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The use of natural antioxidants to combat lipid oxidation in O/W emulsions

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Abstract

This research investigated the efficacy of four natural antioxidants, quercetin, curcumin, rutin hydrate and ascorbic acid in their ability to combat Lipid Oxidation (LO) within different oil-in-water (O/W) emulsion environments. The free radical scavenging and metal chelating ability of the four antioxidants was first assessed through DPPH and Ferrozine assays respectively and used to help explain each antioxidants efficacy in particular environments. Generally, in emulsions with no added iron, compounds that exhibited the greatest levels of DPPH and Ferrozine inhibition provided the best oxidative stability. In the presence of added iron, antioxidant effectiveness reduced dramatically and in some cases resulted in prooxidant activity. It was concluded that the antioxidant metal chelating mechanism of antioxidants in emulsions with added iron was largely insignificant compared to the prooxidant effect gained by these compounds through their interaction with iron. The most non-polar compounds, curcumin and quercetin provided peroxide value (PV) reductions of 65% and 74% respectively in 5% oil phase volume emulsions compared to just 28% and 43% PV reductions in 40% oil phase volume emulsions; thus providing more evidence of the widely reported 'polar paradox'. Combinations of ascorbic acid with quercetin or curcumin resulted in antioxidant synergism, whereas other antioxidant combinations led only to additive or antagonistic effects. This research builds on the understanding of the fundamental behaviour of natural antioxidants within different emulsion formulations.

1 Introduction

Lipid oxidation (LO) can result in changes to the taste, texture and appearance of fat (lipid) containing food products in addition to loss of nutritional value and reduced shelf-life (McClements and Decker, 2000). The process of LO occurs as a free radical chain reaction and remains a major current challenge within the food industry, particularly within O/W emulsions, for a variety of reasons. Firstly, the relentless drive for the replacement of saturated fats with healthier, unsaturated ones in food products greatly increases their susceptibility to LO due to the reduced bond dissociation energies harboured within unsaturated lipid molecules (Domínguez et al., 2019). Secondly, the food industry is focused on the replacement of synthetic, and often highly effective antioxidants with natural alternatives (Caleja et al., 2017) due to safety concerns and consumer acceptance (Waraho et al., 2011). Thirdly, O/W emulsions are commonplace within food products, with everyday examples including milk, cream, soups, sauces and a variety of processed foods. In O/W emulsions, a tremendous interfacial area between the oil and water phases is generated through emulsification, and it is here where LO in O/W emulsions is believed to predominantly occur through reactions between lipid hydroperoxides located at the surface of oil droplets, and transition metals such as ferrous iron located in the aqueous phase (Waraho et al., 2011). This large interfacial area provides numerous sites for LO to occur and greatly accelerates LO within O/W emulsions.

The addition of highly potent, natural antioxidants provides a potential solution to the aforementioned challenges and consequently there has been increased recent research into their assessment for combatting LO (Zahid et al., 2018, Glodde et al., 2018, Ghorbani Gorji et al., 2019). However, there has yet been limited research into fundamental understanding of the behaviour of natural antioxidants in many different O/W emulsion environments (e.g. differing antioxidant/prooxidant concentrations, oil phase volumes, choice of emulsifiers, antioxidant combinations etc.). This research paper was produced to build knowledge in this

area though assessment of four antioxidants found widespread in nature, namely quercetin, curcumin, rutin hydrate and ascorbic acid.

2 Materials and methods

2.1 Materials

Distilled water was used as the continuous phase within emulsions, this was obtained by pumping water through a reverse osmosis unit followed by milli-Q water system prior to usage. Consumer grade sunflower oil was used as the dispersed phase in emulsions; a large batch of this oil was purchased from a local Aldi supermarket to avoid batch-to-batch composition variations. Polysorbate 20 (P20) was obtained from Acros organics. Quercetin, curcumin, rutin hydrate, L-ascorbic acid, Ferrozine and para-anisidine were all obtained from Sigma Aldrich. sodium dodecyl sulphate (SDS), iron (II) sulphate heptahydrate, ammonium thiocyanate, 2,4-decadienal aldehyde, anhydrous barium chloride, hydrochloric acid, and glacial acetic acid were all provided by Fisher Scientific. Mirenat-D (CAT) cationic surfactant was provided by Vedeqsa. Cumene hydroperoxide was provided by Scientific Laboratory Supplies Ltd.

2.2 Antioxidant activities

2.2.1 DPPH assay

The 1,1-diphenyl-2-picryl hydrazyl (DPPH) assay was taken from a study by Olugbami et al, 2015. This assay was used to determine the free radical scavenging activity of antioxidant compounds. Briefly, 1ml of prepared 0.1mg/ml DPPH solution was added to 1.5ml of antioxidant solutions dissolved in ethanol at concentrations ranging from 0-80μg/ml. This mixture was shaken and then stored in the dark at room temperature for 20 minutes before having its absorbance measured at 517nm using a UV spectrophotometer.

$$DPPH\ inhibition\ (\%) = 100 \times \frac{A_{control} - A_{sample}}{A_{control}}$$

Where A_{sample} and $A_{control}$ are the absorbance with and without antioxidants respectively.

88 2.2.2 Ferrozine assay

89 The Ferrozine assay was a modified version taken from a study by Dinis et al, 1994. This
90 assay was used to determine the ferrous iron chelation activity of antioxidant compounds.
91 10µl of 4mM ferrous sulphate solution was added to 2ml of antioxidant solutions in ethanol at
92 different concentrations. This mixture was shaken and left for 3 minutes. Then 10µl of 20mM
93 Ferrozine solution was added, the mixture was shaken and left for 10 minutes in the dark at
94 room temperature prior to having its absorbance measured at 562nm using a UV
95 spectrophotometer.

$$Ferrozine\ inhibition\ (\%) = 100 \times \frac{A_{control} - A_{sample}}{A_{control}}$$

96 Where A_{sample} and $A_{control}$ are the absorbance of DPPH solution with and without antioxidants
97 respectively.

98 2.3 Emulsion formulation

99 1% (w/w) emulsifier (either polysorbate 20, sodium dodecyl sulphate or Mirenat-D) was
100 added to distilled water to form the aqueous phase of the emulsion. The desired amount of
101 sunflower oil (either 5, 10, 20 or 40% (w/w)) was then added to this to make up a total
102 emulsion weight of 100g. This mixture was emulsified through use of a Silverson L5M high
103 shear mixer fitted using a fine emulsion screen, 57mm diameter head, and homogenisation
104 speed of 7000rpm for 5 minutes. 25g of homogenously dispersed emulsion was then poured
105 into individual sample pots to which the desired mass of antioxidants or iron sulphate
106 heptahydrate were added if required. These 25g samples were used throughout the duration
107 of the LO study (7 days). All emulsions were formulated at native pH.

108 2.4 Size Measurement

109 The Sauter mean diameter (D [3,2]) of emulsified oil droplets was calculated using a
110 Mastersizer 2000. It was ensured that the Mastersizer was measuring >77% laser light
111 transmittance prior to its operation. The stirring speed for all size measurements was
112 selected at 1300rpm and sample was added via pipetting until laser obscuration reached a

value of 5%. A refractive index value of sunflower oil (1.47) was selected for all emulsions. All measurements were performed in triplicate.

2.5 Lipid Oxidation Study

The levels of primary and secondary oxidation products generated throughout a 7 day period were measured. Hydroperoxides served as the primary oxidation product, the quantity of which was measured via the peroxide value method. Secondary oxidation products were measured by the P-anisidine method which predominantly measured the quantity of unsaturated aldehydes. Emulsions were stored at 40°C throughout the experiment to accelerate the lipid oxidation process. Control emulsions were formulated in all experiments except in the antioxidant combination study to compare to emulsions without added antioxidants.

