The effect of the composition of polysorbate 80 grades on their physicochemical properties.

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ABSTRACT

Polysorbate 80 is one of the most commonly used surfactants in the formulation of biotherapeutics, particularly those administered intravenously. It comprises a mixture of fatty acids but is not a precisely defined chemical entity. Hence, there are a range of different grades available in the market, all meeting compendial specifications. Polysorbate 80 is known to undergo auto degradation producing protein-damaging by-products, and to contain residual impurities that can have an impact on the stability and integrity of the active ingredients in the formulation. Given the variety of chemical compositions that polysorbate 80 can comprise, the degradation pathway and extent could vary depending on the grade used in the formulation. This study compared the physical and chemical properties of four commercially available polysorbate 80 grades with different degrees of purity and oleic acid content and investigated their degradation profiles. This study did not find any significant differences between the properties or degradation profiles of the four grades investigated. Further studies are underway to understand the formation of other reactive impurities and their impact on the model protein formulations.

KEY WORDS: Polysorbate 80, peroxide impurities, biotherapeutics, excipients, quality, auto-degradation

INTRODUCTION

Since 1982, when the first biopharmaceutical product Humulin (recombinant human insulin), was approved for therapeutic use, there has been exponential growth in biologic products, which have been employed to treat a broad spectrum of diseases (1, 2). Biologic products on the market include monoclonal antibodies (mAbs), recombinant growth factors, purified proteins, recombinant proteins, recombinant hormones and vaccines, and comprise the fastest growing sector in the pharmaceutical market with a global revenue of 228 billion USD in 2016 (3, 4). Amongst the various biologic products, protein therapeutics and in particular mAbs are the fastest growing segment, comprising 53% of first-time approvals in the period between 2015 and 2018, having a market value of 115 billion USD in 2018. This market value is expected to reach as high as 300 billion USD by 2025 (5-7). Many biologic products are now nearing their patent expiration date and the end of the market exclusivity period, thereby propelling a surge of generic products and biosimilars. This calls for great attention to be paid towards formulation development, since generic versions are to be delivered at prices much lower than the originator counterparts (4).

Non-ionic surfactants are extensively used in the formulation of protein therapeutics to prevent undesirable outcomes such as surface adsorption and aggregation. These commonly arise during processing steps such as filtration, pumping, agitation, thawing

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and lyophilisation, all of which create interfacial interactions (7, 8). These undesirable effects could result in decreased therapeutic activity and, in a worst case scenario, evoke an immunogenic response (9). Monoclonal antibodies are often administered by the sub-cutaneous route, with therapeutic doses as high as 1-3 mg/kg body weight which need to be formulated into concentrated formulations. This is a particular concern due to the high propensity of mAbs towards aggregation (10). Surfactants play a crucial role in producing stable formulations of these highly concentrated mAb drug products (7). Polysorbate 80 and polysorbate 20 are the most used surfactants in the formulation of mAbs, with almost 70% of marketed products formulated using at least one of the two surfactants (11).

Polysorbates are synthetic fatty acid esters of polyoxyethylene (POE) sorbitan, idealised chemically homogenous polysorbate 80 is an oleic acid (OA) ester. However, the commercially available polysorbate 80 is a mixture of different fatty acid esters. To be used in clinical products the composition must be ≥ 58% OA ester, as stipulated by the United States, European, and Japanese Pharmacopoeias (12). This means that there is scope for the precise composition to vary across different manufacturers and within different grades supplied by the same manufacturer, and still meet the specifications set by the different pharmacopoeias (13, 14). The NOF Corporation supplies a polysorbate 80 with 99% oleic acid, and the super-refined polysorbate 80 provided by Mallinckrodt Baker is subjected to an additional chromatographic purification step allowing the removal of impurities such as formaldehyde and peroxides (15). This leaves the pharmaceutical industry with a choice of polysorbate grades that can potentially be used in the formulation of drug products.

