingly, the increase was significantly more pronounced for the mAb drug product samples. This observation might however be linked not only to PS80 oxidation but also to peroxidation derived from the mAb formulation.

# Evaluation of Polysorbate Content by FMA (Fluorescence-Micelle-Assay)

FMA is a flow injection assay (without separation/stationary phase) able to quantify the total polysorbate content without discrimination between PS20 and PS80. The quantification is based on the micelle-forming abilities of the surfactant. Upon addition of the fluorescent dye N-Phenyl-1-Naphtylamine (NPN) and incorporation into the hydrophobic core of micelles, the fluorescence is greatly increased and can be used for quantification of polysorbate. As a requirement, the surfactant concentrations must be above the CMC (Critical Micelle Concentration). The CMC is defined as the concentration of surfactants above which micelles can form so that all additional surfactants added to the system will form micelles. Therefore, the FMA is special in its ability to assess not only the content, but also a functional aspect (i.e., micelle formation) of polysorbates in solution.




Quantification of polysorbate micelles was performed on a HPLC instrument with fluorescence detection. The fluorescence detector was set to an emission wavelength of 420 nm and an excitation wavelength of 350 nm. The mobile phase was based on sodium chloride with Trizma Base at pH 8.0 and a low amount of organic solvent (acetonitrile), Brij35, and the fluorescent dye NPN. A reaction coil at a column temperature of 35 °C was used. A flowrate of 1.5 mL/min was applied. Quantification was performed based on a linear calibration curve of a polysorbate standard diluted to concentrations in the range of 0.025 g/L to 0.3 g/L using purified water.

Measurements were performed for different samples containing either polysorbate 20 or polysorbate 80 both stressed by light and unstressed. Negative controls were measured side-by-side. Results are summarized in Table 10. The impact of light stress on PS20 is shown in Figure 16. The impact of light stress on PS80 is shown in Figure 17.

**Table 10:** Results of Polysorbate 80/20 quantification of samples with and without light stress by FMA.

| Test Sample | Determined Mean Conc. [g/L] | | Theoretical Conc. Polysorbate [g/L] | Recovery [%] |
|---|---|---|---|---|
| | Polysorbate 80 | Polysorbate 20 | | |
| LC/MS Water | n.d.* | | | |
| LC/MS Water light stressed | n.d. | | | |
| LC/MS Water + PS80 | 0.093 | - | 0.100 | 93 |
| LC/MS Water + PS80 light stressed | 0.078 | - | 0.100 | 78 |
| mAb | 0.216 | - | 0.200 | 108 |
| mAb light stressed | 0.184 | - | 0.200 | 92 |
| mAb + PS80 | 0.279 | - | 0.002 | 93 |
| mAb + PS80 light stressed | 0.243 | - | 0.300 | 81 |
| LC/MS Water + PS20 | - | 0.104 | 0.100 | 104 |
| LC/MS Water + PS20 light stressed | - | 0.091 | 0.100 | 91 |