2.5.1 Peroxide value (PV) method

The PV method was a modified version taken from a study by Shantha and Decker, 1994. Briefly, emulsions were gently shaken to form a homogenous mixture. Then 100µl of emulsion sample was taken and added to 1.5ml of isooctane/propan-2-ol mixture (3:1 v/v). This mixture was vortexed for 10s intervals, 3 times in order to extract the hydroperoxides into the solvent phase and then the mixture was centrifuged at 4000rpm for 20 minutes. Post centrifugation, 200µl of the upper solvent layer was taken and added to 2.8ml of methanol/butan-1-ol (2:1 v/v) mixture along with 15µl of 0.072M ferrous iron solution and 15µl of 3.94M ammonium thiocyanate solution. Ferrous iron solution was obtained from the supernatant of a mixture of 25ml $BaCl_2$ solution (0.132M $BaCl_2$ in 0.4M HCL) and 25ml of 0.144M $FeSO_4$ solution. These samples were then placed in the dark for 20 minutes prior to having their UV-absorbance measured using a spectrophotometer at a wavelength of 510nm.

To quantify the amount of hydroperoxides within emulsion samples, a standard curve was produced using cumene hydroperoxide (CH). For this, a 21Mm CH solution in isooctane/propan-2-ol (3:1 v/v) was centrifuged at 4000rpm for 20 minutes. The supernatant

was taken and diluted into several known concentrations. These different concentrations of CH then underwent the same method as emulsion samples for measurement of their UV-absorbance. All measurements were performed in triplicate. The relationship was found to be linear among all PV's encountered in this study.

2.5.2 P-anisidine value (AV) method

The AV method was a modified version taken from a study by O'Dwyer et al, 2013. Briefly, emulsions were gently shaken to form a homogenous mixture. Then 2ml emulsion samples were taken and added to 2ml of ethanol. Ethanol was used to help break-up the interfacial layer and allow for more effective extraction of unsaturated aldehydes to the solvent phase upon vortex mixing. 8ml of isooctane was then added to this mixture and the sample was vortexed for 10s intervals, 3 times in order to extract the unsaturated aldehydes into the solvent phase and then the mixture was centrifuged at 4000rpm for 20 minutes. The absorbance of the supernatant was measured at a wavelength of 350nm using the spectrophotometer to give the A_1 value. Then, 5ml of the supernatant was mixed with 1ml of P-anisidine solution (2.5mg/ml p-anisidine concentration in glacial acetic acid). After keeping the samples for in the dark for 10 minutes, the absorbance was measured at 350nm using the spectrophotometer to give the A_2 value. Anisidine value (AV) was then calculated using the equation below:

$$AV = 25(1.2A_1 - A_2)$$

A standard curve using 2, 4-decadienal aldehyde (DA) was used to verify the accuracy of this method. To obtain the standard curve, a 1mM solution of DA in isooctane was prepared and suitable dilutions made up. These dilutions then underwent the same method as emulsion samples to measure their UV-absorbance. All measurements were performed in triplicate. The relationship was found to be linear among all AV's encountered in this study.

2.5.3 Antioxidant combinations

Four different antioxidant combinations were investigated to assess potential synergistic or antagonistic effects, these were, quercetin-rutin hydrate (Q +RH), quercetin-ascorbic acid (Q+AA), curcumin-rutin hydrate (C+RH) and curcumin-ascorbic acid (C+AA). Antioxidants were always added in equal measure to a total of 1g antioxidant mass (500mg of each antioxidant) which was then added to emulsions formulated as described previously. Experimental values (Exp) obtained were compared with additive values (Add) predicted if antioxidants did not hinder or complement each other. For this study only, PV's and AV's are given as cumulative values over a 7-day period (measurements taken on the same days as previous experiments and added together). This was done to enable greater clarity of any synergy/antagonism which occurred between antioxidant compounds. Single factor ANOVA was used to assess statistical difference between measurements, a probability value of <0.05 was deemed as significant.

2.6 Statistical analysis

All experiments were carried out in triplicate, with error reported as plus/minus a single standard deviation. Any additional statistical analysis is detailed within individual methodology descriptions.

3 Results and discussion

3.1 Antioxidant activities

The antioxidant activity of ascorbic acid as well as polyphenols including quercetin, curcumin and rutin hydrate is believed to stem predominantly from their ability to free radical scavenge and chelate prooxidant metals (van Acker et al., 1998). Therefore, initial experiments were performed to quantify the free radical scavenging ability and metal (ferrous iron) chelation ability of investigated compounds using DPPH and Ferrozine assays respectively. The Ferrozine assay assesses a compounds ability to chelate ferrous metal ions which are one of the most prevalent and prooxidant metals found in food products (Waraho et al., 2011). To assess why these antioxidants performed like they did, it is useful to consider their chemical structures, shown in Figure 1.

As shown in Figure 2, in terms of DPPH inhibition, curcumin was found to be the least effective as it took the longest time to reach an end value of inhibition which was the joint lowest with rutin hydrate. This makes sense considering that radical scavenging ability is dependent on the ability of these compounds to donate hydrogen atoms (Ammar et al., 2009) which is especially related to the number of hydroxyl (OH) and to a lesser extent other moieties such as methyl (CH_3) groups (Pekkarinen et al., 1999). As shown in Figure 1, curcumin has the lowest number of hydroxyl groups, hence it possesses poorer DPPH scavenging ability. Rutin hydrate is a glycated version of quercetin, and it is believed that the added rutinose group serves to block hydroxyl groups present on the flavonoid backbone structure which reduces hydrogen donating potential (de Araújo et al., 2013). This explains why rutin hydrate displayed poorer DPPH inhibition compared to quercetin. Quercetin and ascorbic acid were found to be the best DPPH inhibitors overall with ascorbic acid showing the greater maximum inhibition than quercetin which was also found in another recent study by Rahim et al, 2017 .The smaller size of ascorbic acid molecules compared to quercetin could allow for easier hydrogen atom donation and be the reason for its superior DPPH inhibition.

All four antioxidants studied, possessed multiple hydroxyl (OH) groups along with carbonyl moieties, which meant they each possessed multiple sites for metal chelation (Leopoldini et al., 2006). Rutin hydrate exhibited the lowest ferrous iron chelation activity, which was found to be significantly lower than the closely structurally related quercetin; a finding which was echoed in another recent study (Yi et al., 2017). Rutin hydrate possesses one less chelating site than quercetin due to the replacement of an OH group with a rutinose dissacharide group (C. Hider et al., 2001). In addition to blocking free radical scavenging activity, the presence of the rutinose group is responsible for lowering metal chelation activity through blocking access to hydroxyl groups where chelation could occur (Jo et al., 2009). Curcumin is understood to possess three possible chelation sites, two of them being OH groups situated on either end of the molecule, and one being the di-ketone moiety in the centre of

the molecule (Daniel et al., 2004). Again however, the greater number of OH groups of quercetin and ascorbic acid are likely why these molecules showed the greatest level of Ferrozine inhibition.

3.2 Antioxidant concentration

The concentration of 'antioxidant' compounds is known to be an important factor in determining whether they possess antioxidant or prooxidant activity (Zhou and Elias, 2013). However, all four compounds in this study enhanced oxidative stability and their ability to do so was only aided by increasing concentration up to the maximum value examined in this study. This can be seen in Figure 3 which shows samples exhibited consistently lower PV's and/or AV's when antioxidants were added compared to P20 control samples in which they were not.