An extensive volume of literature is available on the composition of polysorbate 80 and how impurities such as peroxide radicals and biproducts associated with the auto degradation of the polysorbates could affect the stability and integrity of proteins when used in a formulation (8, 15-20). These studies suggest that the composition of polysorbates could have an impact on, either or both, the physical and chemical stability of protein formulations, and could even cause an immunogenic response in the patients. The exact mechanism behind these effects is not quite clear, although various hypotheses have been suggested in the literature. Further, there is no literature establishing a scientific rationale behind the selection of polysorbate grades to be used in pharmaceutical formulations, and if or how the super refined or purer versions are superior in their functionality when compared to the normal grades. To help begin to understand these issues, this work compares the physical and chemical properties of four commercially available polysorbate grades with different degrees of purity and OA content, and investigates their degradation profiles.

MATERIALS AND METHODS

Materials

Tween 80-LQ-(CQ) (STD), Tween 80 HP-LQ-(MH) (HP) and super refined polysorbate 80-LQ-(MH) (SR) were received as samples from Croda Europe Limited (United Kingdom). Polysorbate 80(HX2) (HX) was sourced from NOF Europe GmbH (Germany). The major constituents of these four grades of polysorbates, as detailed in the manufacturer specifications, are given in Table 1. A Pierce™ quantitative peroxide assay kit was supplied by ThermoFisher Scientific (United Kingdom) and methanol-d_4 was sourced from Sigma-Aldrich (United Kingdom).

Peroxide assay

A peroxide assay was performed on all the, as-received

Table 1 Composition of the different polysorbate grades, as given in the supplier certificate of analysis

CATEGORY	STD	HP	SR	HX
Peroxide content (meq/Kg)	0	0.3	0.2	1.0
Water content (% w/w)	2.9	0.1	0.0	0.0
Oleic acid (%)	80.6	76.4	86.7	99.2
Palmitic acid (%)	4.6	8.1	2.5	0.2
Stearic acid (%)	1.4	1.9	2.6	0.1
Linoleic acid (%)	0	0	0.2	0.2
Ethylene glycol (mg/Kg)	8	0	0	NA

and the samples collected at predetermined time points during stability studies, to quantify the free peroxide radicals present in the samples. Serial dilutions of an aqueous 30% hydrogen peroxide solution were used as reference standards to build a calibration curve. The procedure for the peroxide assay is as follows: initially the working reagent was prepared by mixing reagent A and B from the assay kit at a 1:100 v/v ratio. Then, 20 µL of the sample to be analyzed and 200 µL of working reagent were added to each well in a microplate. The assay reactions were mixed and allowed to incubate for 15-20 minutes followed by measurement of absorbance at 595nm using a plate reader. Three independent experiments were performed, and two replicate measurements were taken for each sample. The concentration of peroxide in the samples was calculated with reference to the calibration curve.

Thermal analysis

Differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA) were used to evaluate the presence of solvent and the degradation profile of the polysorbates. DSC measurements were performed with a Q2000 DSC (TA Instruments, Waters LLC, U.S.A.) at a heating rate of 10°C/min. Calibration for cell constant and enthalpy was performed using indium (T_m = 156.6°C, ΔH_f = 28.71 J/g) according to the manufacturer instructions. Nitrogen was used as a purge gas, with a flow rate of 50 mL/min. The data were collected using TA Advantage software for Q series (version 2.8.394) and analysed using TA Instruments Universal Analysis 2000. TA aluminium pans and lids (Tzero) were used, with a typical sample mass of 8−10 mg. For TGA analysis, samples were heated at 10°C/min in open aluminium pans with a Discovery TGA (TA Instruments, Waters LLC, U.S.A.). Nitrogen was used as a purge gas at a flow rate of 25 mL/min. Data collection and analysis were performed using the TA Instruments Trios software, and percent mass loss or onset temperature were calculated.

