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An investigation into the effect of polysorbate 80 grade in biotherapeutic formulations

Sebastian J Gurgul[a], Gayathri Kollamaram[a], Cheng Zhang[b], Gareth R Williams[*a]

[a]UCL School of Pharmacy, University College London, 29-39 Brunswick Square, London WC1N 1AX, UK
[b]UCL Department of Biochemical Engineering, University College London, Bernard Katz Building, Gower Street, London, WC1E 6BT, UK

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ABSTRACT

Polysorbate 80 is a commonly used excipient in the food, cosmetics and pharmaceutical industries. In pharmaceutical formulations, the only requirement in the British Pharmacopeia and US Pharmacopeia is for the composition to be ≥ 58% oleic acid ester. However, there is a tendency for companies to prefer more refined materials. This study focused on whether this is necessary, and whether less refined materials could be used instead. To this end, we probed the chemical stability of four different polysorbate 80 samples and then explored the stability of the active ingredients in formulations of biologics containing the different polysorbate 80s. No chemical degradation could be identified by nuclear magnetic resonance or IR spectroscopy over storage for 44 weeks in the fridge or under accelerated aging conditions (30 °C/65% RH; 40 °C/75% RH). All the samples contain some water, with the water content greater than stated in the supplier datasheet (possibly because of adsorption upon storage at the supplier sites). No change in water content is noted upon the storage of polysorbate 80. With the super-refined grades, autoxidation can be seen to occur upon storage, with hydrogen peroxide, aldehydes and ketones produced. This is less noticeable with the less refined grades, likely because these already contain autoxidation products and there is an equilibrium in place. Increasing the temperature and humidity of the aging conditions causes an increase in the rate of autoxidation. We find that the grade of polysorbate 80 used as excipient does not affect the stability of biologic formulations, with no differences observed in terms of activity after combination with any of the polysorbates. It hence appears that less-refined polysorbates could be used in place of the more-expensive super-refined materials, reducing the cost and providing wider access to medicines.

KEY WORDS: Polysorbate, stability studies, biotherapeutics, excipients

INTRODUCTION

Polysorbates (otherwise known as Tweens) are oily liquids originating from ethoxylated sorbitan (a derivative of sorbitol) and esterified with fatty acids. They are water-soluble non-ionic surfactants which are divided into several groups depending on their composition (1, 2). The main materials used are termed polysorbate 80, 60, 40 and 20 (3, 4). The key difference between them is the type of major fatty

*Corresponding address: UCL School of Pharmacy, University College London, 29-39 Brunswick Square, London WC1N 1AX, UK, E-Mail: g.williams@ucl.ac.uk

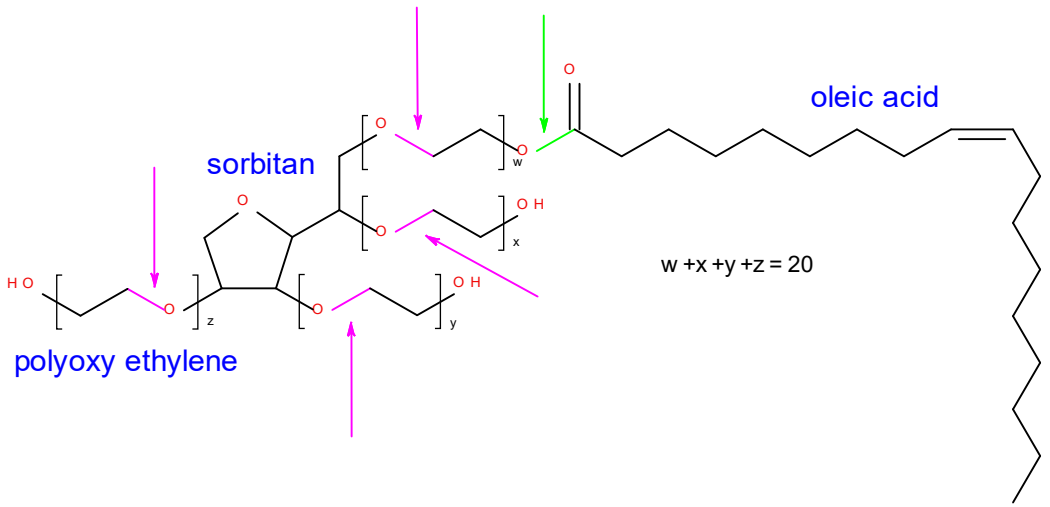

Figure 1 Structure of polysorbate 80 with oxidation/hydrolysis sites marked: POE chain (→) and fatty acid moiety (→).

acid. In polysorbate 20 this is sorbitan monolaurate, whereas in polysorbate 80 it is sorbitan monooleate. The number on the end represents the total amount of oxyethylene groups. Polysorbate 80 is composed of sorbitan and polyoxyethylene (POE) together with an oleic acid chain (above 58%) (Figure 1) (5). Polysorbates have high hydrophile-lipophile balance (HLB) numbers (6) and low critical micelle concentration (7, 8) which makes them efficient surfactants even at low concentration. They are commonly used in food products and the preparation of cosmetics due to their low toxicity, biocompatibility, and potent emulsifying, wetting and stabilizing properties (9). Polysorbate 80 is also widely used in the pharmaceutical industry (10). It is an excipient in many medications including for chemotherapy (e.g., Taxotere®, Lemntrada, Avastin) and autoimmune diseases (e.g., Remicade®), and also in the Janssen SARS-CoV-2 vaccine. It can prevent protein aggregation, increase colloidal stability (11), decrease filter adsorption (12) and reduce the surface turnover of protein (13). Polysorbates are primarily used to stop surface adsorption, to prevent protein aggregation and also to aid solubility. Although they have been used widely for more than 70 years, most of the previous research and knowledge in monographs has focused on formulations of small molecule drugs.

There is very little guidance available in terms of the purity of the polysorbate required for use in pharmaceutical formulations. The British Pharmacopoeia and US Pharmacopeia (14, 15) specifications for polysorbate 80 composition are shown in the Supplementary Information, Table S1 (5). For polysorbate 80 to be used in clinical products the most important specification is that the composition must be ≥ 58% oleic acid ester (5). There is a tendency for manufacturers to use high-purity grades of polysorbate 80, but it is unclear if this is in fact necessary. If lower purity grades could be used, there could be distinct improvements in patient access to biosimilars. It is also important to understand the degradation of the polysorbates. As shown in Figure 1 polysorbate can undergo autoxidation on the POE chain (→) or hydrolysis at the fatty acid moiety (→) producing aldehydes, ketones, acids, peroxides, fatty acid esters and other degradation produces (Figure S1). These could have an impact on either, or both, physical and chemical stability of protein formulations (9, 16).

In this work four different polysorbate 80 materials were investigated (shown in Table 1). These are identified as CQ (standard polysorbate 80 from Croda), MH (refined polysorbate 80 from Croda), Super (super refined polysorbate 80 from Croda) and NOF (super refined polysorbate 80 from NOF Corporation). The composition varies between the grades (CQ, MH, Super and NOF). As seen in Table 1, not only is the

water content different (0 - 2.9% w/w) but also the amount of hydrogen peroxide present (0 – 1 meq/kg). H_2O_2 is left after manufacturing but is a strong oxidant.

Table 1 Composition of the different grades of poy80, taken from the supplier specification documents

COMPONENT	CQ	MH	SUPER	NOF
Water (% w/w)	2.9	0.1	0.0	0.0
H_2O_2 (meg/kg)	0	0.3	0.2	1.0
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 (meg/kg)	8	0	0	n/a

Some studies in the literature have looked into how different grades of polysorbate influence active pharmaceutical ingredient (API) and formulation stability. One showed no distinguishable differences in polysorbate 80 functionality (assessed through measurements of protein aggregation and particle formation during mechanical stress) between two different grades (J. T. Baker-Avantor which is compliant with multi-compendial pharmacopoeia and Croda, compliant with the Chinese Pharmacopeia) (11). However, with an increasing degree of polysorbate 80 hydrolysis, greater numbers of subvisible particles were measured. Additionally, higher levels of polysorbate 80 degradants were found to destabilize IgG monoclonal antibodies (11).

In another study IgG1 antibodies were formulated with various grades of polysorbate. Aggregation was found to be minimized with super-refined polysorbate 80 when compared with NF and ultra-pure grades (17). The indication is that optimal properties are obtained with intermediate grades of polysorbate 80, rather than very high or low purity materials. However, Larson *et al.* found that monoclonal antibody loss was comparable in formulations without polysorbate 80 to those with esterase hydrolyzed polysorbate 80. Monomer loss for oxidized polysorbate 80 was similar to that of nondegraded polysorbate 80 (18).

Another study looked at the stability of four different polysorbate 80 grades: Tween 80-LQ-(CQ), Tween 80 HP-LQ-(MH) and super refined polysorbate 80-LQ-(MH) from Croda, and polysorbate 80(HX2) from NOF. The authors determined that there are variations in water content but no difference in peroxide content, surface tension and degradation profile upon storage. However, it has to be mentioned that this research covered only a period of fourteen weeks and the polysorbate 80 samples were considered without the presence of a protein (19).

This study explores in more detail the effect of polysorbate grade on the degradation of both the pure excipient and protein formulations. The first part of the study is focused on probing the stability of different polysorbate 80 materials (Table 1). The second part examines the influence of the polysorbate on the stability of three different biologic APIs: (the enzyme alkaline phosphatase (ALP), monoclonal antibody Bevacizumab (Beva) and a fragment antibody (Fab).

MATERIALS

All materials used in this project are listed in Table S2 in the Supplementary Information. The Fab protein was expressed and purified based on a previously reported protocol (20, 21).

METHODS

Sample preparation

Stability studies

Three different grades of polysorbate 80 from Croda (CQ (standard), MH (refined), and Super (super refined)) and one from NOF (super refined HX2, non-GBP) were used in this project (see Table 1). Once the polysorbate 80 samples were received from the supplier the content was divided between amber glass vials and capped. Each vial contained 1.5-2 mL of polysorbate 80. The transfer was done in a nitrogen-filled glove bag to prevent the samples interacting with air and moisture. The glove bag was filled with

nitrogen gas and emptied 5 times before the vials were opened. The polysorbate 80-loaded vials were stored in three different conditions: in the fridge (5-8 °C, approximately 25% relative humidity (RH)), and under two sets of accelerated aging conditions (30 °C, 65% RH and 40 °C, 75% RH). The latter experiments were performed in LEEC SFC2C RH humidity cabinets. A vial from each set was recovered and measurements performed on day 0, week 1, week 2, week 3, week 4 and then monthly until month 11.

Formulations

Formulations were prepared in deionised (DI) water as shown in Table 2. Those of Beva and Fab were prepared to match the composition of the anti-cancer medicine Avastin®. Formulation of ALP was performed similarly but omitting ethylenediaminetetraacetic acid (EDTA) and succinic acid. This is because ALP contains Zn^{2+} and Mg^{2+} in its structure and EDTA can chelate these ions. Succinic acid also interacts with the metal ions, causing precipitation and deactivating the ALP. As a result, if these excipients were included, within 24 hours the ALP formulations become cloudy and no enzyme activity could be detected.

Analytical techniques

Proton and carbon NMR

200 µL of polysorbate 80 and 400 µL of 4d-methanol were mixed in a vial then transferred into an NMR tube. A Bruker Avance 400 MHz NMR spectrometer equipped with a broadband and selective (^{1}H and ^{13}C) inverse probe was used for measurement.

FTIR

A droplet of each of the polysorbate formulations was measured on a Perkin-Elmer Spectrum 100 instrument, at a resolution 4 cm^{-1} and from 4000 to 550 cm^{-1}. 8 scans per sample were recorded.

Table 2 Full compositions of the biologic formulations prepared with CQ, MH, Super and NOF. Quantities are given per 1 mL of DI water. Control A5, B5 and C5 – formulations containing all compounds of the formulation without the polysorbate 80. Controls A6, B6 and C6 – formulations contained API in water only.

FORMULATION	API 10 mg	POLYSORBATE 80 0.2 mg	EDTA 0.05 mg	SUCCINIC ACID 2.63 mg	SUCROSE 85 mg
A1	ALP	CQ	-	-	√
A2	ALP	MH	-	-	√
A3	ALP	Super	-	-	√
A4	ALP	NOF	-	-	√
A5	ALP	-	-	-	√
A6	ALP	-	-	-	-
B1	Beva	CQ	√	√	√
B2	Beva	MH	√	√	√
B3	Beva	Super	√	√	√
B4	Beva	NOF	√	√	√
B5	Beva	-	√	√	√
B6	Beva	-	-	-	-
C1	Fab	CQ	√	√	√
C2	Fab	MH	√	√	√
C3	Fab	Super	√	√	√
C4	Fab	NOF	√	√	√
C5	Fab	-	√	√	√
C6	Fab	-	-	-	-

Thermogravimetric analysis (TGA)

TGA was performed on a TA Instruments Discovery instrument to determine the amount of volatile compounds (mainly water) in each sample. Samples were heated from 40 to 150 °C with a heat ramp of 10 °C/min under a 25 mL/min nitrogen flow rate. The percentage mass loss at 130 °C was calculated and taken to represent the amount of volatile compounds in the sample. At this temperature polysorbate 80 is not degraded (this does not take place until around 200 °C) and the mass loss will largely be a result of water loss (6). All measurements were done in triplicate.

Karl Fischer titration (KF)

The amount of water in each sample was also calculated using an Auto Karl Fischer Titri Meter (Max Electronics ME883) instrument. All measurements were performed in triplicate. The procedure is shown in Scheme S1.

Peroxide/aldehyde/formate assays

These assays were performed using commercial kits and following the manufacturer instructions (peroxide oxide assay (22), aldehyde assay (23) and formate assay (24)).