*n.d.: not detected

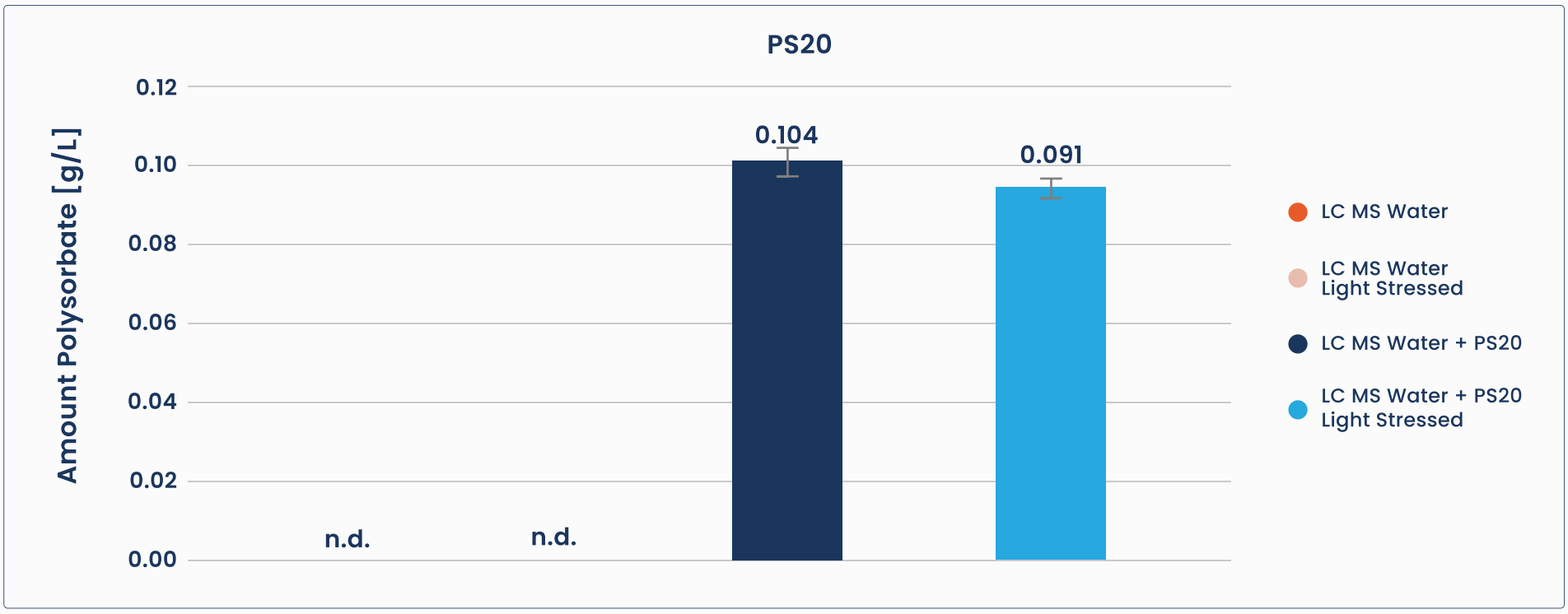



**Figure 16:** Impact of light stress on the PS20 content by FMA.

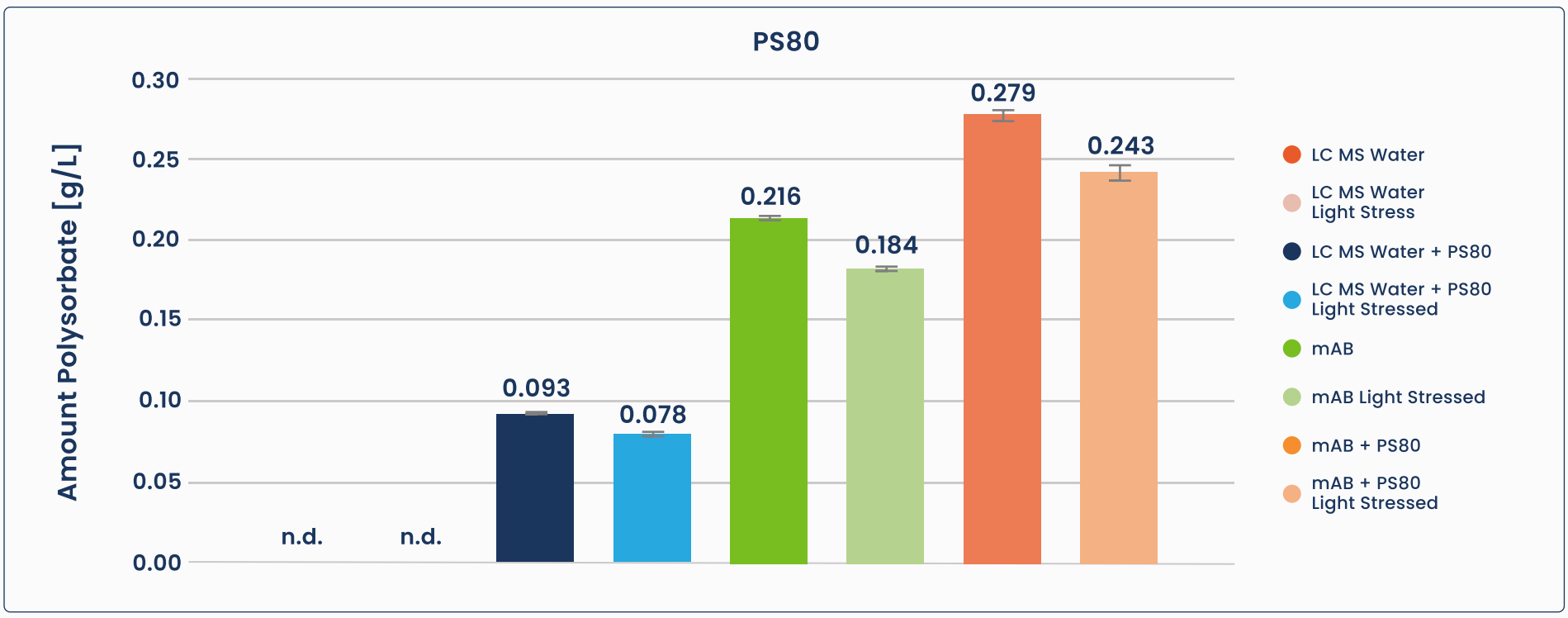



**Figure 17:** Impact of light stress on the PS80 content detected by FMA.

In summary, the polysorbate content was determined with 90% recovery for the unstressed samples. A decrease of 4% to 12% of the total polysorbate content has been observed for light-stressed material.

## Discussion

Polysorbate 20 and Polysorbate 80 are frequently used in biopharmaceutical formulations to prevent protein aggregation. As polysorbates are chemically highly diverse mixtures of sorbitol, ethylene oxides and fatty acids, there is a variation of quality between different manufacturers. Further, polysorbates are susceptible to degradation by light-induced autooxidation or hydrolysis facilitated by enzymes or pH. These degradation products may affect product quality and safety,[10] as degradation products can oxidize the proteins or form subvisible particles. Therefore, a close monitoring of the polysorbate quantity and quality is requested by health authorities.

As presented in this whitepaper, a comprehensive analytical product package is available at ProtaGene to assess polysorbate quantity and quality. The polysorbate quantity can easily and precisely be assessed by the three orthogonal methods HPLC-CAD, HPLC-ELSD and FMA. As shown in the result section, the methods show a recovery deviating not more than 10% from the theoretical concentration. The methods were capable to indicate even a slight loss in polysorbate 20 content in a range < 5% and 8-12% for PS80 with the FMA showing slightly lower content after stressing than the Mixed-Mode chromatographic methods. The FMA is known to slightly underestimate the polysorbate content[11] because no free but only micellar polysorbate is detected by that assay. Nonetheless, all orthogonal content methods have proven their ability to monitor minor changes in Polysorbate content, making them potential indicators of polysorbate stability.