NMR studies

Solutions were prepared by adding 200 µL of the sample to 400 µL of methanol-d_4 in NMR tubes. Solution ^{1}H and ^{13}C NMR spectra were recorded at 300K using a Bruker Avance III 500 MHz NMR spectrometer (Bruker, United Kingdom) equipped with a 5 mm QNP cryoprobe (CP QNP 500S2 P/C/N-H-D-05 Z). Data acquisition and processing were performed using TopSpin (version 2.1) software (Bruker UK Limited, the UK). ^{1}H and ^{13}C chemical shifts were calibrated using methanol-d_4 as an internal reference (^{1}H 4.87(1), 3.31(5) PPM, ^{13}C 49.1(7) ppm). NMR spectra of the four grades of polysorbate 80 samples were acquired after deliberate incubation with hydrogen peroxide and used as a reference to analyse the spectra of the samples during stability studies. These reference samples were prepared by adding 20 µL of 30% H_2O_2 to 180 µL of polysorbate and allowed to incubate for 3 hours.

Stability studies

Stability studies were carried out at three temperatures, that is at 5°C, 30°C and 40°C. Samples were placed in air-tight vials and stored in a refrigerator (5°C) or stability chambers maintained at 30°C/65% relative humidity (RH) or 40°C/75% RH. The sample vials were collected at predetermined time points and analyzed for their physical and chemical properties the same day.

Surface tension measurements

Surface tension measurements of the polysorbate samples were acquired via the pendant-drop method using a First Ten Angstroms (FTA 100 series) instrument (FTA Europe, United Kingdom). Aqueous solutions (0.05% v/v) of the samples were prepared and dispensed through a dispenser unit fitted with an 18-gauge needle with circular tip. A series of images of the droplets while dispensing the sample were collected and processed through the inbuilt Fta32Video 2.0 software. Surface tension was then automatically computed from selected images of the droplets using the Laplace-Young principle.

RESULTS AND DISCUSSION

The as supplied polysorbates were first assessed for their water content. Figure 1 shows the DSC traces

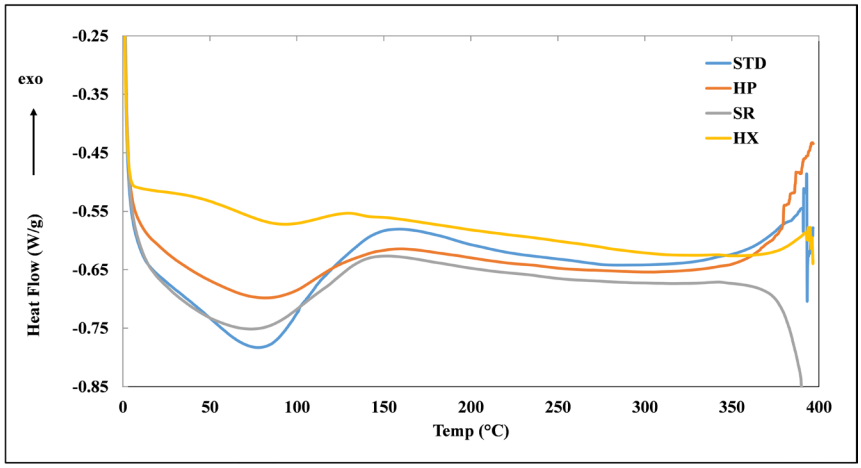

Figure 1 DSC of the polysorbate 80 samples.

of the fresh polysorbate samples. These all present an endothermic event in the temperature range between 60°C and 100°C, which coincides with the temperatures where weight loss is observed in TGA (Figure 2). This is due to the evaporation of water from the samples, and could result from either or both residual water in the formulation or atmospheric moisture taken up during storage. The percentage weight loss in the TGA of the STD grade is relatively higher than for the other grades, which is in accordance with manufacturer specifications (see Table 1). The STD grade is reported to contain 2.9% w/w of residual water whereas the other materials are reported to contain no residual water except the HP grade, which contains 0.1% w/w of residual water. The endothermic and weight loss events observed in the latter grades possibly arise because of atmospheric moisture being adsorbed by the samples during handling. It is evident from the DSC and TGA that all the four grades underwent thermal degradation in the temperature range of 380-400°C, as reported in the literature (18).