ALP activity assay

The samples recovered at each timepoint were diluted to achieve the desired ALP concentration (1 mg/mL). 80 µL of diluted sample and 20 µL of 2-amino-2-methyl-1-propanol (1.5 M) were placed in the wells of a clear 96-well plate. 100 µL of para-nitrophenylphosphate (p-NPP) liquid substrate solution was added to each well. The plate was incubated at room temperature for 5 minutes on a plate shaker at 150 RPM, then 20 µL of NaOH (1 M) was added to stop the reaction. The absorbance was measured at 405 nm on a SpectraMax M2e plate reader (Molecular Devices). A pre-determined calibration curve was used to calculate the activity of the ALP. All measurements were performed in triplicate.

SDS-PAGE

Formulations (containing ALP or Beva) were diluted to achieve the desired API concentration (0.1 mg/mL). 20 µL of diluted formulation, 3 µL of NuPAGE sample reducing agent and 7 µL of NuPAGE LDS sample buffer were mixed with a pipette then placed into a water bath (92 °C) for 5 minutes. A XCell SureLock™ Mini-Cell from ThermoFisher was used for running electrophoresis. 5 µL of protein ladder and 20 µL of each sample were added to individual wells in the gel cassette (NuPAGE 4-12% Bis-Tris gel). The gel was pre-washed with deionized (DI) water in advance and locked inside the tank. NuPAGE MOPS SDS running buffer was added into the tank to a level just above the entrance to the wells. The following parameters were set for electrophoresis: 180 V, 120 mA, 150 W, 55 minutes. After electrophoresis was finished, the gel was removed from the cassette, stained in InstantBlue® Coomassie Protein Stain for 1 hour and then de-stained in DI water overnight. The gel was imagined using a UVP ChemStudio gel and blot imager (Analytik Jena). The data are presented as negative images to clearly show the bands.

Surface plasmon resonance (SPR)

Analysis was performed based on a previously reported procedure (25). Vascular endothelial growth factor (VEGF; the target ligand for Beva) was immobilized on gold chip (Cytiva CM3; BR100541) through a modified dextrin chain at an immobilization level of 61 RU. This was undertaken using standard carbodiimide-mediated coupling (NHS/EDC, 50/50) and ethanolamine (pH 8.5). A series of diluted samples (in HBS-EP running buffer) containing Beva (ranging from 200 mg/mL to 1.5625 mg/mL) was eluted over the VEGF at 25 °C and a flow rate of 30 µL/min, with an association time of 180 seconds and dissociation time of 1200 seconds. Chip regeneration was performed by exposure to 10.0 mM glycine−HCl (pH 2.0) for 1200 seconds. Double-referencing was performed to account for bulk effects caused by changes in the buffer composition or non-specific binding (26). Data were evaluated with the BIA evaluation software (version 2.1) and the best fit (lowest χ^2) was obtained using a 1:1 binding model. The

sensorgram was fitted globally over the association and dissociation phases. This enables us to determine how well the Beva attaches to VEGF on the chip (k_a - rate of adsorption) and how easy it was to remove (k_d - rate of desorption). A binding affinity ($k_D = k_a/k_d$) value was then calculated. The smaller the k_D value is, the greater the binding affinity of the ligand for its target.

Size exclusion chromatography (SEC)

Upon formulation, the Fab stock was thawed from -80 °C, filtered through 0.02 μm syringe filters (Whatman), buffer exchanged to Milli-Q water and concentrated to the desired concentration using 30 kDa cut-off Vivaspin columns (VWR International). The protein stock was then mixed with buffer stock (200 mM sodium phosphate at pH 7) to achieve the final working concentration (1 mg/mL).

SEC-HPLC was used to characterise monomer retention throughout the sample incubation. Experiments were performed at 1 mL/min with a mobile phase of 200 mM sodium phosphate at pH 7 on an Agilent 1200 HPLC system. For each sampling point, 50 μL sample was transferred into a HPLC vial insert, and triplicates of 5 μL were injected into an Agilent Zorbax Bio Series GF-250 column to obtain a monomer peak at around 2.6-2.8 min in a 4.5 min cycle time. Calibration curves were established using pure monomer samples with a range of known concentrations. The monomer concentrations in the samples were determined based on their peak area and calibration curves. Here, the concentration of Fab determined reflects only the non-aggregated molecules.

Nanodrop

A Synergy HTX multimode reader was used to calculate the concentration of Fab in the formulations. A drop of each formulation (3 μL) was placed on the measurement window using a pipette. A pre-set program was used to quantify protein concentration. These values include all protein molecules, both monomeric and aggregated. All measurements were done in duplicate.

RESULTS AND DISCUSSION

Polysorbate 80 stability studies

Proton and carbon NMR

Proton NMR

Before the samples were analysed, each polysorbate 80 sample was spiked with hydrogen peroxide to mimic autoxidation. The proton NMR spectrum for spiked polysorbate 80 was compared to the spectrum of a clean sample (Figure S2 a) to see where changes occur. Two regions were identified to change after oxidation: 10.75-10 PPM and 5-4.3 PPM. NMR spectra were then taken at each timepoint. The features in each were found to be very similar. In all the spectra gathered, there are no marked changes in the region 10.9-10 PPM (Figure S3), suggesting that no degradation has occurred under any of the conditions explored.
Exemplar low-ppm spectra from week 0 (day 0) and the final week of the experiment (week 44) together with the spiked profiles are shown in Figure S4. While there are some peak shifts visible, none of the NMRs resemble the peroxide-spiked NMR in the 5-4.3 PPM region. As no significant changes can be observed between day 1 and week 44 for all samples/conditions, it appears that all the polysorbate 80s are stable in terms of their proton NMR properties.

Carbon NMR

The ^{13}C NMR spectra for H_2O_2 spiked polysorbate 80 was compared to NMR of an untreated sample (Figure S2 b) to see where changes occur after oxidation. Three key regions were identified: 80.8-80.2 PPM (Figure S5), 49-47 PPM (Figure S6) and 30-28.5 PPM (Figure S7). All three regions were carefully analysed in the spectra of the aged samples, but no differences between week 0 and week 44 were identified. None of the spectra resembles that of spiked polysorbate 80.

A closer look at the ^{13}C NMR spectra using the DEPT135 technique was thus applied. In this particular experiment, CH and CH_3 groups give a positive peak while CH_2 gives a negative peak, as illustrated in Figure S2 c. If autoxidation had occurred on any of the

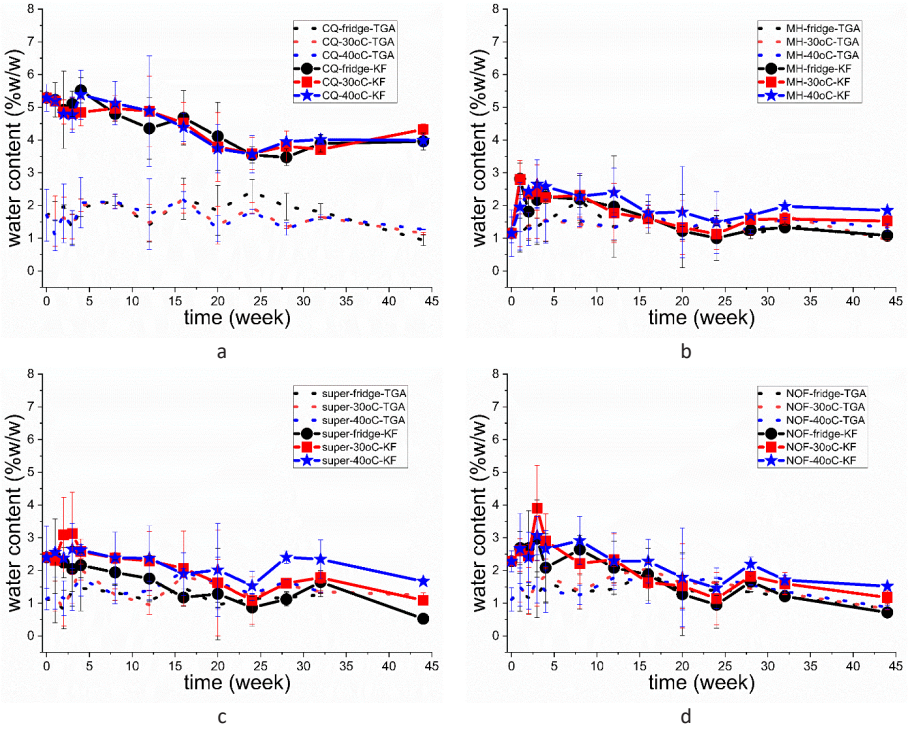

Figure 2 Water content calculated using TGA and KF (a) CQ; (b) MH; (c) Super; and (d) NOF (mean ± S.D; n=3).

chains, there should be a visible shift from a positive to a negative peak (or vice versa) indicating that the chain was broken. After careful analysis (Figure S8) it was clear that none of the spectra showed a visible change (flip) between positive and negative peaks. There are some changes in the spectra (Figures S8 and S9), but these amount only to very small changes in peak position or shape which are not thought to be significant. These data, in combination with the ^{1}H NMR, suggesting that all the polysorbate 80s are stable upon storage.

FTIR

As for the NMR experiments, a comparison between fresh and hydrogen peroxide spiked samples was first performed (Figure S10). Two regions were identified as potential sites where changes in the spectra could be observed: 3700 – 2500 cm^{-1} (O-H stretching) and 1800-1550 cm^{-1} (C=O stretching). Marked changes can be noted here between the fresh and spiked materials. However, no distinct changes were noted in any of the spectra after the aging experiments, which remain virtually identical between the fresh sample and all aging timepoints and conditions explored. Exemplar spectra from week 0 (day 0) and the final week of the experiment (week 44) together with the spiked profiles are shown in Figure S11 and Figure S12.

TGA and Karl Fischer titration

The amounts of water calculated using TGA (mass loss at 130 °C) and Karl Fischer titration are shown in Figure 2. As would be expected, the water content determined is almost the same when measured by both TGA or the KF method in most cases. The exception is the standard polysorbate 80 (CQ). In this case the KF analysis gives a water content almost twice the amount which is seen in TGA. In KF titration the water from polysorbate 80 is completely dissolved (polysorbate 80 is soluble in methanol) and all the water can be measured because of the selective and stoichiometric reaction between the H_2O in the sample and the iodine in the reagent. This might not be the case when using TGA to determine the water content, where some water could remain trapped in the polysorbate 80

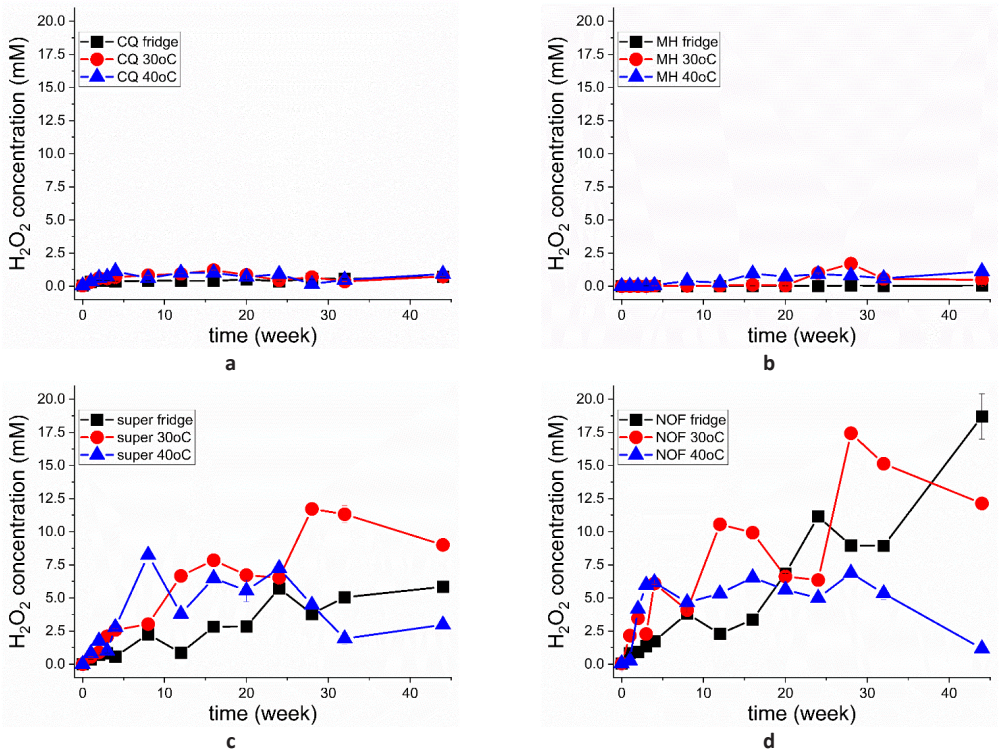

Figure 3 Hydrogen peroxide concentration in (a) CQ; (b) MH; (c) Super; and (d) NOF as a function of time (mean ± S.D; n=3).

matrix. For example, when water content was analysed by TGA and KF in Eudragit RLPO it was found that there was a difference in the water content determined (27).

In the manufacturer specifications (Table 1) the indication is that the water content is very low (CQ 2.9% w/w, MH 0.1% w/w, Super 0.0% w/w and NOF 0.0% w/w). However, the TGA and KF analysis show that the water content is higher (KF: CQ 5.3% w/w, MH 1.2% w/w, Super 2.4% w/w and NOF 2.3% w/w). This could be due to storage time, since the polysorbate 80 used in this project was not sourced immediately after manufacturing. Generally the water content does not change within the 44 weeks period, and in this regard all the samples appear to be stable.

Peroxide assay

To calculate the peroxide amount in the samples a calibration curve was first constructed (Figure S13 a). The peroxide concentration was calculated for all samples and is displayed in Figure 3. The data show that the less refined samples tend to have less peroxide and the concentration does not change over 44 weeks of storage under any conditions. In the refined samples the concentration of peroxide increases with time, and an increase in temperature speeds up this process. Thus, it can be hypothesized that the less refined samples (which contain residues of the autoxidation products) are at an equilibrium and further autoxidation to give H_2O_2 is prevented. In contrast, the more refined samples undergo some extent of oxidation to yield H_2O_2 during storage.