The results in Figure 3 correlate well with earlier results in Figure 2. Ascorbic acid was found to be the most potent antioxidant as this was able to provide greatest oxidative stability when used at lower concentrations (added antioxidant masses of 0.4mg and 0.1mg) compared to other compounds which makes sense as this compound was found to be the most potent of the four investigated in terms of DPPH and Ferrozine inhibition. Interestingly, although curcumin was found to cause significantly lower DPPH and Ferrozine inhibition than quercetin it still exhibited near-identical levels of PV's and AV's to quercetin at all antioxidant concentrations. This is perhaps due to curcumin's highly non-polar nature, allowing it to partition more towards the oil phase and oil-water interface where hydroperoxides are located, which is the predominant location for LO to occur in O/W emulsions (McClements and Decker, 2000). Therefore, although harbouring lower antioxidant activities than quercetin, it could be that the more effective positioning of curcumin molecules within these emulsions due to its reduced polarity is able to make up for this deficit; this is in agreement with the widely reported 'polar paradox' which states that non-polar molecules are more effective in aqueous (polar) systems due to their positioning at the O/W interface and vice-versa (Shahidi and Zhong, 2011). Therefore, increased hydrophobicity is perhaps also why

quercetin combatted LO more effectively than rutin hydrate, in addition to superior antioxidant potency displayed in Figure 2. Furthermore, the smaller molecular size of quercetin compared to rutin hydrate would allow it to locate more efficiently at the O/W interface leading to greater antioxidant concentrations (Yi et al., 2017).

3.3 Iron concentration

Metal contaminants are common in water (Yang et al., 2015), and all food-grade oils are known to contain a degree of iron and other prooxidant metals (Villière et al., 2005). Even trace quantities of these prooxidant metals within emulsions is thought to be sufficient to have a significant impact on oxidative stability (Mozuraityte et al., 2016), particularly in the presence of antioxidants. Ferrous iron (in the form of ferrous sulphate heptahydrate (FSH)) was chosen as the metal contaminant in these experiments as one of the most common, prooxidant and important transition metals in determining LO within oil-in-water emulsions (Waraho et al., 2011). Results are displayed in Figure 4.

Interestingly, control emulsions which varied only in FSH concentrations showed the lowest oxidative stability when no iron was added and no significant difference between samples containing 10 or 50μM FSH. For example, with P20 control emulsions on day 7, as FSH concentration was increased from 0 to 10μM the PV decreases from 9.6 to 8.0mM and AV's decrease from 6.9 to 5.1. This is in contrast to a number of studies which found the addition of iron to P20 emulsions resulted in a prooxidant effect with increasing concentration (Cengiz et al., 2019, Yi et al., 2016). This is possibly because P20 is known to harbour significant quantities of peroxides, which can build up over prolonged periods of storage or, in the case of this study, through emulsion storage at 40°C for 7 days. A study investigating the oxidation of alpha-tocopherol in surfactant micelles by Mancuso et al, 1999 found that addition of higher iron concentrations (50-250μM) caused decomposition of peroxides in P20 samples. This would explain the reduced PV's and AV's in P20 control emulsions with increased FSH concentration from 0 to 10 or 50μM.

Figure 4 results also show that P20 control emulsions exhibited reduced AV's with increased FSH concentration from 0 to 10 or 50μM; a finding which was also observed in a study by Nuchi et al, 2001 which assessed the oxidative stability of methyl linoleate dispersions in response to iron. When higher FSH concentrations of 100μM were used in this study, P20 emulsions were observed to physically destabilise via phase separation after one day; hence addition of FSH appears to result in P20 being removed from the O/W interface, physically destabilising the emulsions and also resulting in the removal of prooxidant species (peroxides) with it making FSH appear to possess a form of antioxidant activity within P20 emulsions.

When no FSH was added to 1% P20 emulsions, all added compounds caused significant reductions in PV's and AV's, enhancing oxidative stability. With the addition of 10μM FSH, all compounds show reduced difference in oxidative stability in terms of PV's and AV's compared to the control; ascorbic acid and quercetin even showed increased AV's. At maximum FSH addition of 50μM there is little to no significant difference in PV's, however at this concentration quercetin and rutin hydrate caused large increases in AV's, illustrating their prooxidant nature under higher ferrous iron concentrations. The prooxidant nature of flavonoids such as quercetin and rutin hydrate in the presence of iron stems primarily from their possession of a catechol moiety, which becomes oxidised by ferric iron to a quinone. This results in the formation of electrophiles which act as potent prooxidants, in addition to the generation of quinone groups which are themselves ineffective at scavenging free-radicals (Keceli and Gordon, 2002). This finding was also described in a study by Osborn and Akoh, 2003 which investigated the behaviour of quercetin in combination with iron. This also explains why curcumin (which was shown to be a less effective chelator of ferrous ions than quercetin or rutin hydrate through a Ferrozine assay) is able to provide greater oxidative stability in the presence of higher FSH concentrations as it does not possess a catechol group that can become oxidised. A study assessing the impact of quercetin on O/W emulsions with added ferrous ions found that the quercetin concentration needs to be high

enough, relative to ferrous iron, in order to exhibit an antioxidant effect (Yi et al., 2017). Another study found that rutin acted as a potent antioxidant with the addition of no iron, but its efficacy was greatly reduced and it exhibited prooxidant behaviour with the addition of 50μM ferric chloride (Yang et al., 2015). All four antioxidants investigated would have also undergone a degree of metal-catalysed oxidation in the presence of ferrous iron causing the generation of hydrogen peroxide which may then reduce to the highly prooxidant hydroxyl radical (Zhou and Elias, 2012) which further explains their increased prooxidant nature under the addition of FSH. From these results it is clear that the efficacy of all antioxidants to combat LO is only hindered by addition of ferrous iron.

It was initially hypothesised that compounds which were found more capable of chelating ferrous iron via the Ferrozine assay would therefore provide greater oxidative stability in the presence of added FSH. However, these results indicate that the prooxidant behaviour of these compounds in the presence of iron has far greater influence on LO than their antioxidant ability to chelate ferrous iron.

3.4 Effect of emulsifier type

The choice of emulsifier is widely regarded as one of the most important factors in determining the oxidative stability of O/W emulsions as it effects surface charge, interfacial thickness and droplet size. These experiments specifically investigated the efficacy of four different antioxidants within emulsions formulated with different emulsifiers and assessed the reasons for this. Three emulsifiers were used in this study which were SDS (anionic), P20 (non-ionic) and CAT (cationic) surfactants and these were chosen to provide different surface charges to emulsion droplets. Droplet sizes of these formulated emulsions together with their native pH and zeta potential (ZP) values along with ZP values of the four antioxidants at the corresponding emulsion pH's are given in Table 1.

From Table 1 it can be seen that CAT caused the formation of acidic emulsions whereas SDS and P20 formed fairly neutral ones. As expected SDS and CAT exhibited strongly negative and positive zeta potential values respectively indicating their droplet charge status.

P20 however also exhibited a significantly negative zeta potential in spite of its non-ionic character; this phenomenon can primarily be attributed to the presence of surface-active free fatty acids within the dispersed phase of sunflower oil which migrate to the O/W interface and impart a negative charge (Waraho et al., 2011). In addition, OH^- ions present within emulsions are known to preferentially adsorb onto the polar head groups of P20 (McClements, 2004) which locate at the oil-water interface and impart further negative charge. All antioxidants possessed negative zeta potentials as particles which decreased under lower pH values encountered with CAT however constantly remained negative; this gives indication of the predominantly negative charge associated with these antioxidants within each emulsion formulation.