Surface tension is a key functional property of polysorbates that underpins their role as a surfactant in protein formulations. This study analyzed the surface tension of the, as-received samples and, samples isolated at different time points during the stability studies. It was observed that the surface tension of all the four grades remained unaffected after storage at all three temperature conditions explored (Table 2). Thus

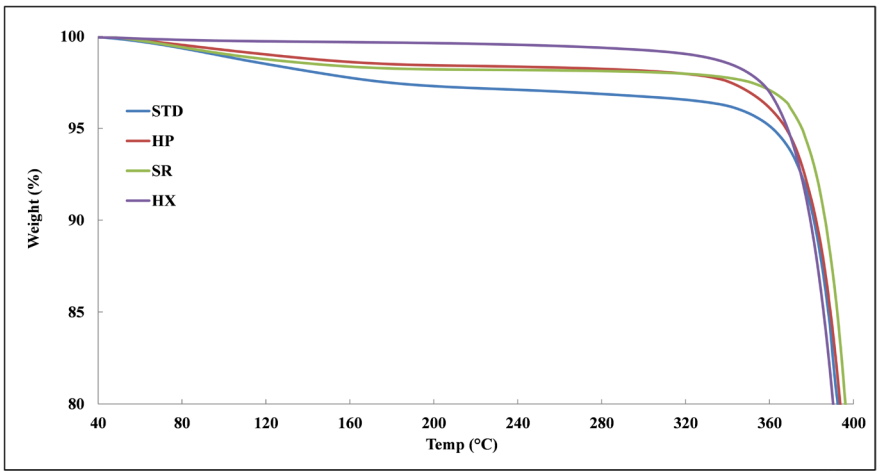

Figure 2 TGA of the polysorbate 80 samples.

it appears that storage at elevated temperatures should not diminish the ability of the polysorbates to stabilize the active ingredients in proteins.

Table 2 Surface tension (mN/m) of 0.5% v/v aqueous solutions of the as-received polysorbate materials and samples stored at 5°C, 30°C and 40°C for two months

GRADE	AS RECEIVED	5°C	30°C	40°C
STD	44.96 ± 0.09	44.35 ± 0.16	44.65 ± 0.25	44.95 ± 0.15
HP	45.30 ± 0.10	45.85 ± 0.32	45.56 ± 0.18	45.32 ± 0.28
SR	46.60 ± 0.06	46.65 ± 0.07	46.80 ± 0.09	46.40 ± 0.38
HX	46.90 ± 0.24	46.70 ± 0.15	46.45 ± 0.08	46.88 ± 0.19

The detrimental effect of residual peroxides on drug molecules has been extensively reported (8, 21). Peroxides present in the polysorbates could cause oxidative damage to proteins by attacking amino acid moieties sensitive to oxidation, such as methionine and tryptophan (22, 23). Polysorbates themselves, undergo autoxidative degradation resulting in the formation of reactive peroxides and aldehydes which could further cause the degradation of proteins (24, 25). Hence it is important to measure the peroxide content in the polysorbates in order to determine appropriate storage and transport conditions, as any degradation of the polysorbates alone will inadvertently be translated into more severe effects in the final protein formulations. In this study we measured the peroxide content using a Pierce quantitative peroxide assay kit. It was observed that the HP grade material contained the smallest amount of peroxides, followed by SR, STD and HX (D1 in Figure 3). The samples stored at 5°C showed a gradual but negligible increase in the peroxide content, reaching no higher than 0.8 mEq in any of the samples after 14 weeks (Figure 3a). Samples stored at 30°C and 40°C showed a relatively steep rise in peroxide content, reaching peak levels at week 8 and week 4 respectively, followed by a drop in the peroxide levels upon further incubation (Figures 3b and 3c). The rise and fall in peroxide levels arises because of the propagation followed by termination of the radical chain autooxidation reaction of the ethylene oxide subunits in polysorbate 80; this is a spontaneous reaction that increases with the rate of temperature,