Aldehyde assay

An aldehyde calibration curve was first prepared and is shown in Figure S13 b. Experimental data are presented in Figure 4. It is clear that the less refined samples (CQ and MH) have more aldehyde at the beginning of the study. In all cases it seems that the tendency is for the aldehyde level to increase with time, reaching around 20 - 34 mM. This is least notable for the less refined

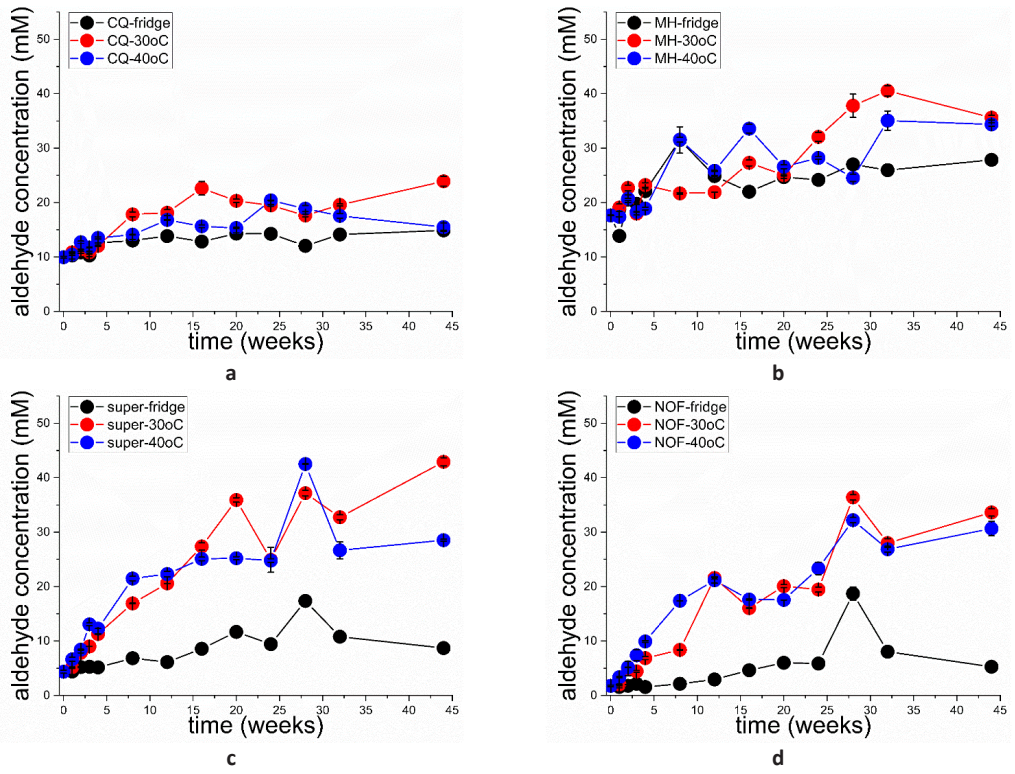

Figure 4 Aldehyde concentration in (a) CQ; (b) MH; (c) Super; and (d) samples as a function of time NOF (mean ± S.D; n=2).

sample (CQ), and most significant for the high-purity polysorbate 80 samples. Storage conditions also seem to have a marked impact on aldehyde production. In fridge storage, the amount of aldehyde produced is the smallest, while with an increase of the temperature the amount of aldehyde rises more rapidly with time.

Formate assay

A calibration curve for the formate assay is shown in Figure S13 c. The change in formate concentration in the polysorbate 80 samples upon storage is depicted in Figure 5. All the grades of polysorbate 80 contain a very low amount of formate at week 0. With time the concentration increases, and the less refined samples (CQ and MH) produce less formate (up to 1.75 mg/mL) than the refined samples (Super and NOF; around 4 and 2.5 mg/mL respectively). Again, storage temperature has a notable influence, and in accelerated conditions at 40 °C the autoxidation of polysorbate 80 is sped up and more formate generated (up to 4 mg/mL). Storage in the fridge seems to inhibit that process, and samples stored at 30 °C show intermediate properties.

Negligible changes in formate amount are seen in the CQ grade. The other polysorbate 80 materials display much greater changes across 44 weeks, reaching formate concentrations of 54 mM (MH), 115 mM (Super) and 1712 mM (NOF). Figure 5 clearly indicates that storing polysorbate 80 in the fridge prevents formation of formate. Overall, it can be seen that autoxidation is less extensive with the less refined samples, while more byproducts are seen to emerge when using a more refined polysorbate 80 material. Storage in the fridge in general helps to slow these processes.

Polysorbate 80 influence on biotherapeutic formulations

Alkaline phosphatase

The results of ALP activity assays are given in Figure 6, with activity calculated based on the calibration curve in Figure S13 d.

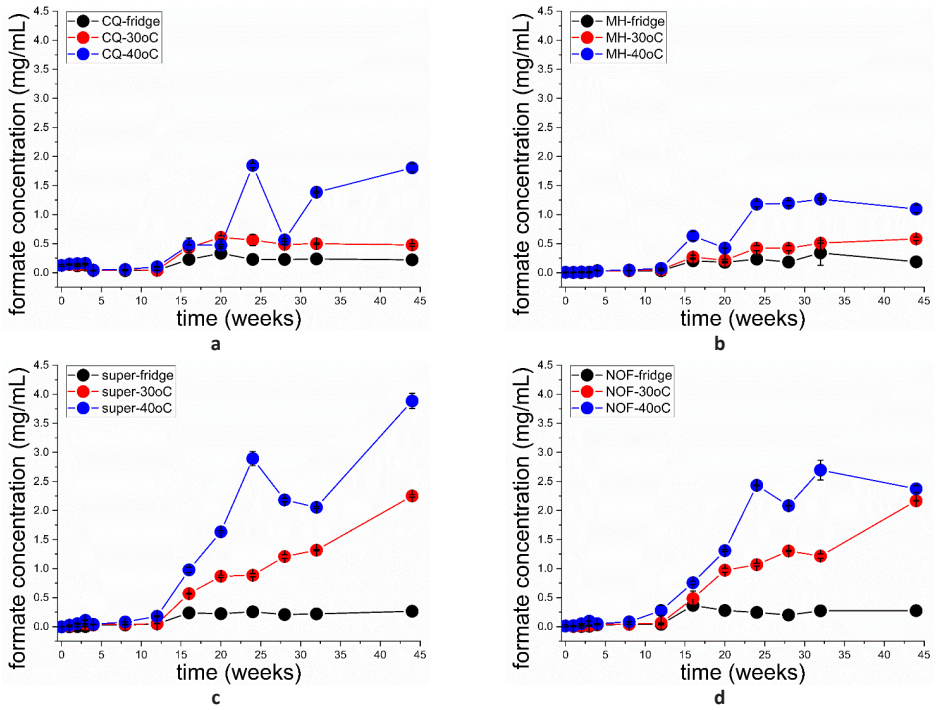

Figure 5 Formate concentration in (a) CQ; (b) MH; (c) Super; and (d) NOF as a function of time (mean ± S.D; n=2).

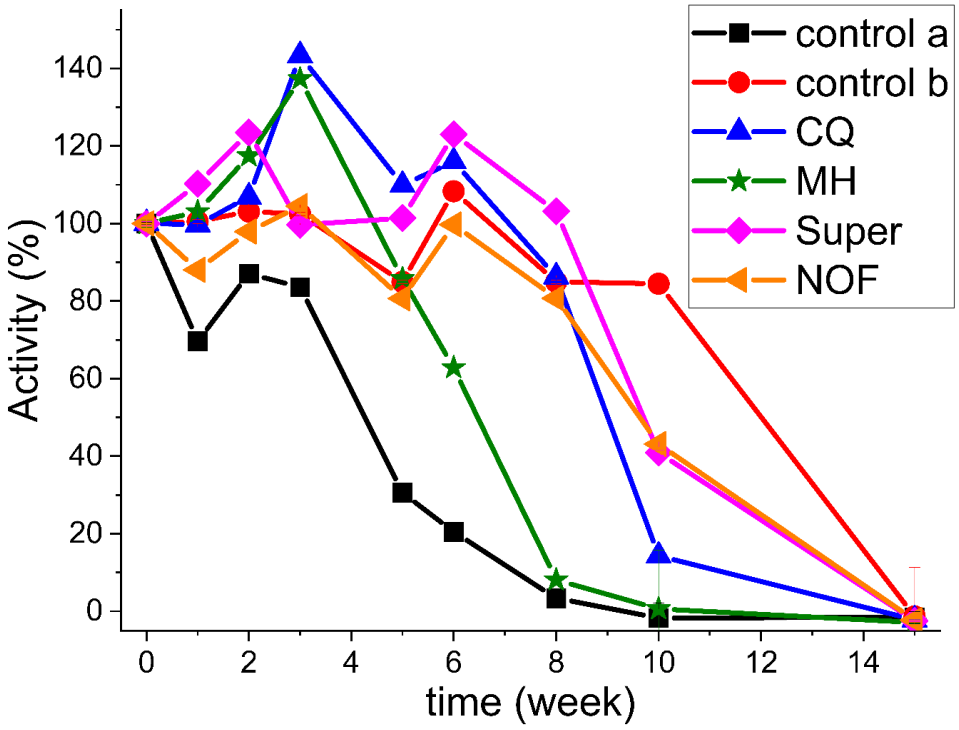

Figure 6 ALP activity assay results (mean ± S.D; n=3).

There was little change during the first four weeks of the study, but at the 4-week time-point two formulations (control A5 and the formulation containing MH) began to lose activity. For the rest of the formulations this decline begun around week 8. In all cases, the decline was rapid and led to no active ALP being present after 10 -14 weeks.

The SDS-PAGE gel images for the API formulations are shown in Figure 7. Monomer ALP arises at 43 kDa and the dimer at 86 kDa. Both bands are more intense at week 1, and some of the bands are missing in the week 15 data, suggesting possible denaturation. The polysorbate 80, particularly the more refined grades, seems to support the retention of these bands, but nevertheless the ALP has lost all its activity after 15 weeks (Figure 6). The presence of the large numbers of bands is likely because the ALP supplied contained a mixture of monomers and aggregates. Overall, the data suggest that neither the presence or absence of polysorbate 80, nor the polysorbate 80 grade, have a significant effect on the ALP activity and structural integrity after storage.

Bevacizumab

The results from SPR measurements over a period of 38 weeks are presented in Figure 8 (exemplary sensorgrams are shown in Figure S14). A straight line was fitted to each set of data and the slope calculated (Table 3 and Figure S15). From the data collected it looks like all the samples are similar , and the KD value does not change with time. This would suggest that the grade and manufacturer of the polysorbate 80 does not change the rate of adsorption and desorption, nor the binding efficiency, of Beva after storage. Two-way ANOVA analysis with a post hoc Tukeys' test (ns = $p > 0.05$) showed that there was no significant difference between the formulations and the controls at each timepoint.

The SDS-PAGE gels containing bevacizumab at week 0 and week 36 are shown in Figure 9. The two visible bands at ~25 and ~149 kDa (corresponding to the light chain and whole molecule) are in agreement with previous publications (28, 29). There are no visible changes in the bands between week 0 and week 36. This is consistent with the SPR data, and again suggests that grade of polysorbate 80 used does not have any effect, with none of those explored causing changes in the Beva formulations. No differences are visible between the polysorbate 80s formulations and the controls.

Fab

The total concentration of Fab (agglomerated and

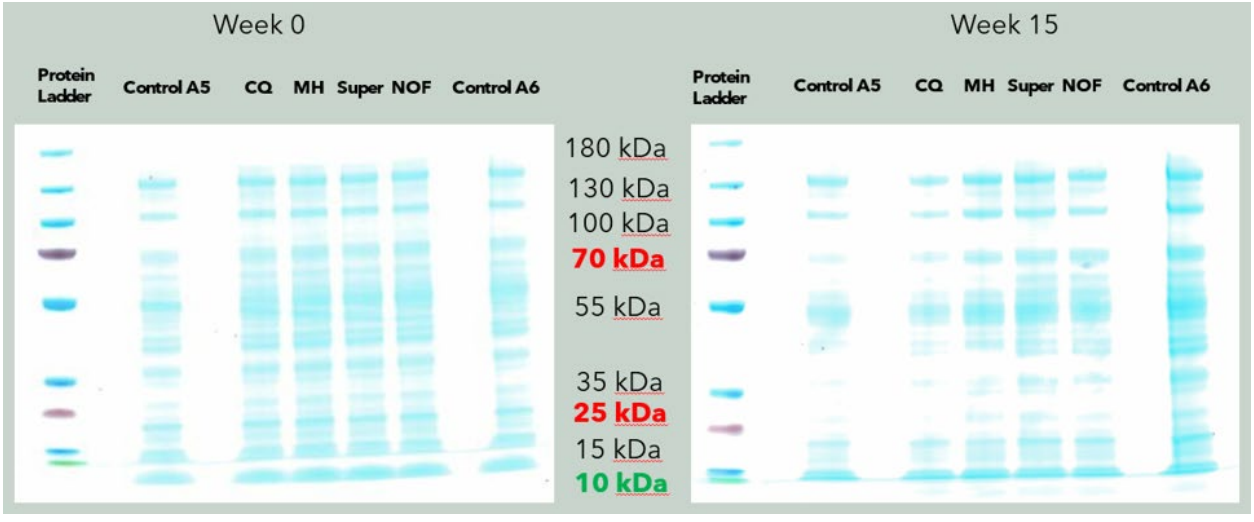

Figure 7 SDS-PAGE gels for ALP formulations. Control A5 – formulation containing all compounds of the formulation without the polysorbate 80. Control A6 – formulation containing API in water

Table 3 Gradient values for rate of adsorption, rate of desorption and biding affinity (mean ± S.D; n=2). Control B5 – formulation containing all compounds of the formulation without the polysorbate 80. Control B6 – formulation containing API in water only