As the degradation pathway for polysorbates depends on the formulation, temperature, pH, light or enzymes,[12, 13] an advanced method panel is necessary to monitor possible degradation in its early stages. LC-MS contributes most valuable information about the polysorbate quality while a determination of free fatty acids might give a hint on the tendency for subvisible particle formation. A determination of peroxides within the sample will yield information about possible oxidation processes on the DS protein level. The methods LC-MC, FFA and Peroxides complement each other yielding deep insight into the degradation processes: As the analysis of the light stressed samples showed a significant amount of peroxide species, the following LC-MS analysis revealed a remarkable increase of oxidized species in light stressed PS80 samples. Further, as the analysis of light stressed PS20 samples by LC-MS showed an increase of non-esterified species, it was expected to find an increase in the most abundant free fatty acid lauric acid. This expectation was confirmed as the FFA-assay showed an increase in free lauric acid for light-stressed PS20 samples (Figure 12). For the light stressed PS80 samples, the results of LS-MC indicated a reduction of unsaturated species as Linoleates and Oleates caused by breakdown of the double bond due to oxidative stress. This has also been confirmed with the FFA-assay, showing a significant reduction of free Linoleic and Oleic acid after stressing of PS80 samples (Figure 13). The degradation methods are, therefore, well-suited to address root-cause analyses of polysorbate degradation. Importantly, analytical methods mentioned here might be applicable under GMP for routine quality control testing of biopharmaceuticals.

## Conclusion

The methods presented here provide a powerful toolbox for the control of polysorbate quality and stability in polysorbate-formulated biopharmaceutical drug products as requested by health authorities.

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