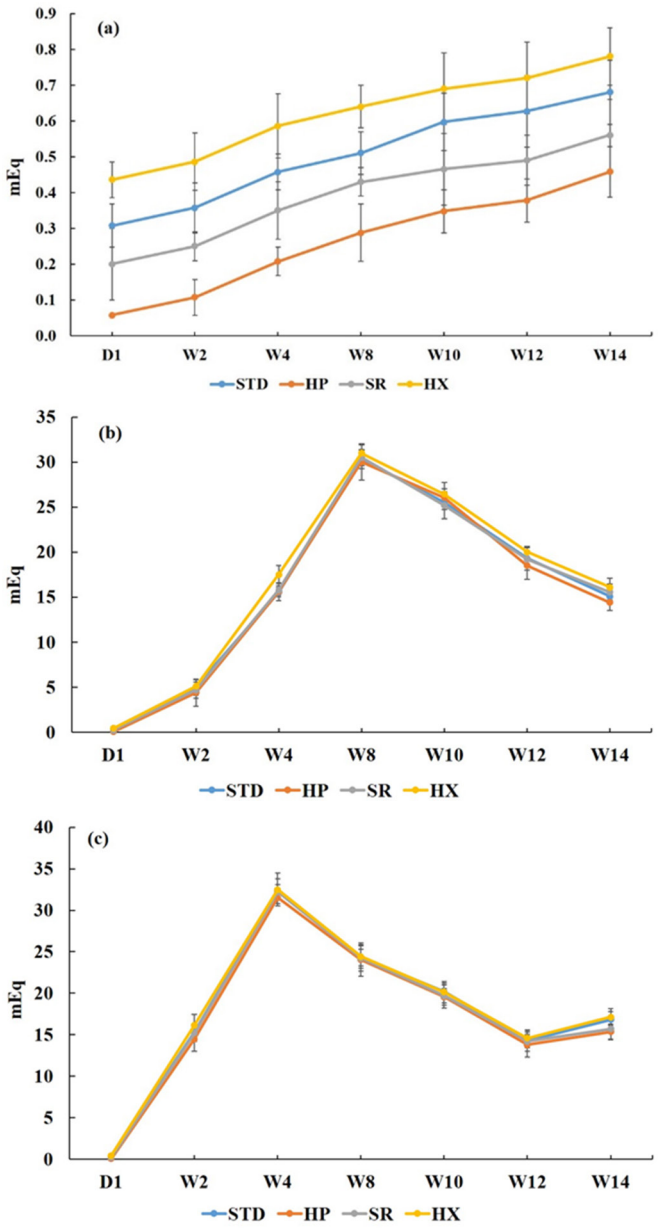

Figure 3 Peroxide content in the as-received samples (D1) of polysorbate 80 and samples stored at (a) 5°C, (b) 30°C, and (c) 40°C

and in the presence of atmospheric air and chemical initiators (Figure 4) (8, 26).

There is a noticeable difference in the peroxide content of the, as received samples, (Figure 3a), but this difference becomes negligible after storage at higher

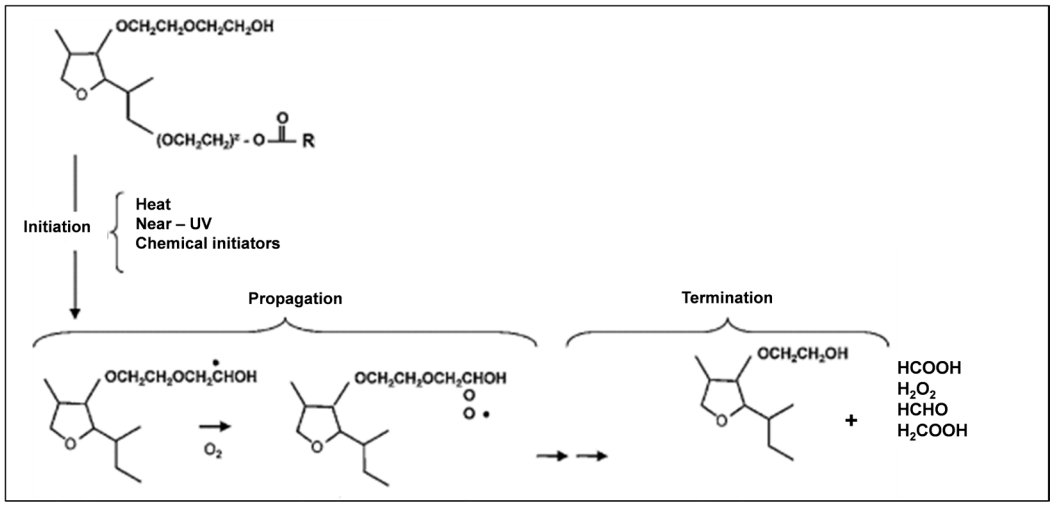

Figure 4 Schematic representation of the auto-oxidation process in the ethylene oxide subunits of polysorbate 80 (R= C17H33) (12).