FORMULATION	RATE OF ADSORPTION [M^{-1} s^{-1} $week^{-1}$] X 10^2	RATE OF DESORPTION [s^{-1} $week^{-1}$] X 10^2	BIDING AFFINITY [nM $week^{-1}$] X 10^{-2}
Control B5	0.17 ± 0.08	5.83 ± 8.72	0.46 ± 2.01
Control B6	-7.25 ± 3.14	38.46 ± 17.45	16.50 ± 6.64
CQ	2.90 ± 1.49	5.02 ± 5.05	-0.55 ± 3.45
MH	-0.72 ± 0.77	1.62 ± 0.99	0.06 ± 0.22
Super	0.11 ± 0.79	5.41 ± 0.74	3.20 ± 0.72
NOF	6.36 ± 2.39	11.30 ± 7.32	10.91 ± 4.64

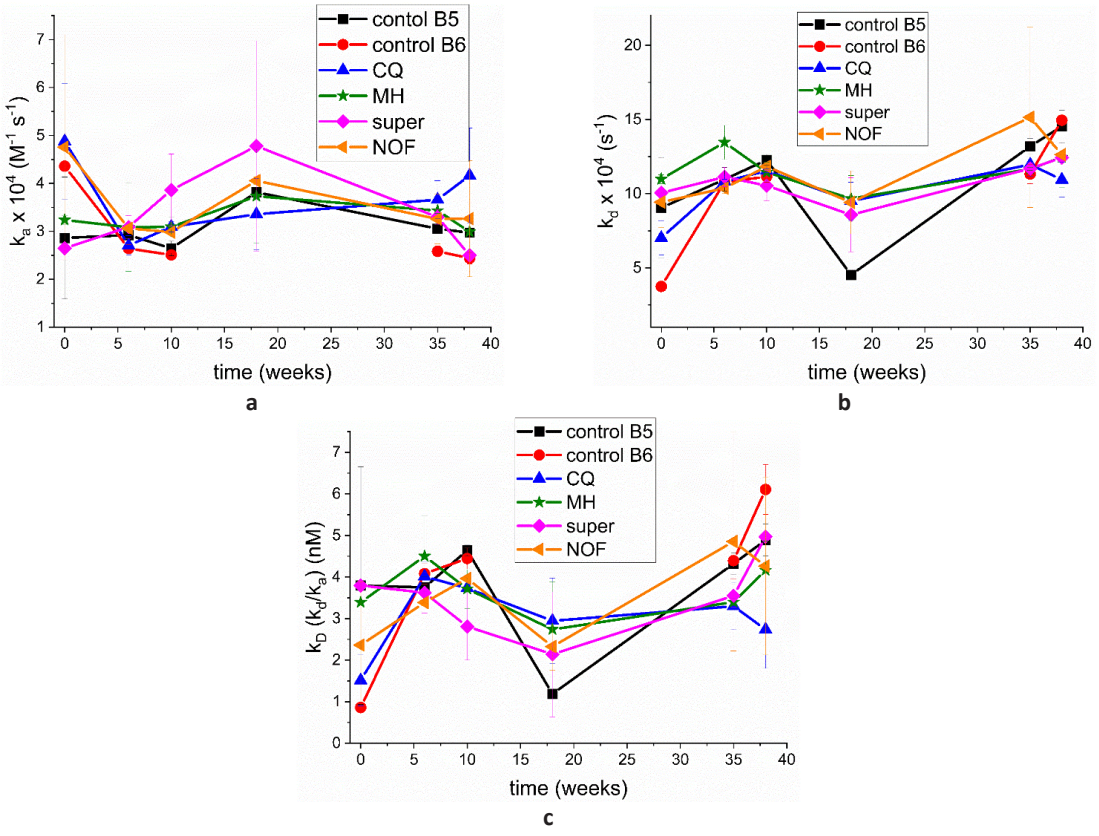

Figure 8 SPR data showing (a) rate of adsorption; (b) rate of desorption; and (c) binding affinity (mean ± S.D; n=2) for Beva formulations after storage in the fridge.

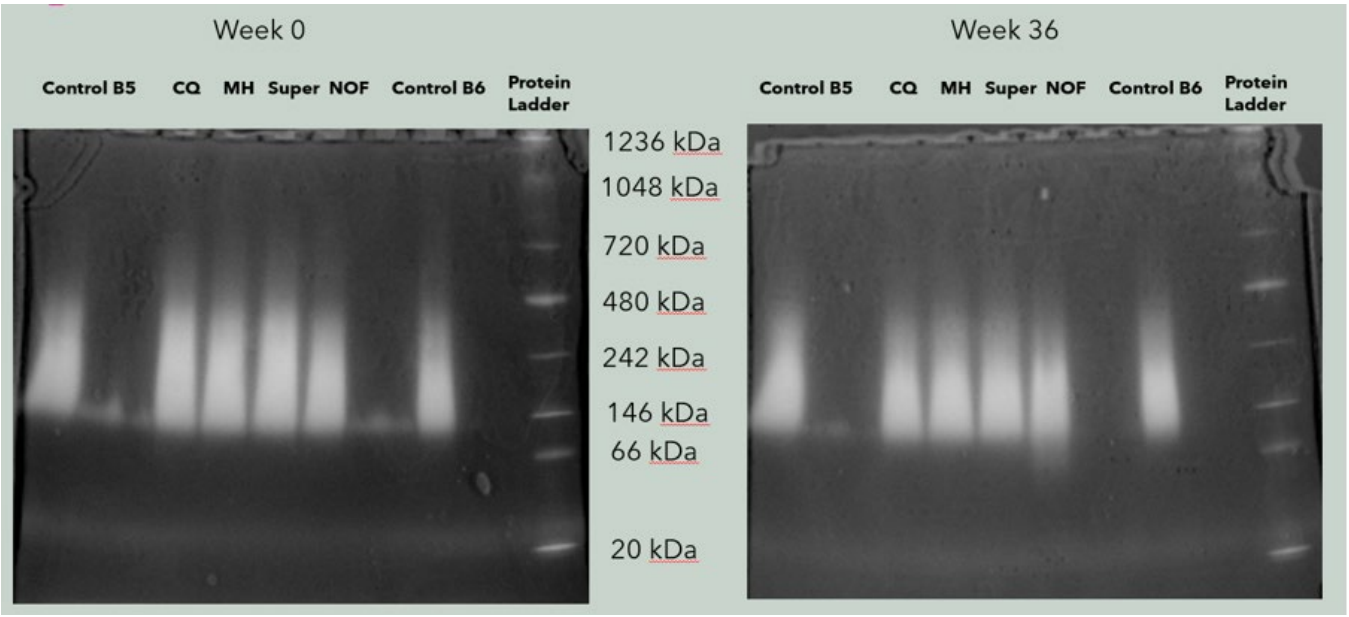

Figure 9 SDS-PAGE gels for Beva formulations. Control B5 – formulation containing all compounds of the formulation without the polysorbate 80. Control B6 – formulation containing API in water only.

non-agglomerated) in the various formulations, calculated using NanoDrop, is shown in Figure 10 a. The concentration of monomer (calculated from SEC analysis) is given in Figure 10 b. The concentration of agglomerates was then calculated using Equation 1 and the result presented in Figure 10 c.

$$C_{agglom} = C_{nano} - C_{SEC} \quad \text{Eq. 1}$$

Once the concentration of agglomerates was calculated at each timepoint a straight line was fitted into each poly 80 dataset and the gradient calculated (Table 4). Again,

Table 4 The rate of formation of agglomerates as a function of time (mean ± S.D.; n=2)

POLYSORBATE	SLOPE [mg mL^{-1} week^{-1}] X 10^{-2}	R^2
Control C5	0.46 ± 2.0	0.012
Control C6	16 ± 6.6	0.673
CQ	-0.55 ± 3.5	0.006
MH	0.06 ± 0.22	0.019
Super	3.2 ± 0.72	0.833
NOF	11 ± 4.6	0.581

there is no notable difference between the polysorbate 80 grades and manufacturer. Some agglomerates of Fab are being formed, but this process appears to be invariant and occurs regardless of the polysorbate 80 grade and/or manufacturer. Two-way ANOVA analysis with a post hoc Tukeys' test (ns = $p > 0.05$) showed that there was no significant difference between the formulations and the controls at each timepoint.

CONCLUSIONS

This study examined the stability of four different polysorbate 80 samples and biologic formulations based on those. The study investigated one standard material (CQ), one refined (MH) and two super-refined (super and NOF). No chemical degradation could be identified over storage for 44 weeks in the fridge or under accelerated aging conditions (30 °C, 65% RH; 40 °C, 75% RH). All the polysorbate 80s contain some water 1.2 – 5.3%), with more present than reported in the supplier datasheet. The discrepancy likely arises from the fact that the samples analysed were not straight from the production line, and thus water could have built up within the storage container. No change in water content is noted upon further storage. The super-refined grades shows evidence that autoxidation

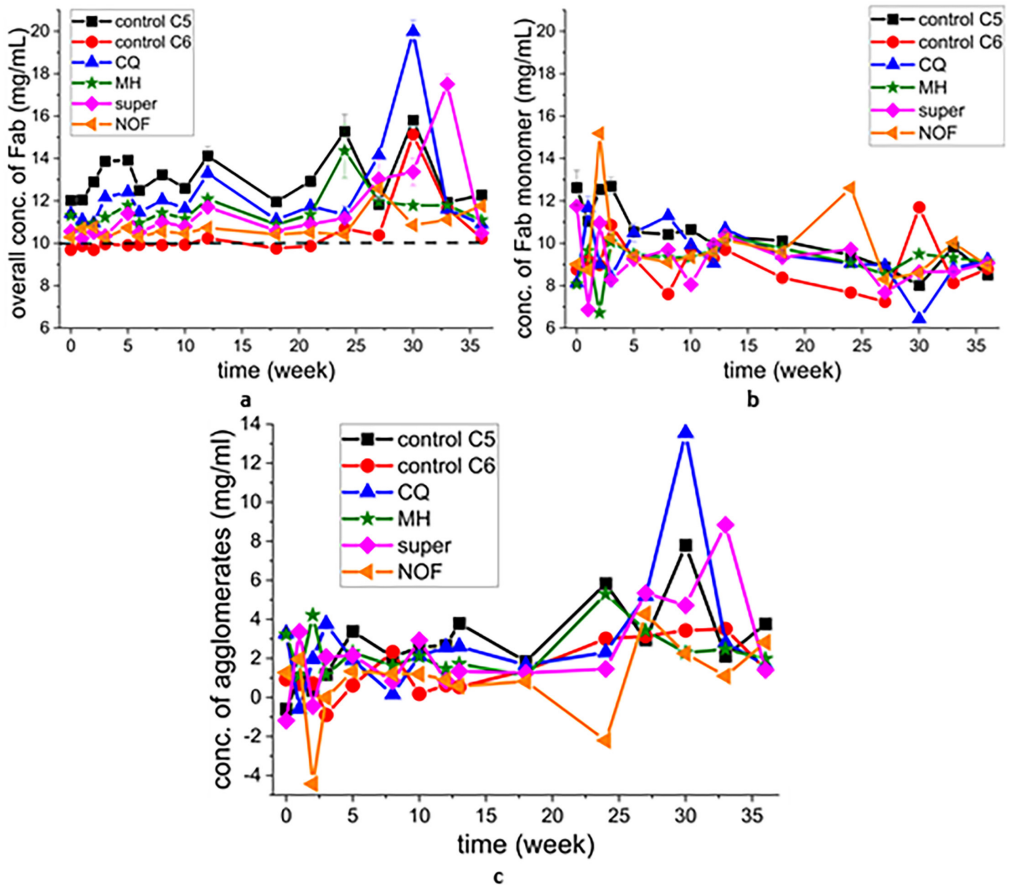

Figure 10 (a) Overall concentration of Fab; (b) monomer concentration; and (c) calculated agglomerate concentration (mean ± S.D.; n=2). Dotted line (panel a) represents original concentration of 10 mg/ml.

and hydrogen peroxide, aldehydes and ketones are produced during storage. This is less noticeable with the less refined grades, probably because these already contain autoxidation products before storage and thus there is an equilibrium in place. The storage conditions play a significant role here, and storage in the fridge slows down the build-up of peroxide, aldehydes and ketones. In general, the grade of polysorbate 80 does not appear to have any influence on the stability of formulations of biologics, with no differences observed in terms of the activity of the biologic after combination with any of the polysorbates. It thus appears that, for the active ingredients explored in this work at least, there are no significant benefits from using more highly-refined polysorbates.

The main objective of this study was to focus on chemical stability rather than physical stability. In future work it would be beneficial to additionally explore the physical stability of these systems. It would also be insightful to perform analysis of endotoxin levels, since these often influence manufacturer decisions, and consider the influence of residual host cell proteins with enzymatic activity on the polysorbate stability.

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(UCL) for their valuable suggestions and guidance throughout this research.