Oxidative stabilities of emulsions formulated with the three different emulsifiers in the presence and absence of each antioxidant are shown in Figure 5. In SDS emulsions, ascorbic acid acted as a prooxidant which was illustrated through its high AV's compared to the control. This is due to the ability of ascorbic acid to reduce ferric iron to the far more potent prooxidant ferrous iron (Choe and Min, 2009) which is then strongly attracted to the oil-water interface due to the negative charge which SDS imparts. Quercetin, curcumin and rutin hydrate in SDS emulsions were all however able to exert substantial antioxidant effect in spite of a negative emulsion surface charge; this is perhaps because these compounds are still able to be solubilised and incorporated into SDS micelles via hydrophobic interaction which was detailed with quercetin in one study (Liu and Guo, 2006). Furthermore, even when these antioxidants are kept away from the interface they are still able to exert antioxidant activity through other mechanisms such as through chelation of prooxidant metals in the aqueous phase.

Antioxidants had either no significant effect or a prooxidant effect on CAT emulsions. This initially seemed counter-intuitive as these antioxidants possessed a negative charge so were expected to be attracted to positively-charged emulsion droplets and locate at the oil-water interface; however this was not the case. The lower pH of CAT emulsions meant that the

solubility of ferric ions was higher, and all antioxidants used were capable of reducing ferric ions to more potent, prooxidant ferrous ions. However, again it seemed that ascorbic acid yielded the greatest potential to convert ferric iron to ferrous iron as this 'antioxidant' was found to be the strongest prooxidant in CAT emulsions.

As was seen earlier in the section on antioxidant concentration in P20 emulsions, all antioxidants were able to exhibit a significant antioxidant effect in contrast to the emulsions which used ionic emulsifiers. This is likely due to a more neutral pH than CAT, a lower negative surface charge than SDS and the larger polar head group of P20 molecules which will situate at the oil-water interface and be able to accommodate more antioxidants within their micellar structure (Huang et al., 1997) bringing antioxidants to the interface where they can exert greater antioxidant effect.

3.5 Oil phase volume

The four antioxidants used in this study were known to differ in their polarity, which is understood to affect partitioning behaviour within emulsions. As it was widely reported that the partitioning of antioxidant molecules within emulsions has substantial impact on their efficacy (Berton-Carabin et al., 2014, López-Martínez and Rocha-Uribe, 2018) it was of interest to see how each antioxidant performed at combatting LO within emulsions of different phase volumes. Experiments were first performed to assess the partitioning behaviour of the four antioxidants in a water-octanol mixture to obtain an indication of their preference for the polar or non-polar phase, results are displayed in Table 2.

As can be seen from Table 2, quercetin and curcumin exhibited highly hydrophobic nature with Log P values >> 0, whereas ascorbic acid exhibited a hydrophilic nature. Curcumin was found to be by far the most hydrophobic molecule, and rutin hydrate showed only slight hydrophobicity with a Log P value close to 0.

Firstly, considering emulsions created with no added antioxidants in Figures 6 and 7 it can be seen that increasing oil phase volume leads to an increase in both PV's and AV's.

Evidently however, looking at AV's, there is a certain quantity of secondary oxidation products (mainly unsaturated aldehydes) contained within the sunflower oil prior to experiments being performed as on day 0 the AV roughly doubles when the oil phase volume is doubled. Therefore, it is pertinent to instead consider the increase in AV from day 0 to day 7 with each oil phase volume used which were 3.2 ± 0.4, 4.3 ± 0.2, 2.7 ± 0.3, and 3.1 ± 0.8 for 5%, 10%, 20% and 40% oil phase volumes respectively which shows no correlation for AV's with increasing oil phase volumes in real terms. However, this is not the same for PV's which always began at around 0mM and only ended up at higher values with increased oil phase volumes which means that the overall impact of oil phase volume on LO is that it increases with oil phase volume. This is because, increasing oil concentration will increase the amount of lipids available for oxidation, in addition to decreasing the separation distance between oil droplets which increases potential for labile species (such as free radicals and hydroperoxides) on nearby oil droplets to react and propagate LO (Berton-Carabin et al., 2014). Interestingly however, other studies have reported the opposite effect, saying that increasing oil phase volume will (1) lead to the generation of larger oil droplets (as shown in this study in Table 3) which has been detailed to retard LO through generation of larger oil droplet surface areas (Gohtani et al., 1999), (2) supress creaming which has been held responsible in a previous study for enhanced oxidative stability as it lowered the amount of oil droplets in contact with the air (Sun and Gunasekaran, 2009) and (3) will decrease aqueous phase volume, thereby decreasing the amount of water soluble prooxidants such as transition metals and enhancing oxidative stability, as was noted in a study by Kargar et al, 2011. The earlier finding in this study that AV's roughly doubled with doubling oil phase volume confirmed that the sunflower oil used in these experiments contained significant amounts of secondary oxidation products; meaning that a great amount of LO had taken place prior to its measurement. Due to the known freshness of sunflower oil used in these experiments, it is likely that this particular type of oil contains large amounts of prooxidants such as hydroperoxides which have resulted in significant generation of secondary oxidation products over a limited storage time. It is therefore believed that the

high amount of prooxidants contained within the sunflower oil was the main reason for the trend exhibited in this study with respect to control emulsions.

The well referenced 'polar paradox' states that more polar antioxidants (such as ascorbic acid) are more effective in non-polar media (such as bulk or high oil phase volume oils), whereas non-polar ones are more effective in polar media (such as aqueous or high aqueous phase volume emulsions) (Shahidi and Zhong, 2011) as this helps their orientation at the oil-air/oil-water interface where LO is thought to predominantly occur. In terms of AV's, the addition of antioxidants was able to prevent any significant increases compared to control emulsions over a 7 day period, and hence no differences in efficacy with oil phase volume could be established from this data. However, in terms of PV's, substantial differences could be found when antioxidants were added to emulsions with different amounts of oil. As PV's only ever increased, the percentage reduction of PV's compared to control emulsions are given in Table 4 on day 7 only, as this day gives clearer indication of each antioxidants efficacy.

From Table 4, it can be seen that the two antioxidants with the highest Log P values (most hydrophobic), curcumin and quercetin, were able to prevent significantly more PV formation in 5% and 10% oil phase volumes than at 20% and 40% and thus behave in accordance with the 'polar paradox'. It is likely that when these highly hydrophobic molecules partition, they 'bury' themselves inside the core of oil droplets and away from the interface where LO is most prevalent; this phenomenon is further exacerbated by the larger droplet sizes with increasing oil phase volume meaning hydrophobic antioxidants are able to position even further away from the interface (deeper in the oil droplet core). This explains why the decrease in PV reduction with oil phase volume exhibits the most extreme change in the case of curcumin, the most hydrophobic molecule. This also explains why rutin hydrate, a molecule with both hydrophilic and hydrophobic nature, exhibits an initial enhancement in PV reduction from 5-10% oil phase volume, followed by a decrease thereafter; as there is an optimal oil phase volume of 10% to allow for its most effective partitioning for combatting LO.

As a hydrophilic molecule, ascorbic acid did not follow the opposite trend, and following the same logic it is probable that this is because water serves as the continuous phase, so even when there is less water at high oil phase volumes, there is less possibility of ascorbic acid being able to distance itself from the oil-water interface.