temperatures for extended periods of time. All the given grades of polysorbate 80 show no difference in their impact and could have equally detrimental effects when used in formulations that are stored or handled at higher temperatures. However, there could be a difference in the impact of polysorbate grade on the formulations that are developed below 5°C. Overall, based on the results of this study, it is suggested that it is best practice to store polysorbate 80 at 5°C as it will result in least degradation. It is expected that storage in cool conditions and away from light would minimize the degradation. All four grades of polysorbate (ranging from standard to high purity/super refined) showed very similar levels of peroxides after storage at higher temperatures, contrary to the expectation that super refined grades might show lower peroxide levels. Of course, peroxide levels cannot be considered alone as the determinant of purity and other possible by-products of auto-oxidation such as formaldehyde and formic acid will also be important. However, these findings suggest that in terms of peroxide levels there is no advantage in using the higher purity grades of polysorbate, the standard grade performs very similarly in this assay.

The stability data clearly show that peroxide content tends to increase with storage time. NMR was thus used to examine the effect of peroxide formation on the different grades of polysorbate. Initially all four grades of polysorbate 80 were deliberately incubated with hydrogen peroxide to identify the structural differences that arise from the presence of peroxide. The ^{13}C NMR spectra of the H_2O_2 treated samples showed downfield shifts of the peaks and splitting in the region at 83 PPM (Figure 5). This is possibly because of the interaction of peroxide with the secondary alcohol and ether functional groups (Figure 4).

The results from ^{13}C NMR analysis of the samples subjected to stability studies show changes in the spectra similar to those caused by deliberate incubation with hydrogen peroxide. All four grades of the samples stored at 5°C, even at the very first time point of two weeks, showed signs of peroxide induced degradation with a split peak at 83 PPM (Figure 6).

As the peroxide levels quantified at 30°C and 40°C were far greater than at 5°C, it was expected that all four grades would show similar degradation profiles in their NMR spectra. Samples of STD stored at all three temperatures for four weeks showed similar peak splitting and downward shifts of the signals (Figure 7). Similar spectra were observed for the other greades. These data are fully consistent with the earlier peroxide assay results (see Supplementary Information, Figures S1 and S2).

The results from this study suggest that the STD grade

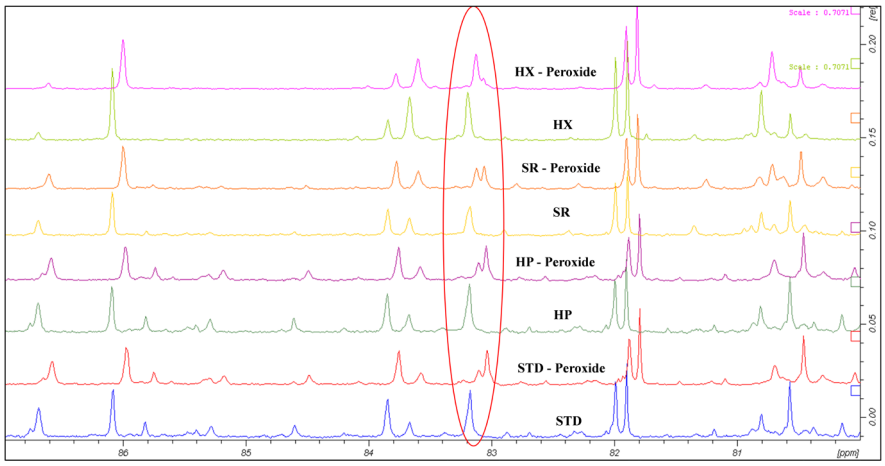

Figure 5 ^{13}C NMR spectra of the as received polysorbate 80 samples, and of samples after incubation with hydrogen peroxide.