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Suppelemental Files to:

An investigation into the effect of polysorbate 80 grade in biotherapeutic formulations

FIGURES S1-S15

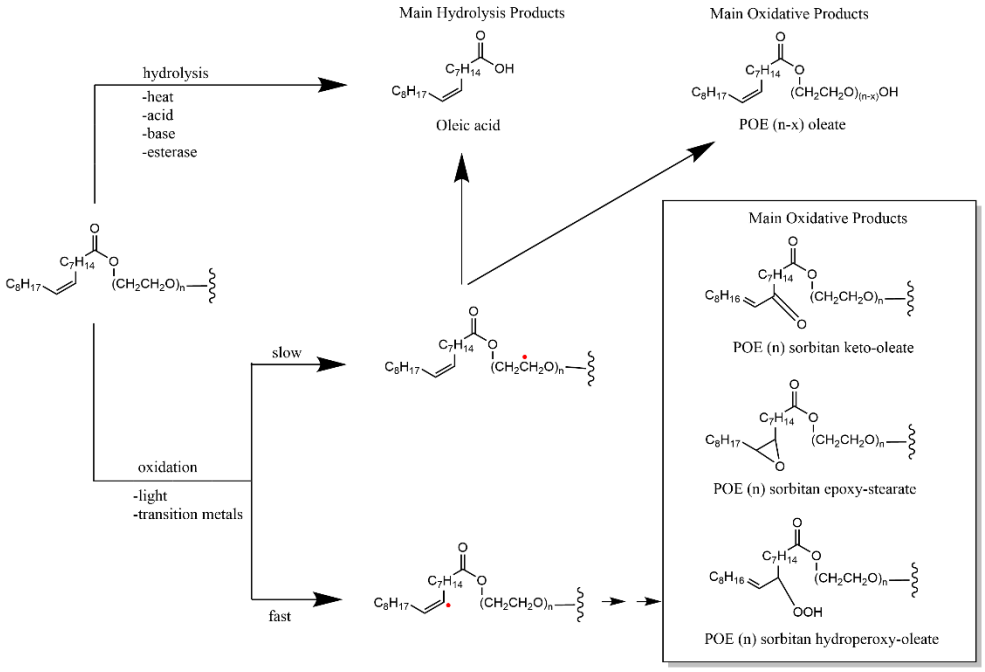


Figure S1 Reaction scheme for polysorbate 80 oxidation/hydrolysis on POE and moiety chains. Adapted from (18)

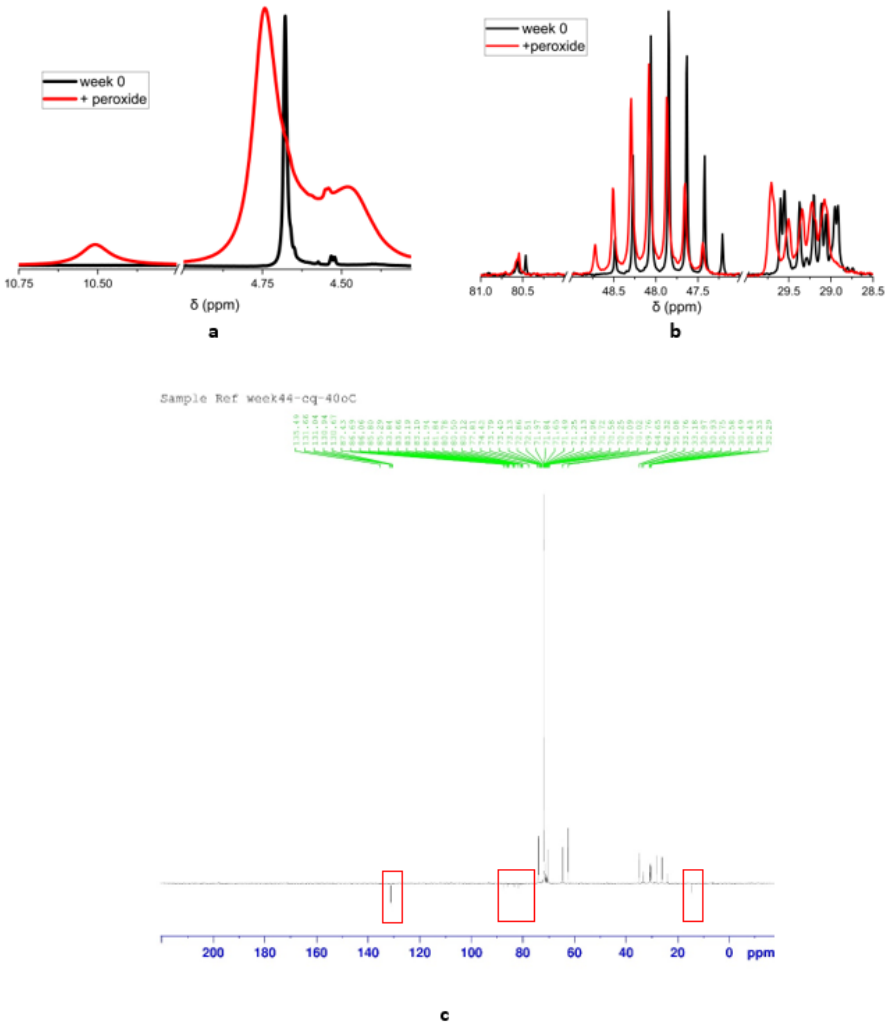


Figure S2 (a) ^{1}H NMR; (b) ^{13}C NMR; and (c) DEPT135 NMRs for spiked and clean samples of CQ.

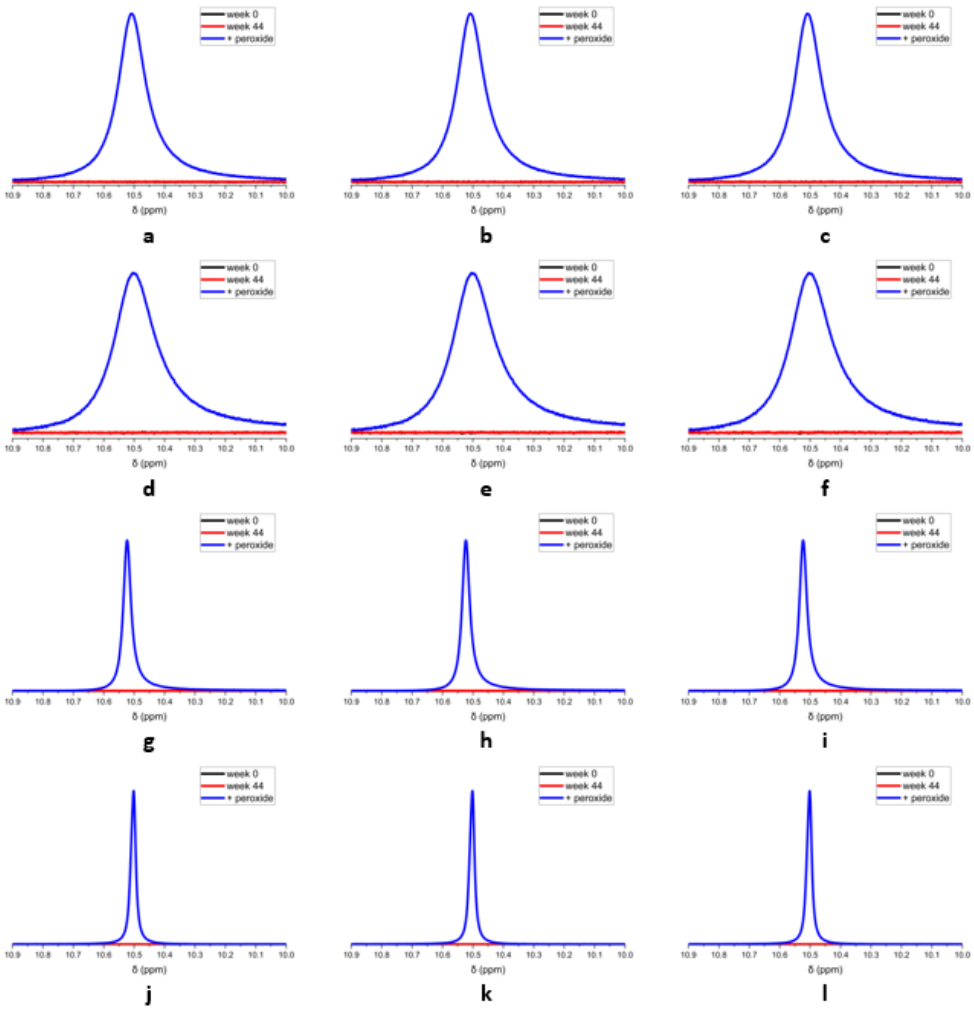


Figure S3 Proton NMR spectral changes in the region of 10.75-10 PPM for CQ (**a ,b ,c**); MH (**d, e, f**); Super (**g, h, i**); and NOF (**j,k,l**) stored at 5-8 °C, 25% RH (**a, d, g, j**), 30 °C, 65% RH (**b, e, h, k**); and 40 °C, 75% RH (**c, f, i, l**).

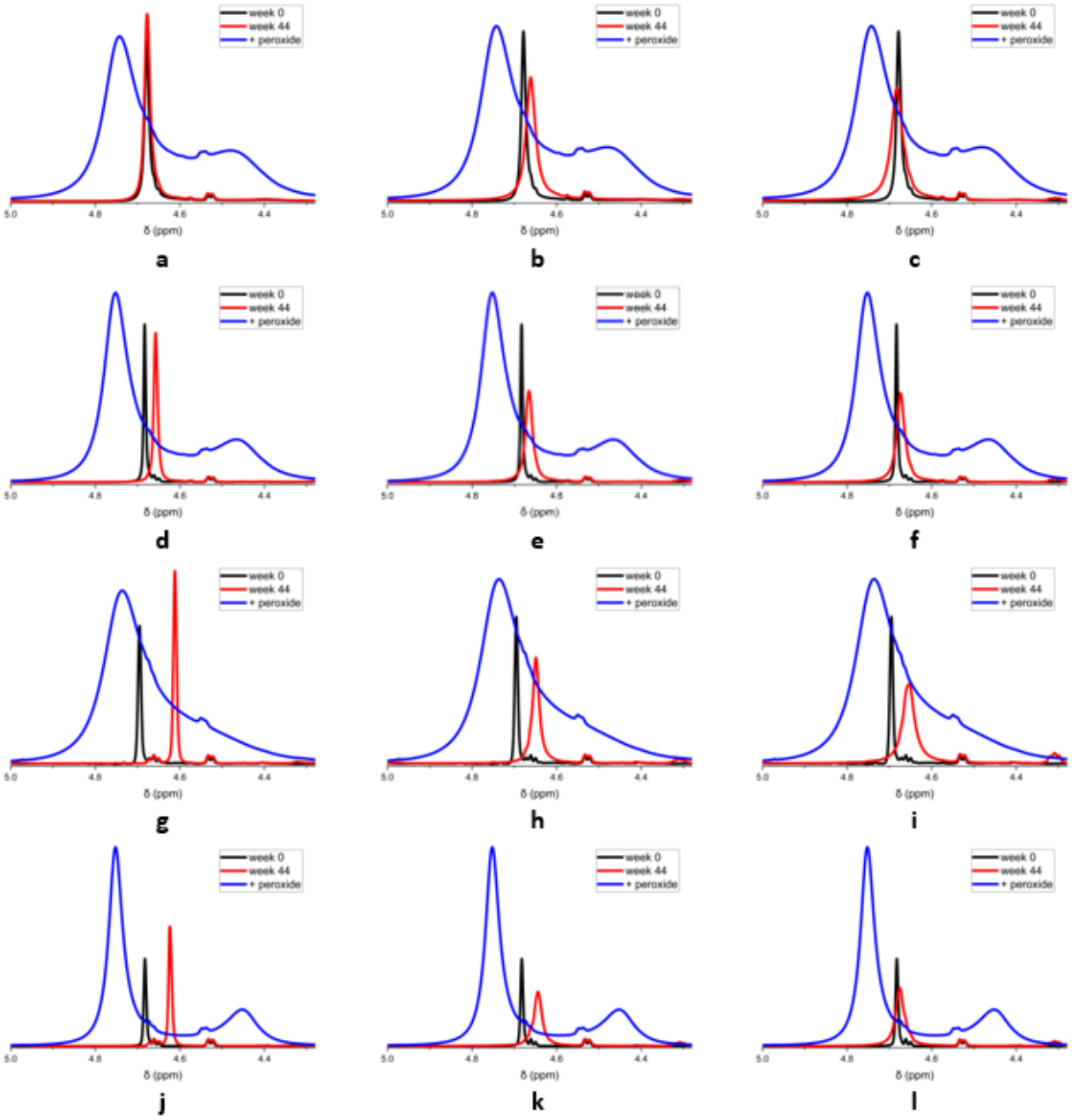


Figure S4 Proton NMR spectral changes in the region of 5-4.3 PPM for CQ (**a, b, c**); MH (**d, e, f**); Super (**g, h, i**); and NOF (**j, k, l**) under storage in the fridge (**a, d,g, j**), at 30 °C/65%RH (**b, e, h, k**); and at 40 °C / 75% RH (**c, f, i, l**).