3.6 Antioxidant combinations

Quercetin-rutin hydrate emulsions were found to generally exhibit antagonistic behaviour however the effect only became statistically significant with the addition of 10μM FSH, as this caused a significant increase in AV's and no change to PV's. One study reported that the use of quercetin and rutin in combination caused a synergy in their ability to reduce ferric (Fe^{3+}) to the more prooxidant ferrous (Fe^{2+}) iron ions (Hajimehdipoor et al., 2014) which could be the reason for this antagonistic effect. This antagonism is likely to be due to quercetin harbouring higher reduction potential than rutin hydrate (Bors et al., 1995), meaning quercetin acts as a primary antioxidant, and rutin hydrate acts as a secondary antioxidant by regenerating quercetin from its radical form; as quercetin is known to reduce ferric ions to the more potent prooxidant ferrous ions more effectively than rutin hydrate, this causes an antagonistic effect on oxidative stability. Curcumin-rutin hydrate emulsions showed only additive behaviour in combination which likely means these compounds do not interact with each other and perhaps share similar dominant antioxidant mechanisms.

Quercetin-ascorbic acid mixtures acted synergistically in combatting LO at all FSH concentrations, significantly increasing either PV's or AV's; and curcumin-ascorbic acid emulsions showed synergism in the presence of 10μM FSH with a significant increase in experimentally obtained AV's compared to PV's. Ascorbic acid has been reported to regenerate quercetin from its oxidised quinone structure through reducing it, and is widely reported to possess a protective/enhancing effect on polyphenolic compounds (Inoue et al., 2006, Skaper et al., 1997) such as quercetin and curcumin. However, as earlier results showed ascorbic acid was able to combat LO either at least as effectively if not more effectively than quercetin and curcumin this cannot be the reason for the synergy.

Furthermore, as quercetin and ascorbic acid were shown to perform similarly in terms of their abilities to chelate ferrous via Ferrozine inhibition, it is likely that their synergism is owed to the fact that their different antioxidant strengths lie with mechanisms other than free radical scavenging or metal chelation. One study showed that ascorbic acid was capable of quenching singlet oxygen more effectively than quercetin (Fatima et al., 2016), whereas another concluded that quercetin was more effective at inhibiting lipoxygenase than ascorbic acid (Silva et al., 2000). Therefore, through combining antioxidants with different antioxidant mechanistic strengths, it is likely that a synergistic effect will occur as one antioxidant can account for the mechanistic shortfall of the other and enhance their overall effectiveness.

4 Conclusions

This study has shown the efficacy of four natural antioxidants to enhance oxidative stability of O/W emulsions under a range of different emulsion environments. In P20 emulsions with no added ferrous ions, ascorbic acid and quercetin were found to serve as the most potent antioxidants which was in line with what initial DPPH and Ferrozine assays predicted. Curcumin however was found to reduce the formation of primary and secondary oxidation products much more effectively than rutin hydrate, despite rutin hydrate being more effective at inhibiting DPPH and this was attributed to the highly hydrophobic nature of curcumin enabling more effective partitioning behaviour. The prooxidant effect of ferrous iron on these antioxidants was concluded to be of far greater importance to these compounds than their ability to chelate ferrous iron as they all lost their antioxidant activity at higher ferrous iron concentrations. Antioxidants performed less effectively in ionic emulsions compared to non-ionic emulsions which was believed to be due to a variety of reasons including changes in emulsion pH and reduced antioxidant presence at the oil-water interface. Higher emulsion oil phase volumes were found to promote LO and reduced the efficacy of antioxidants, particularly highly hydrophobic ones, which was believed to be due to these compounds partitioning deep within oil droplets where they could not function as effectively at combatting LO. Synergy between compounds in combatting LO was exhibited when ascorbic acid was combined with either quercetin or curcumin; a finding attributed to different compounds performing particular antioxidant mechanisms more effectively than the other and hence making up for the antioxidant deficiencies of the other compound. Ultimately, this work has shown how key formulation parameters impact upon the efficacy of common, naturally occurring antioxidant compounds and thus will be highly useful in assessing their suitability for specific food applications.

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Figure captions

Figure 1- Chemical structures of investigated compounds

Figure 2- Antioxidant activities of investigated compounds

Figure 3- Oxidative stabilities of 1% (w/w) P20 emulsions under varying antioxidant concentrations. Where Q= quercetin, C=curcumin, RH=rutin hydrate, and AA= ascorbic acid.

Figure 4- Oxidative stabilities of emulsions under varying FSH concentrations. Where Q= quercetin, C=curcumin, RH=rutin hydrate, and AA= ascorbic acid.

Table 1- Droplet sizes and native emulsion pH values with different emulsifiers along with zeta potentials of these emulsions and antioxidants at the corresponding emulsion pH

Figure 5-Oxidative stability of emulsions formed with different emulsifiers. Where Q= quercetin, C=curcumin, RH=rutin hydrate, and AA= ascorbic acid.

Table 2- Partition coefficients of the four antioxidant compounds studied

Table 3- Droplet size (D [3,2]) of emulsions formulated with different oil phase volumes

Figure 6- Oxidative stability of emulsions with oil phase volumes of 5% and 10%. Where Q= quercetin, C=curcumin, RH=rutin hydrate, and AA= ascorbic acid.

Figure 7- Oxidative stability of emulsions with oil phase volumes of 20% and 40%. Where Q= quercetin, C=curcumin, RH=rutin hydrate, and AA= ascorbic acid.

Table 4- Reduction in PV's of emulsions containing different oil phase volumes and antioxidants on day 7 of LO measurement

Figure 8- Effect of antioxidant combinations on oxidative stability. Experimental values given to the right of predicted values as solid black bars. Where Q= quercetin, C=curcumin, RH=rutin hydrate, and AA= ascorbic acid.

Emulsion	Emulsion droplet size D [3,2] μm	Emulsion pH	Emulsion zeta potential (mV)	Antioxidant zeta potential at emulsion pH (mV)			
				Quercetin	Curcumin	Rutin hydrate	Ascorbic acid
Anionic (SDS)	5.74 ± 0.20	6.94 ± 0.12	-110.0 ± 2.1	-44.1 ± 1.1	-59.7 ± 1.1	-32.8 ± 1.2	-29.4 ± 2.0
Non-ionic (P20)	5.86 ± 0.07	5.99 ± 0.07	-31.4 ± 0.2	-44.1 ± 1.3	-43.8 ± 2.8	-32.5 ± 0.4	-11.5 ± 1.5
Cationic (CAT)	6.82 ± 0.14	3.29 ± 0.10	86.0 ± 2.4	-8.6 ± 0.4	-9.1 ± 1.5	-10.4 ± 0.8	-19.9 ± 0.5

Table 1- Droplet sizes and native emulsion pH values with different emulsifiers along with zeta potentials of these emulsions and antioxidants at the corresponding emulsion pH

Sample	Log P
Quercetin	1.95 ± 0.07
Curcumin	3.55 ± 0.07
Rutin Hydrate	0.40 ± 0.01
Ascorbic Acid	-1.86 ± 0.09

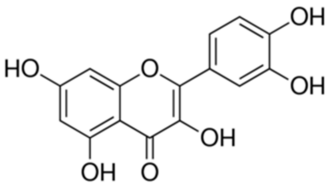
Table 2- Partition coefficients of the four antioxidant compounds studied

Oil phase volume (%)	Droplet size D[3,2] µm
5	3.9 ± 0.2
10	5.9 ± 0.1
20	8.5 ± 0.5
40	9.2 ± 0.6

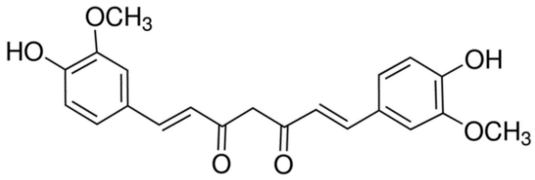
Table 3- Droplet size (D [3,2]) of emulsions formulated with different oil phase volumes