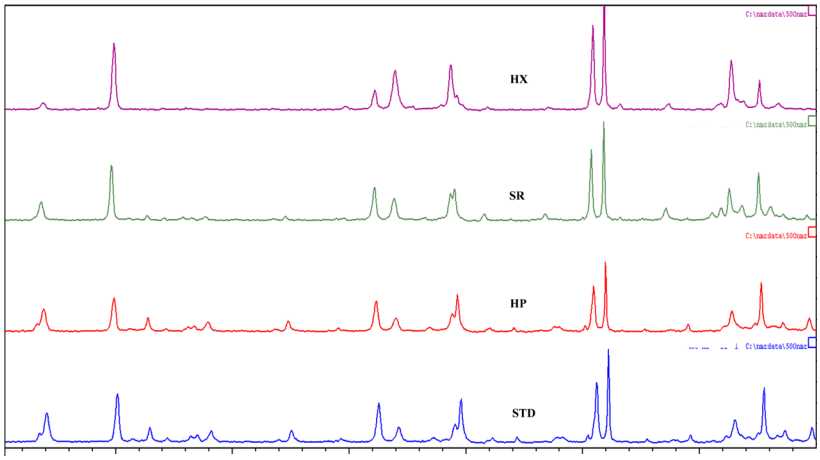

Figure 6 ^{13}C NMR spectra of the polysorbate 80 samples after two weeks stored at 5°C.

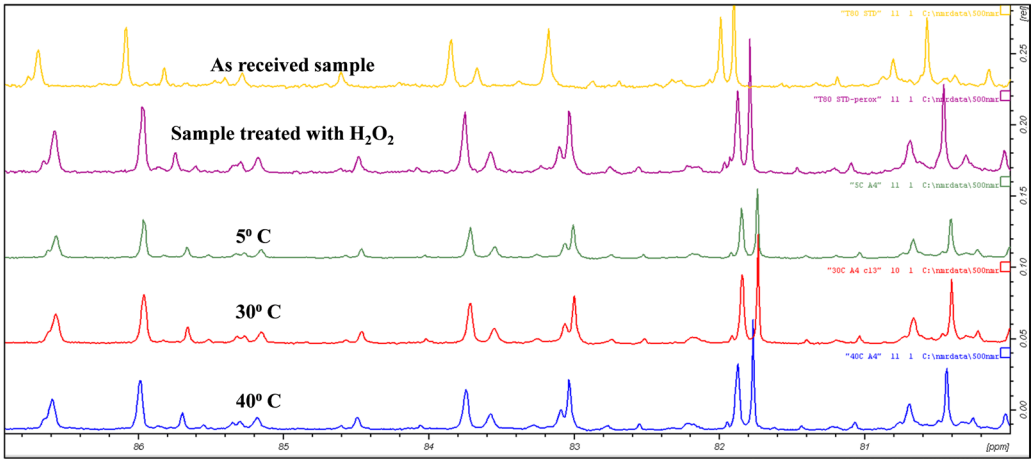

Figure 7 ^{13}C NMR spectra of the STD samples after four weeks of storage at 5°C, 30°C and 40°C.

has relatively more water content than the others (though still within the limits set by the pharmacopoeias) but performs identically to the higher-purity polysorbates when it comes to surface tension measurements and peroxide induced degradation. Hence, it is possible to speculate that the STD grade could be an economic choice to use in generic formulations and make biotherapeutics more affordable, particularly in low- and middle-income countries. However, further assays are needed to confirm this, and this study will be continued in the future to explore the physico-chemical stabilities of protein formulations prepared using different polysorbate grades.

CONCLUSION

This study suggests that, while there are variations in water content, differences in the composition of polysorbate 80 grades have minimal impact in terms of the surface tension, peroxide content, and degradation profile upon storage. The authors cannot categorically suggest that lower-purity grades are just as effective as the higher-purity grades based on the current findings, but it is believed this information could be of benefit to generic drug manufacturers by reducing the cost of biosimilars thereby widening access to patients. The authors are currently exploring issues around the production of other reactive intermediates during storage and preparing formulations with model proteins to validate these promising findings.

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