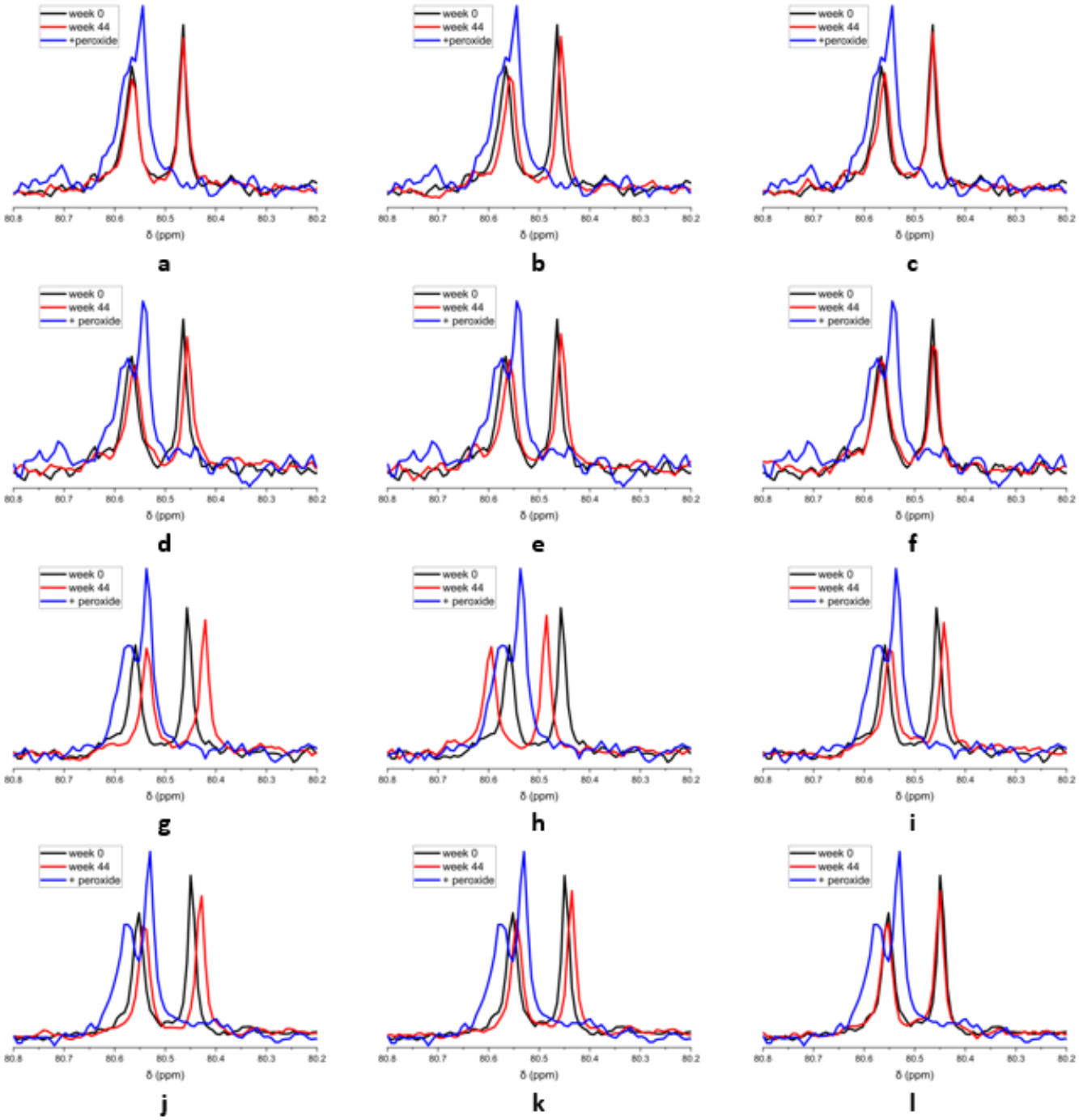


Figure S5 Carbon NMR changes in the region of 47-49 PPM for CQ (**a, b, c**); MH (**d, e, f**); Super (**g, h, i**); and NOF (**j, k, l**) after storage at 5-8 °C, 25 % RH (**a,d,g,j**), 30 °C, 65% RH (**b, e, h, k**); and 40 °C, 75% RH (**c, f, i, l**).

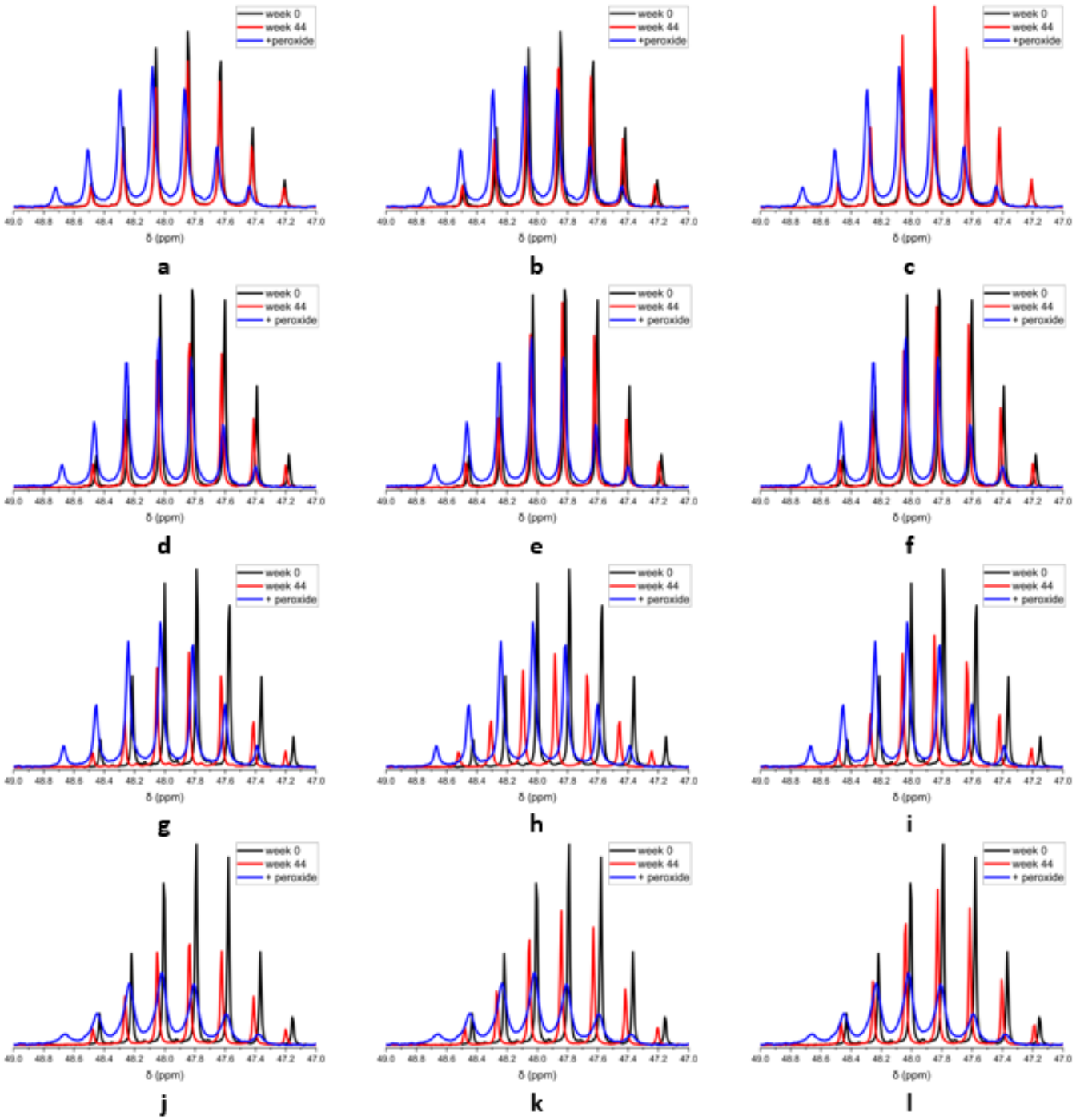


Figure S6 Carbon NMR changes in the region of 47-49 PPM for CQ (**a, b, c**); MH (**d, e, f**); Super (**g, h, i**); and NOF (**j, k, l**) after storage at 5-8 °C, 25% RH (**a, d, g, j**), 30 °C, 65% RH (**b, e, h, k**); and 40 °C, 75% RH (**c, f, i, l**).

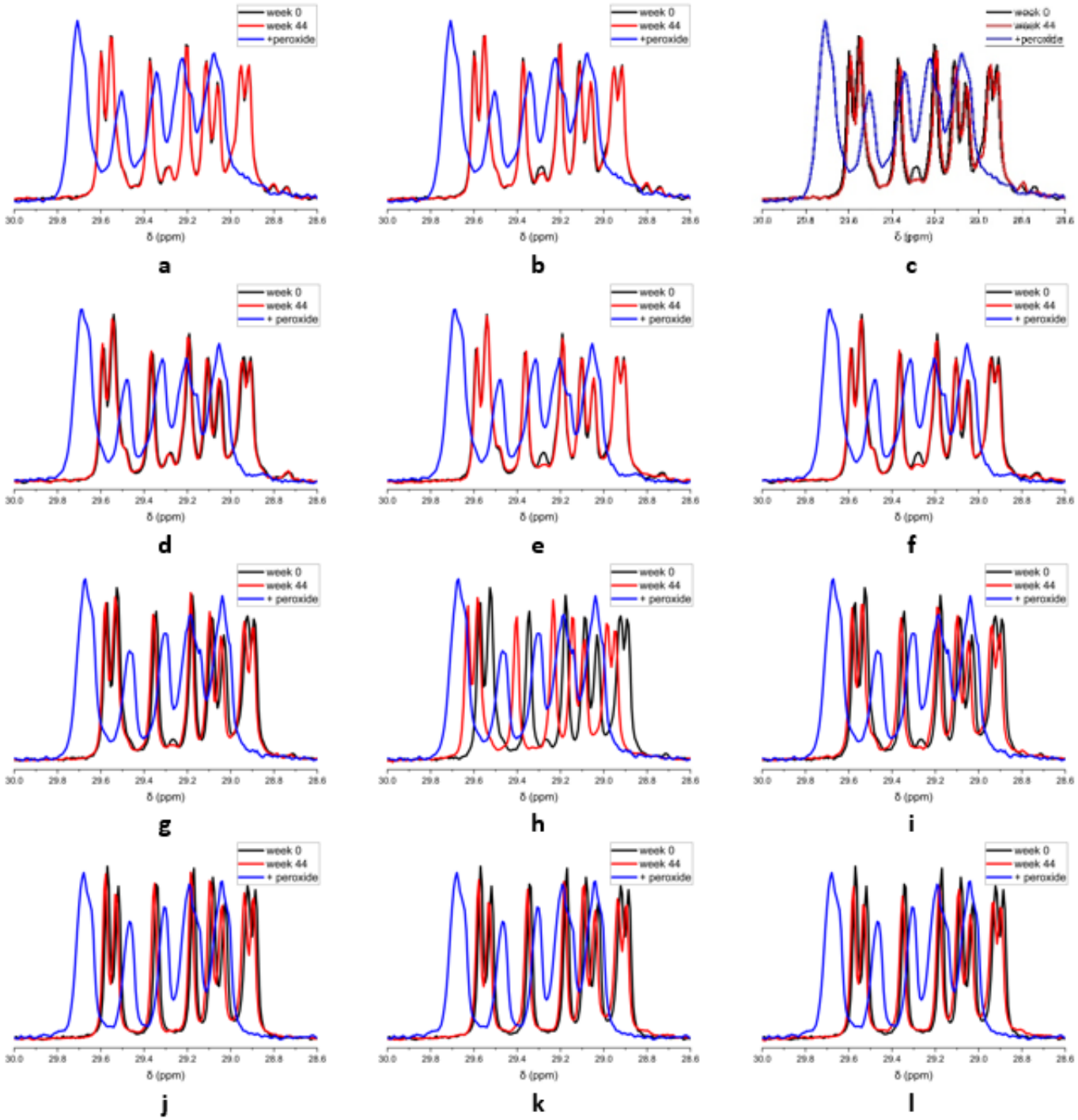


Figure S7 Carbon NMR changes in the region 30-28.6 PPM for CQ (**a, b, c**); MH (**d, e, f**); Super (**g, h, i**); and NOF (**j, k, l**) after storage at 5-8 °C, 25% RH (**a, d, g, j**), 30 °C, 65% RH (**b, e, h, k**); and 40 °C, 75% RH (**c, f, i, l**).

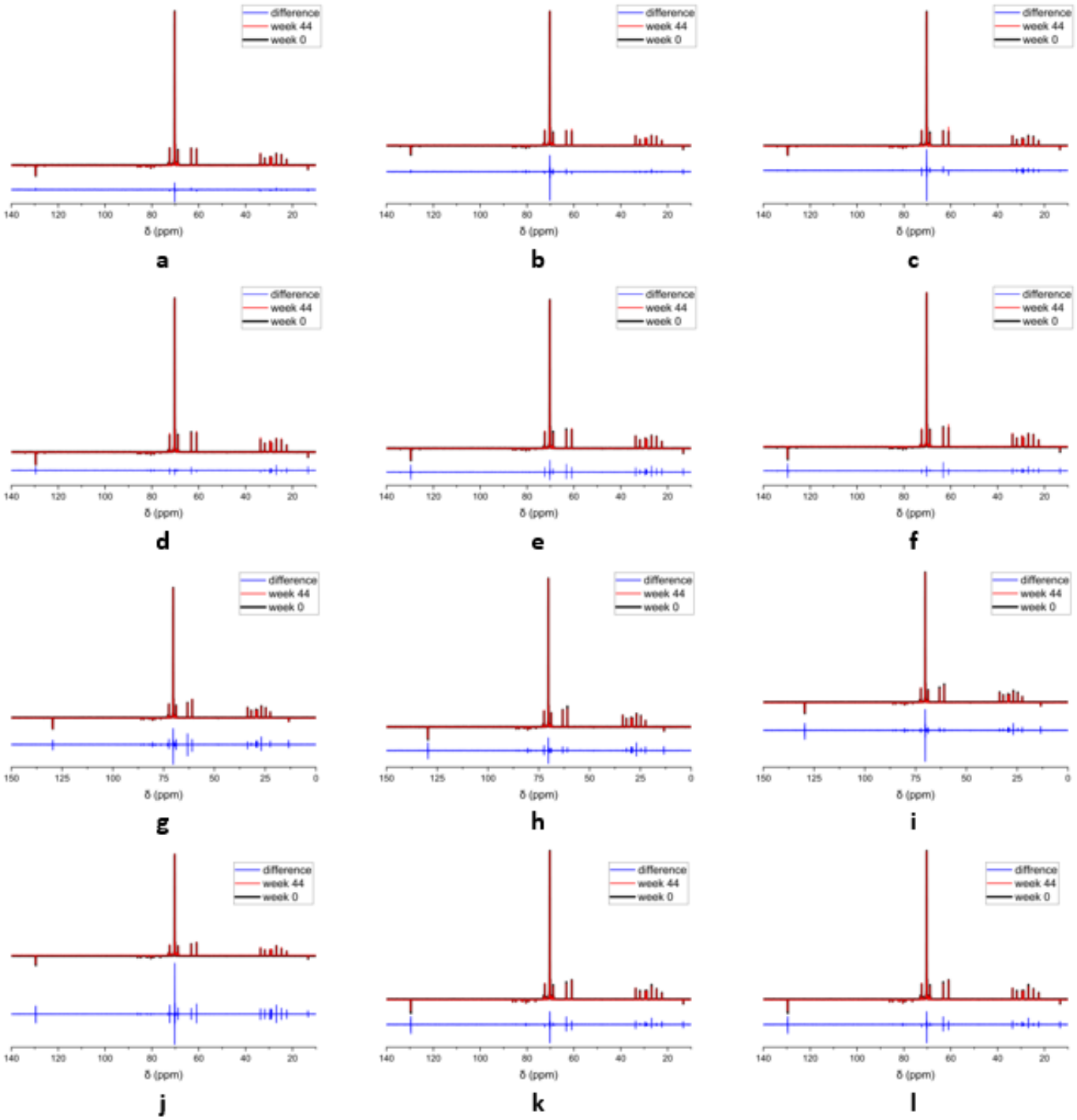


Figure S8 Carbon DEPT135 NMR changes for CQ (**a, b, c**); MH (**d, e, f**); Super (**g, h, i**); and NOF (**j, k,l**) after storage at 5-8 °C, 25% RH (**a,d,g,j**), 30 °C, 65% RH (**b, e, h, k**); and 40 °C, 75% RH (**c, f, i, l**).