Antioxidant	PV reduction on day 7 (%) in:			
	5% Oil	10% Oil	20% Oil	40% Oil
Quercetin	74.4 ± 5.5	69.7 ± 2.2	54.8 ± 5.2	43.3 ± 2.3
Curcumin	64.6 ± 5.5	64.7 ± 1.8	38.8 ± 5.4	28.2 ± 3.9
Rutin hydrate	24.3 ± 5.7	36.5 ± 2.6	13.0 ± 6.6	18.5 ± 7.1
Ascorbic acid	60.6 ± 1.0	62.8 ± 6.0	52.9 ± 2.6	42.8 ± 3.9

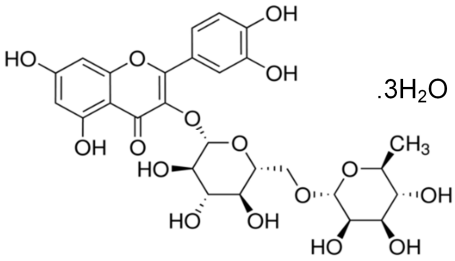
Table 4- Reduction in PV's of emulsions containing different oil phase volumes and antioxidants on day 7 of LO measurement



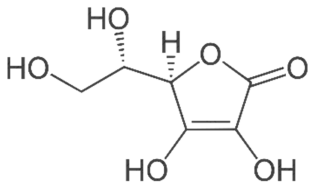
Quercetin



Curcumin

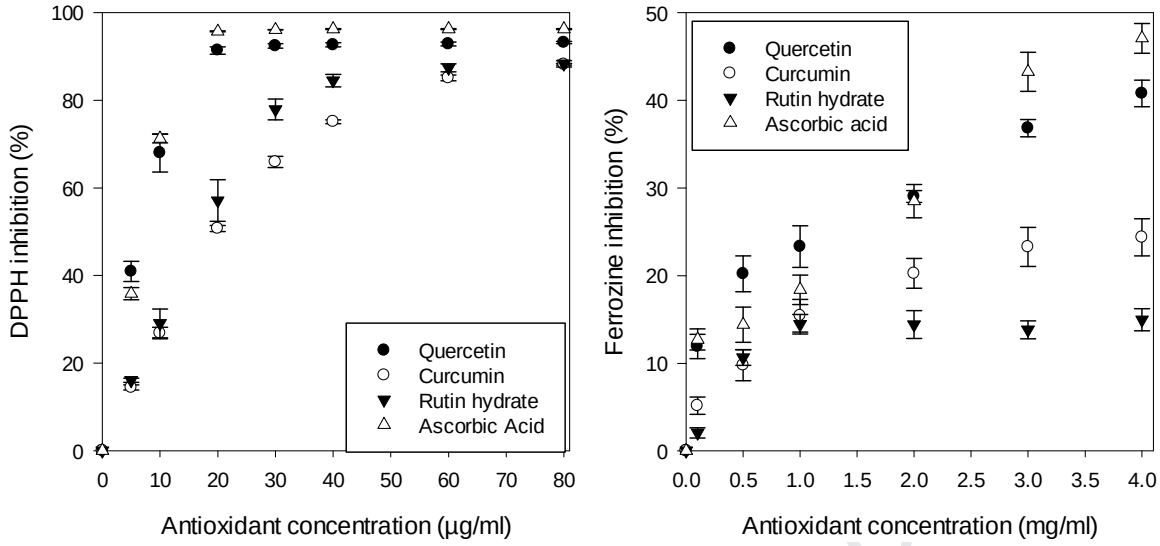


Rutin hydrate



Ascorbic acid

Figure 1- Chemical structures of investigated compounds



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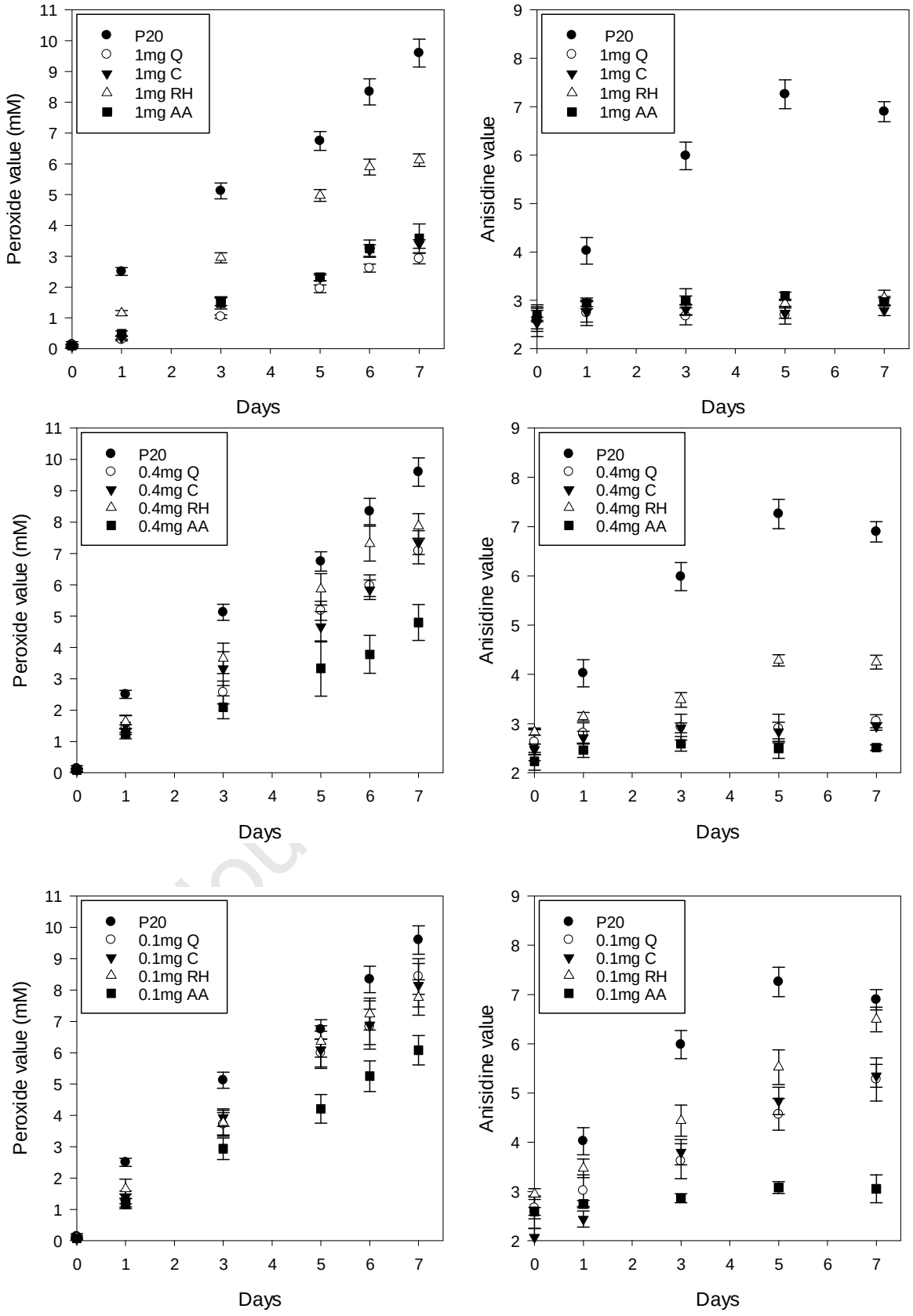