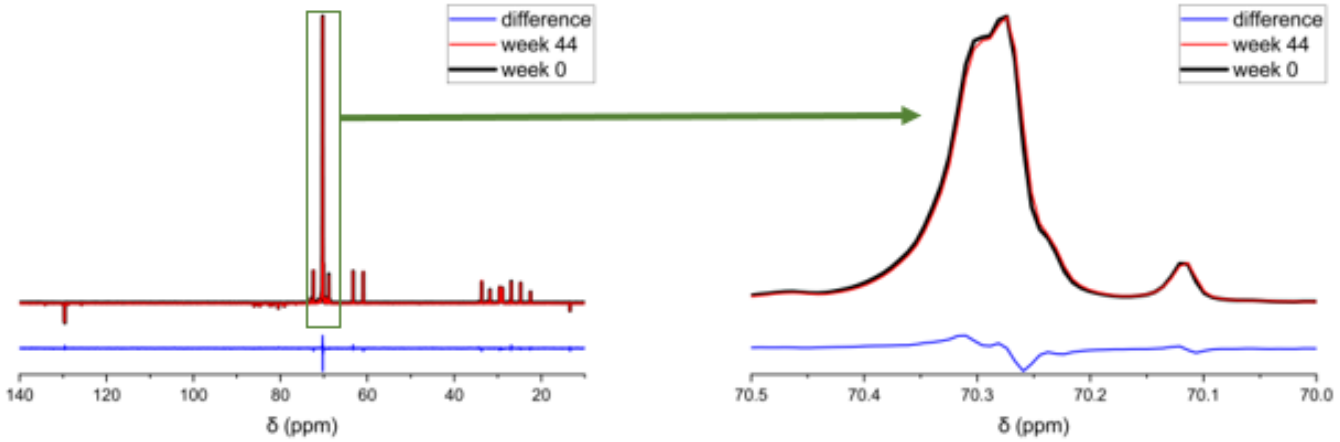


Figure S9 Close up on the spectral changes in DEPT135 carbon NMR for CQ stored in the fridge.

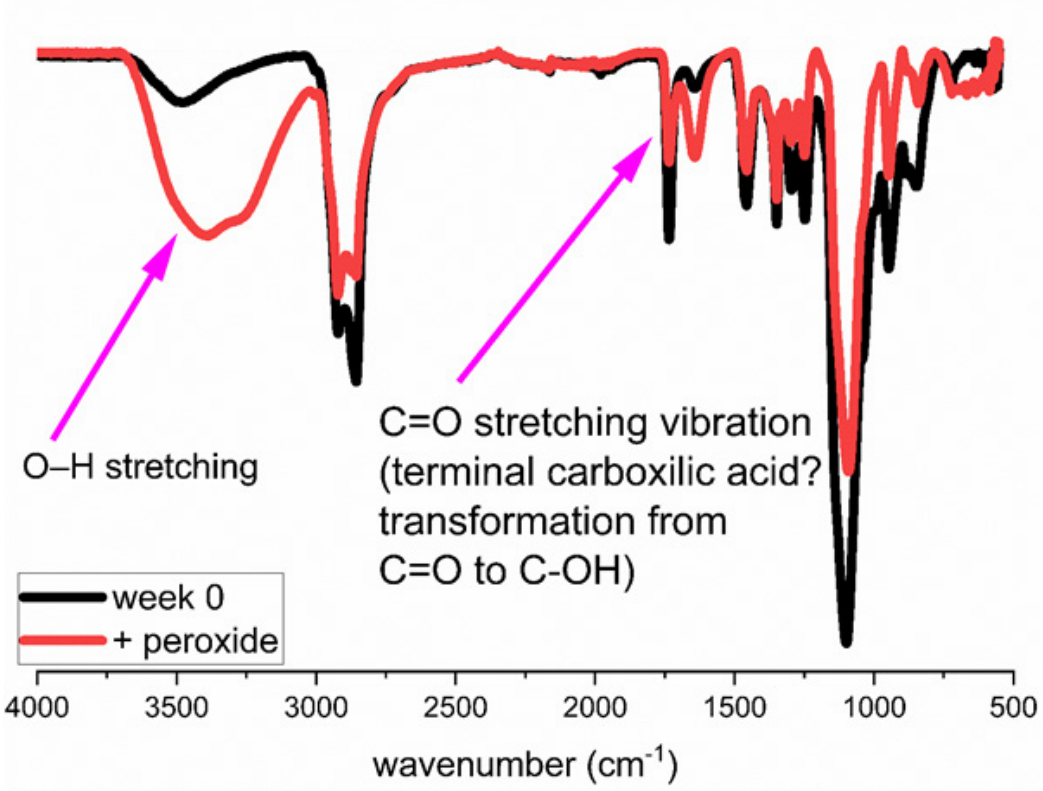


Figure S10 FTIR spectra for fresh and spiked polysorbate 80 (CQ).

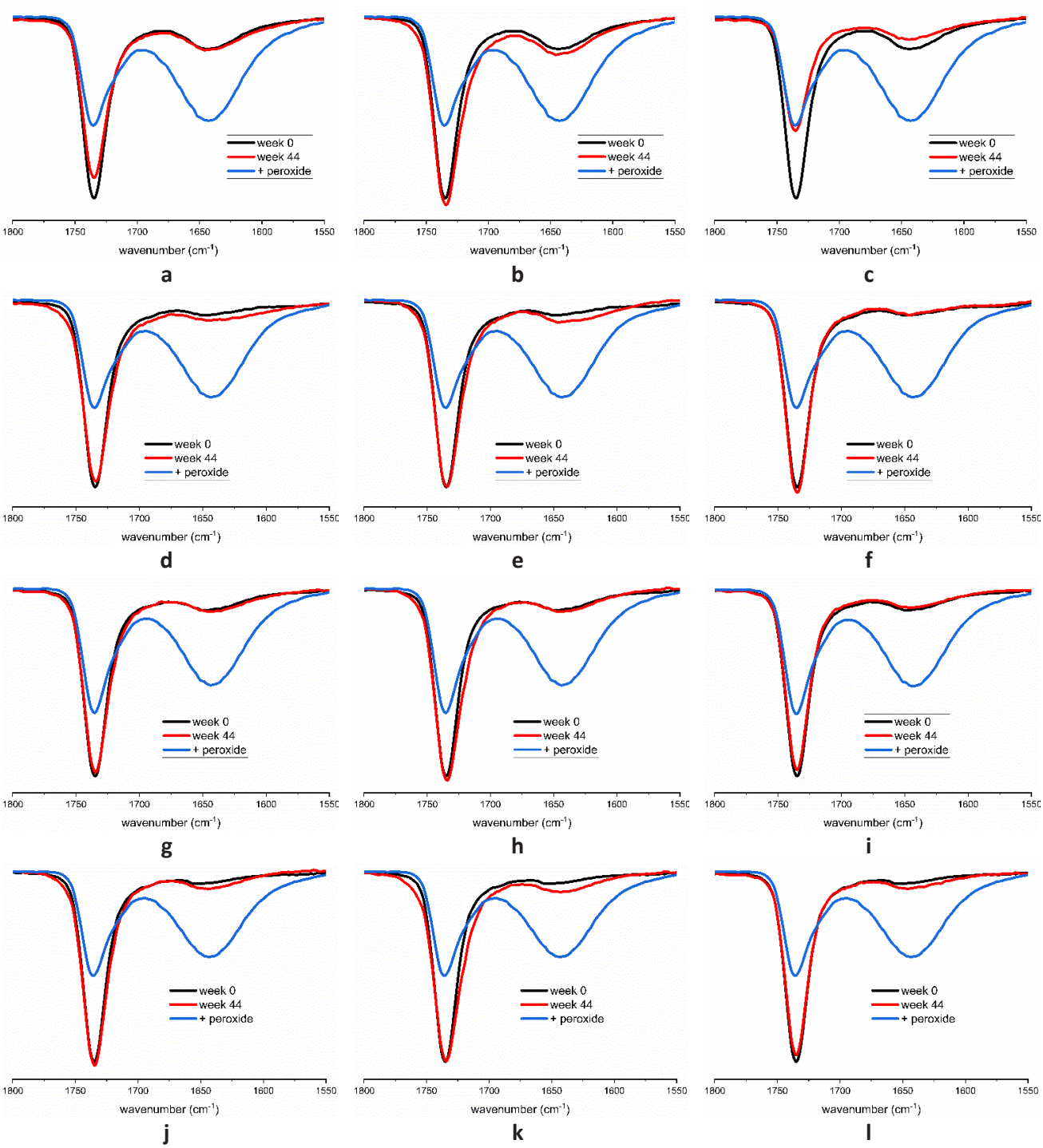


Figure S11 Changes in FTIR spectra in the region of 3800-2500 cm^{-1} for CQ (**a, b, c**); MH (**d, e, f**); Super (**g, h, i**); and NOF (**j, k, l**) stored at 5-8°C, 25% RH (**a, d, g, j**), 30 °C, 65% RH (**b, e, h, k**); and 40 °C, 75% RH (**c, f, i, l**).

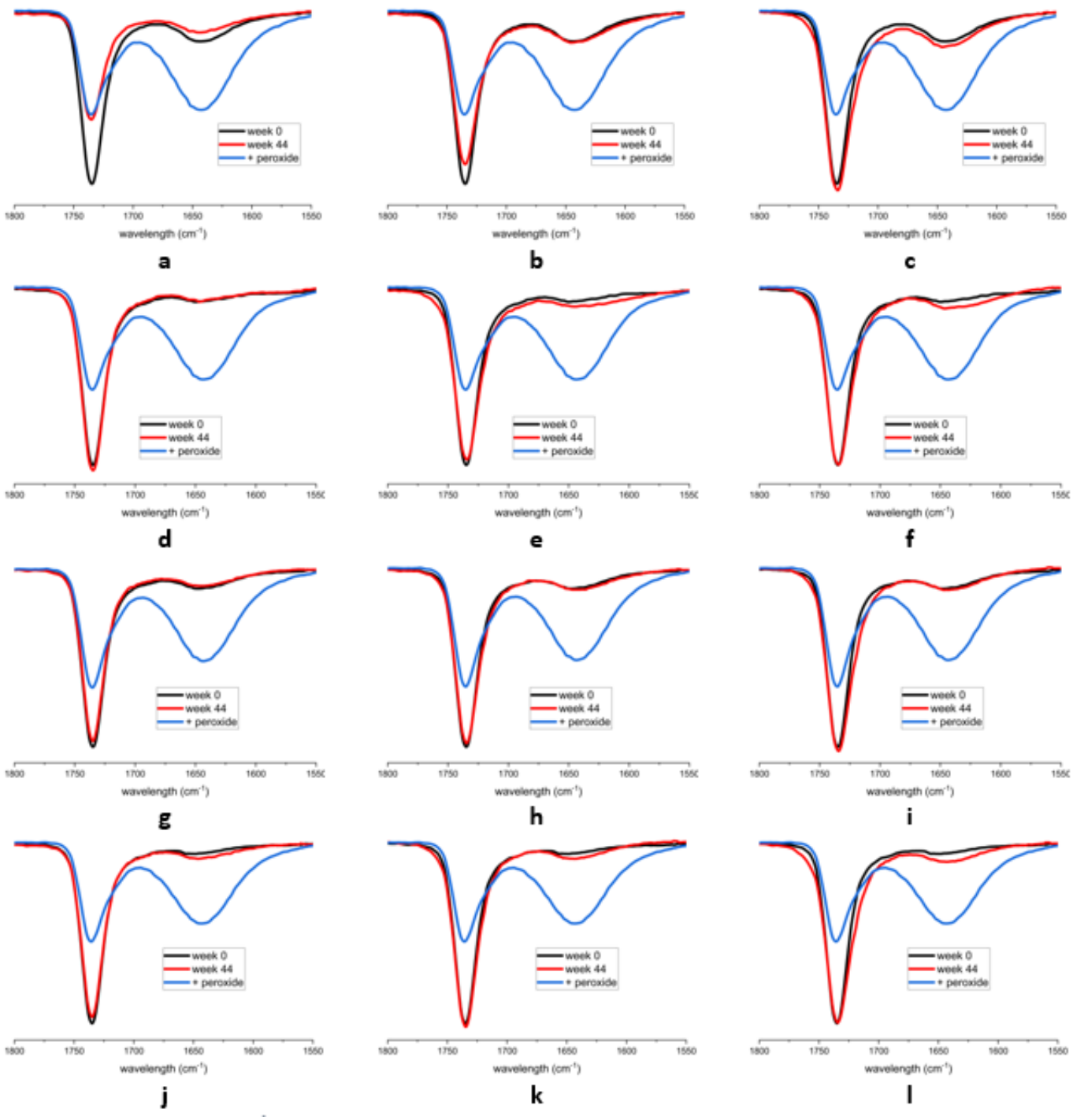


Figure S12 Changes in FTIR spectra in the region of 1800-1550 cm^{-1} for CQ (**a, b, c**); MH (**d, e, f**); Super (**g, h, i**); and NOF (**j, k, l**) stored at 5-8 °C, 25% RH (**a, d, g, j**), 30 °C, 65% RH **(b, e, h, k**); and 40 °C, 75% RH (**c, f, i, l**).

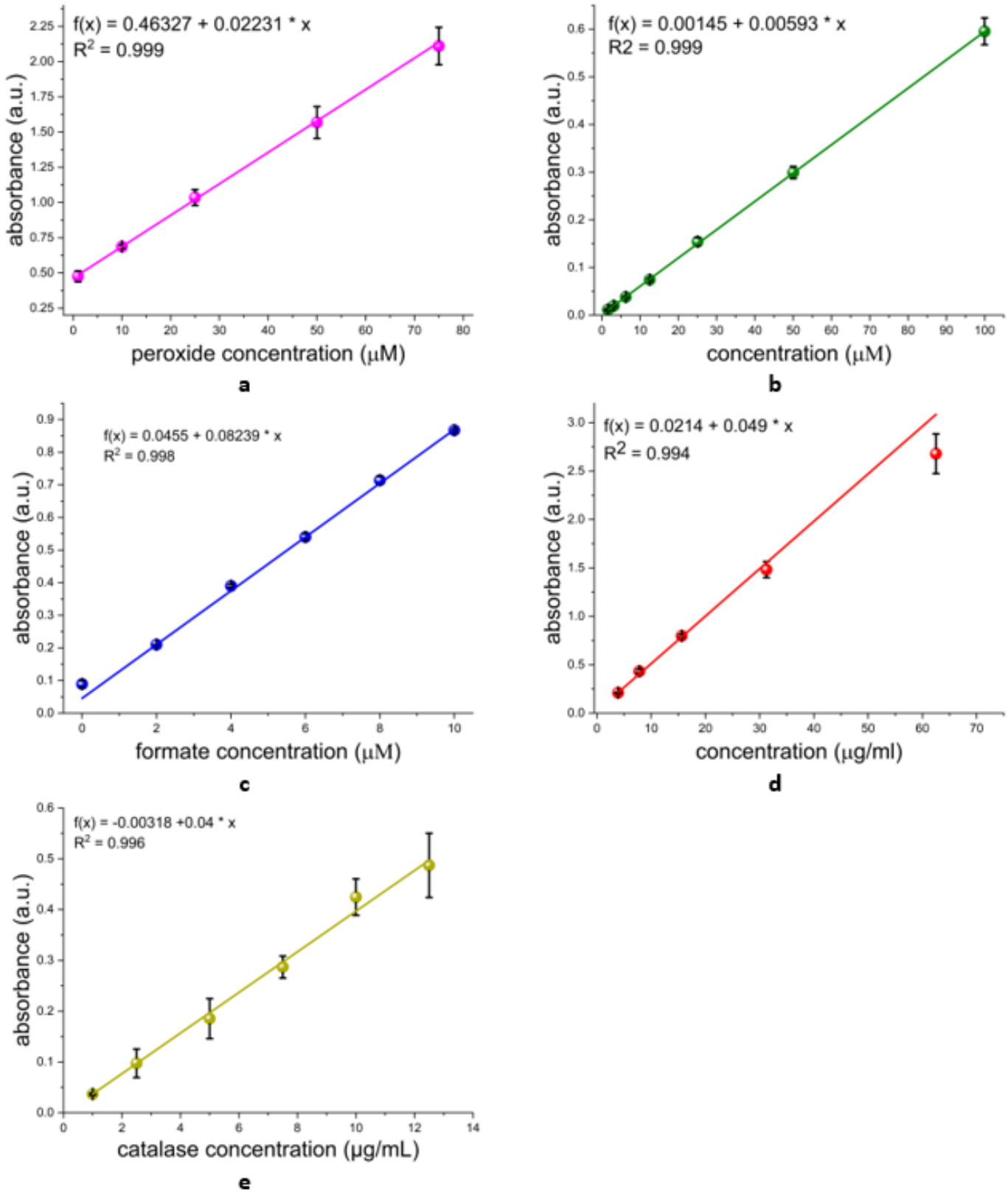


Figure S13 Calibration curves for (a) peroxide; (b) aldehyde; and (c) formate assays; (d) alkaline phosphatase activity assay; and (e) catalase activity assay.

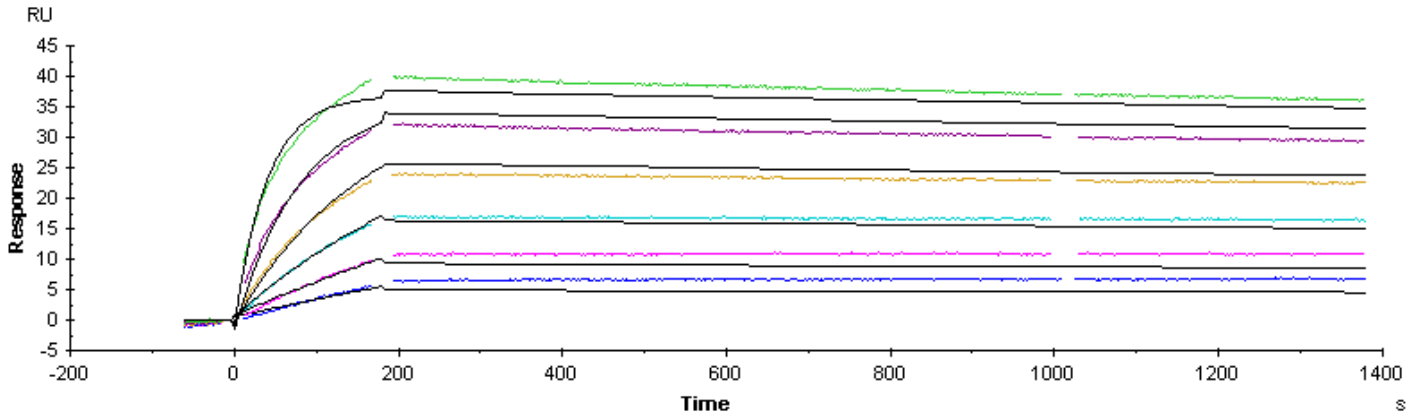
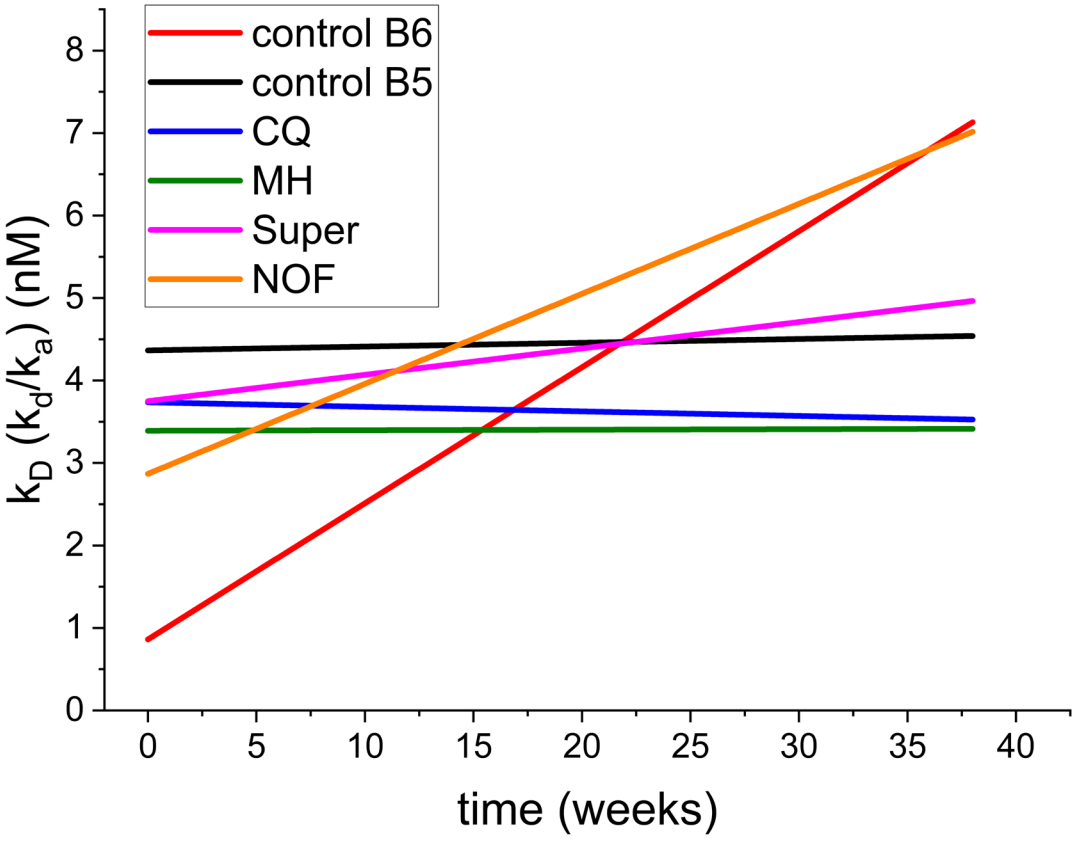


Figure S14 Exemplar sensorgrams for Beva in water, obtained at week 0. The coloured lines represent the response of different concentrations of Beva over time. The green line represents the most concentrated Beva (200 µg/ml) and the blue line the least concentrated Beve (1.56 µg/ml) responses. Black lines represent fitted lines.



R^2 values:

Control B5 = 0.11
Control B6 = 0.67
CQ = 0.00
MH = 0.14
Super = 0.83
NOF = 0.58

Figure S15 Linear fits for changes in k_D values as a function of time. No change in k_D with time is seen: where there is an apparent change based on the slope of the fit line, the R^2 is very poor.

Table S1 Polysorbate 80 composition and test values as detailed in reference (5)

TEST	VALUE	FATTY-ACID FRACTION COMPOSITION	
Acid value	Maximum 2.0	Myristic acid	Maximum 5.0 per cent
Hydroxyl value	Maximum 10	Palmitic acid	Maximum 16.0 per cent
Peroxide value	Maximum 10	Palmitoleic acid	Maximum 8.0 per cent
Saponification value	45-55 (on 4 g)	Stearic acid	Maximum 6.0 per cent
Ethylene oxide	Maximum 1 ppm	Oleic acid	Minimum 58.0 per cent
Ethylene dioxan	Maximum 10 ppm	Linoleic acid	Maximum 18.0 per cent
Water	3.0 per cent (on 1.00 g)	Linolenic acid	Maximum 4.0 per cent
Ash	0.25 per cent (on 2.0 g)		

Table S2 List of materials used in this work

PRODUCT	COMPANY	PRODUCT	COMPANY
2-amino-2-methyl-1-propanol	VWR International Ltd	NuPAGE LDS sample buffer	Life Technologies Corporation
Aldehyde assay kit	Merck	NuPAGE MOPS SDS running buffer	Life Technologies Corporation
Alkaline phosphatase	Sigma	NuPAGE sample reducing agent	Life Technologies Corporation
Alkaline phosphatase Yellow (pNPP) liquid substrate system for ELISA	Sigma-Aldrich	PageRuler prestained proteine ladder	ThermoScientific
Bevacizumab	Hetero Biopharma	Pierce Quantitative peroxide assay	ThermoScientific
Bovine serum albumin	Sigma-Aldrich	Potassium hydroxide	Fisher
catalase	Sigma	Potassium periodate	Scientific Laboratory Suppliers Ltd
D4-Methanol	Sigma-Aldrich	Purpald	Sigma-Aldrich
EDTA	Acros Organics	Refined polysorbate 80 (MH) (BN 0001481978)	Croda
Fab		Sensor Chip CM3	Cytiva
HBS-EP+ buffer	Cytiva	Sodium dihydrogen phosphate monohydrate	Sigma-Aldrich
Hydranal composite 5	Honeywell	Sodium hydroxide	Fisher Scientific
Hydrochloric acid	Honeywell	Sodium phosphate dibasic dihydrate	Fluka
Hydrogen peroxide	Sigma-Aldrich	Standard polysorbate 80 (CQ) (BN 2905YP0171)	Croda
InstantBlue® Coomassie Protein Stain	Abcam	Succinic acid	Sigma-Aldrich
Keton assay kit	Merck	Sucrose	Sigma
Methanol	Sigma-Aldrich	Super-refined polysorbate 80 (NOF) (BN 903358)	NOF
methanol anhydrous	VWR International Ltd	Super-refined polysorbate 80 (Super) (BN 0001779440)	Croda
NativeMark unstained protein standard	Life Technologies Corporation	Tween 80 (BN L17032304)	bioWORLD
NativePAGE 4-16 % Bis-Tris gel	Life Technologies Corporation	Tween 80 (BN 0024048912)	BASF
NativePAGE running buffer	Life Technologies Corporation	Tween 80 (BN 8.22187.0050)	Merck
NativePAGE sample buffer	Life Technologies Corporation	Tween 80 (BN 2022_001)	PCC
NuPAGE 4-12 % Bis-Tris gel	Life Technologies Corporation	VEGF	Merck

Scheme S1 Karl Fischer titration process and calculations.

- The burette was filled with a titrant (hydranal composite 5) and the reaction vessel with anhydrous methanol. A magnetic stirrer was added to the reaction vessel and the stirrer switched on (50% speed). The time was set to 45 seconds, the time at which the change of the current between the pairs of electrodes was stable.

- Anhydrous methanol without the sample was titrated first to ensure that all water residue had reacted with I_2 and SO_2 (Equation S1). The titrant was added dropwise in intervals of 10-15 seconds. Once the liquid become amber and a change in current occurred (all water has reacted) the instrument automatically stopped the titration.

- Next the sample was transferred quantitatively to the reaction vessel containing titrated methanol.

- The level of titrant in the burette was noted. The sample was titrated until no water was left and the system automatically stopped the titration. The level of titrant was noted again.

$$I_2 + SO_2 + H_2O \rightarrow 2HI + SO_3 \qquad \text{Eq. S1}$$

The water content in the sample was calculated using Equation S2.

$$MC = (KF \times MDF \times 100)/m \qquad \text{Eq. S2}$$

MC (%) – moisture content; KF (mL) – volume of titrant used (difference in titrant level in the burette before and after titration); MDF (mg/mL) – moisture determining factor (as provided by the supplier); m (mg) – weight of sample.