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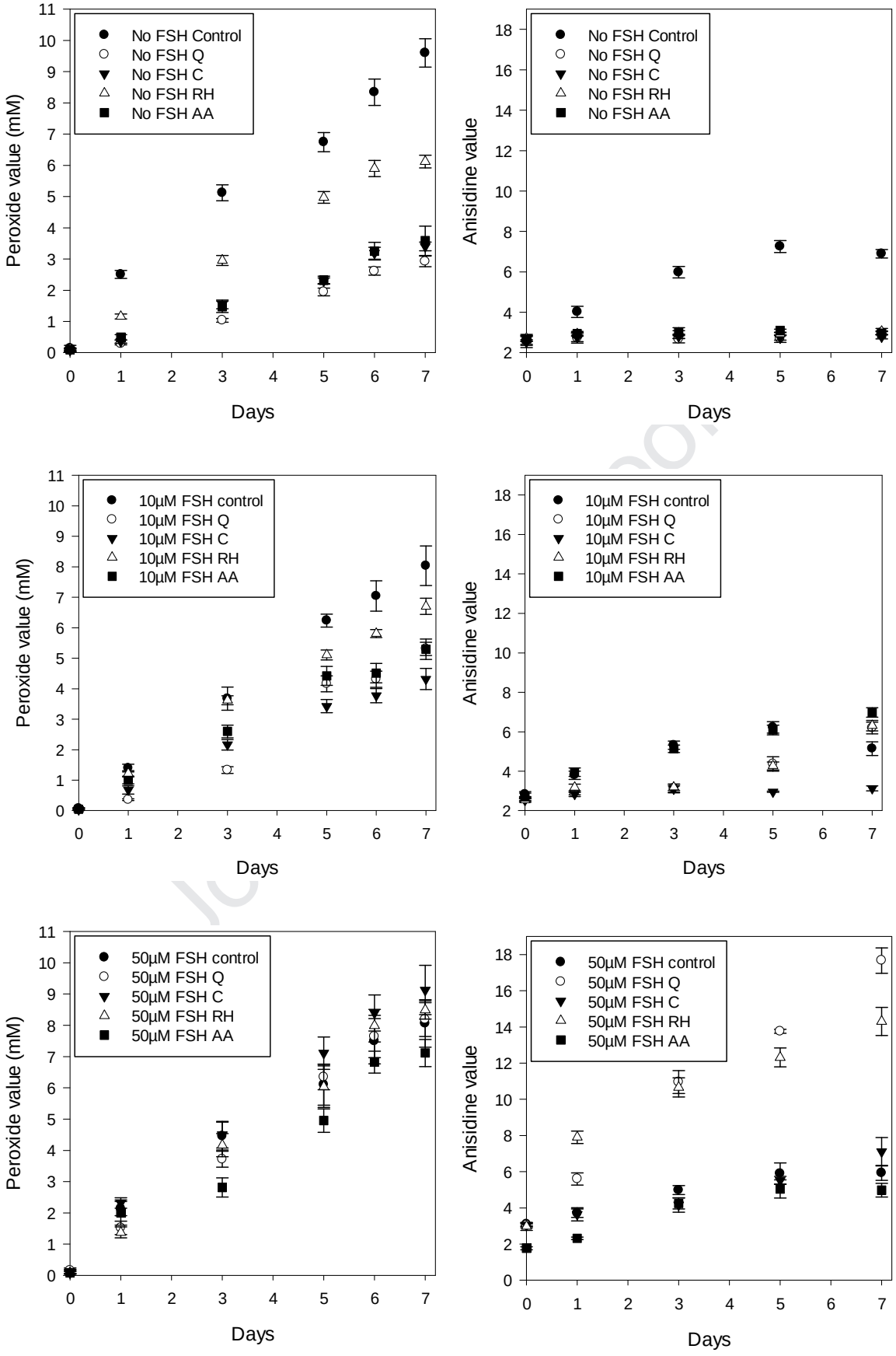

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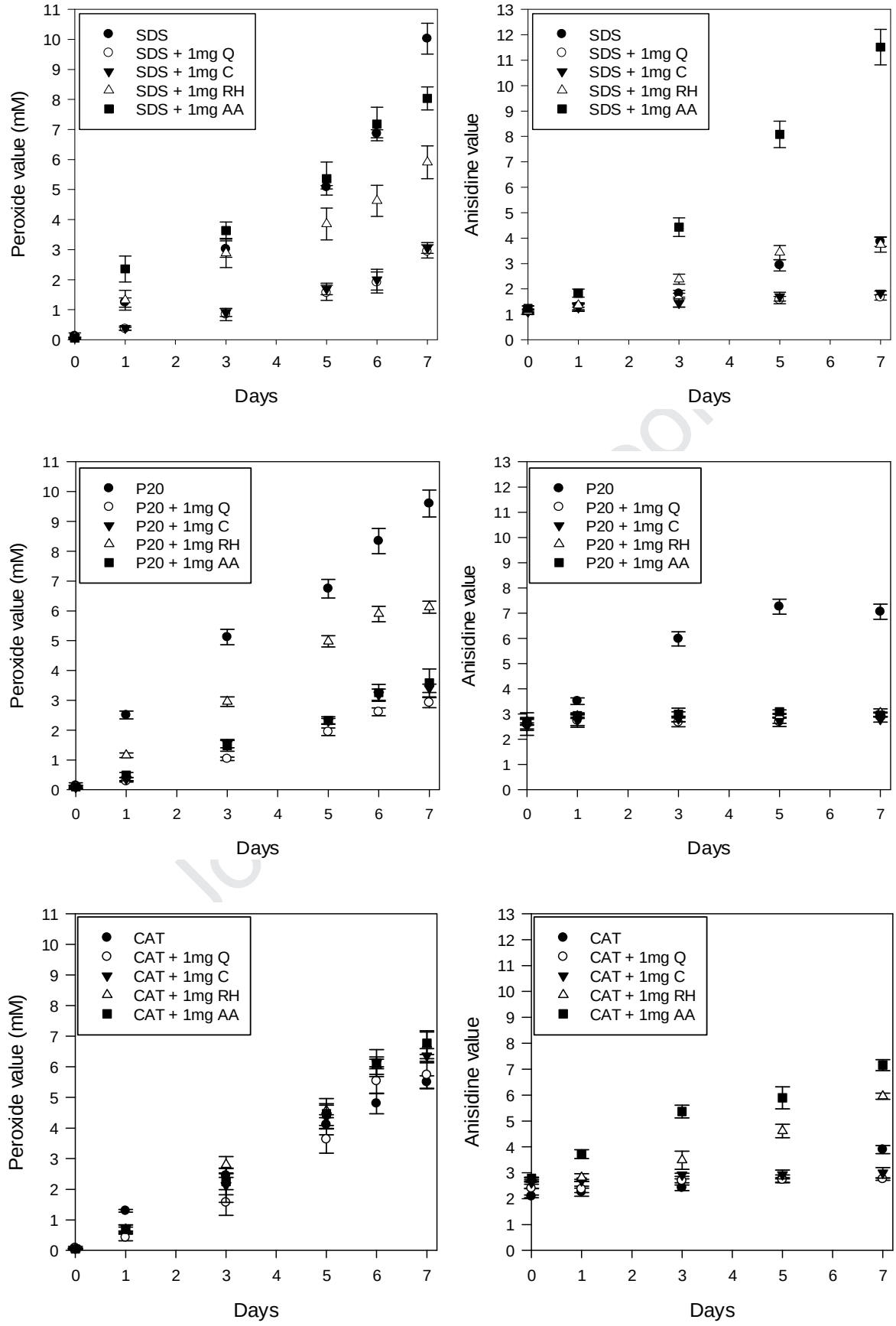

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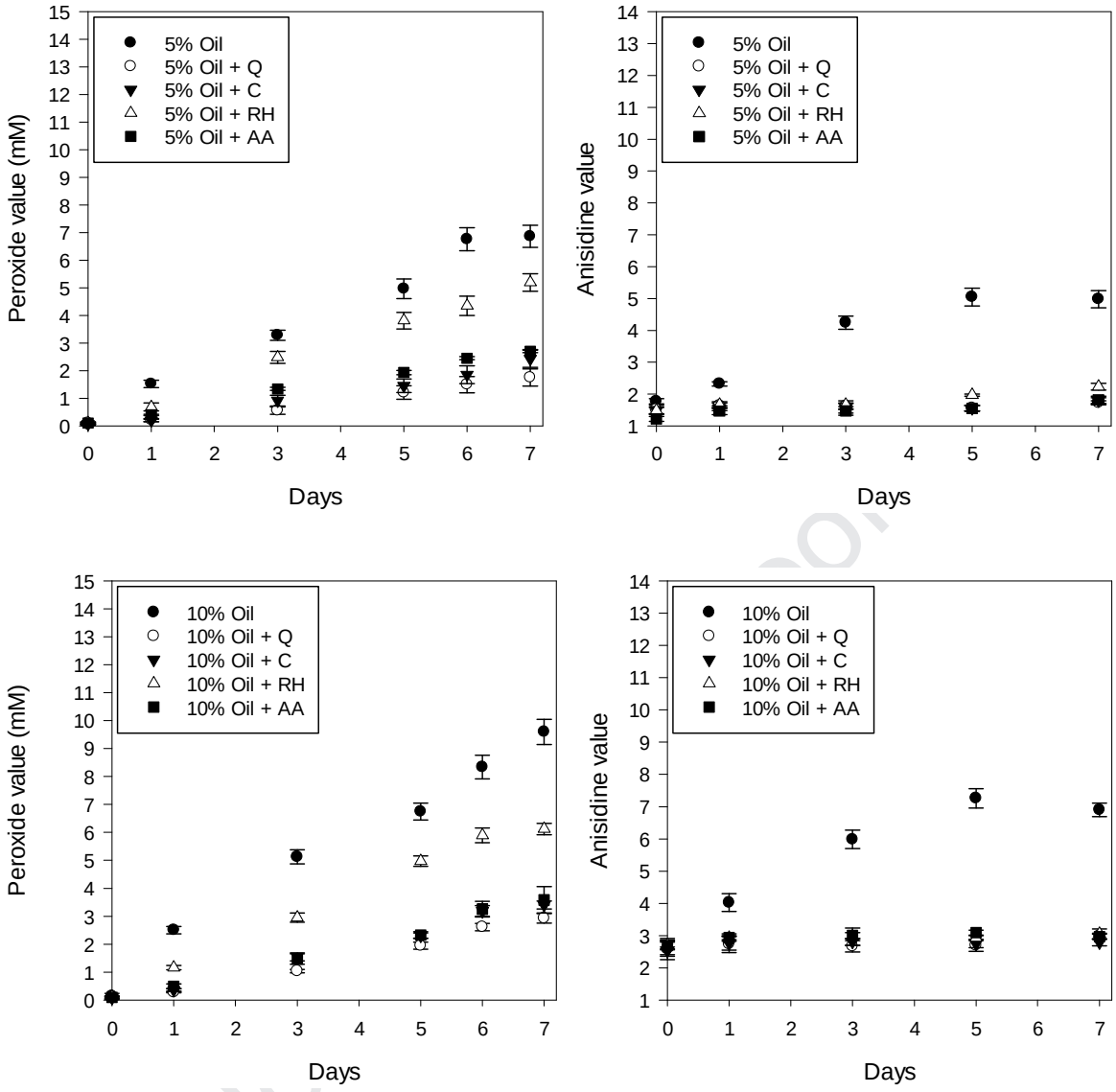

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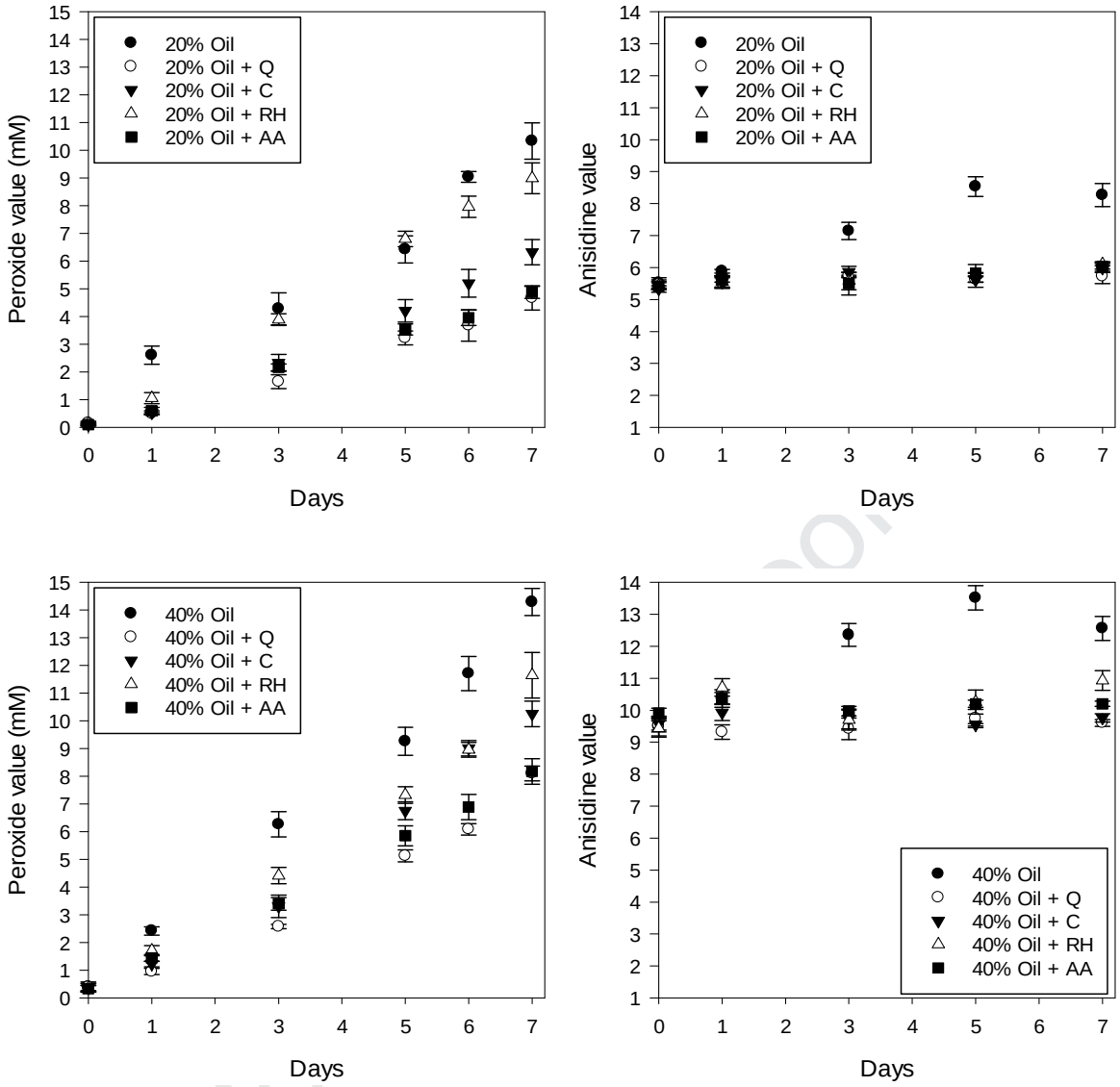

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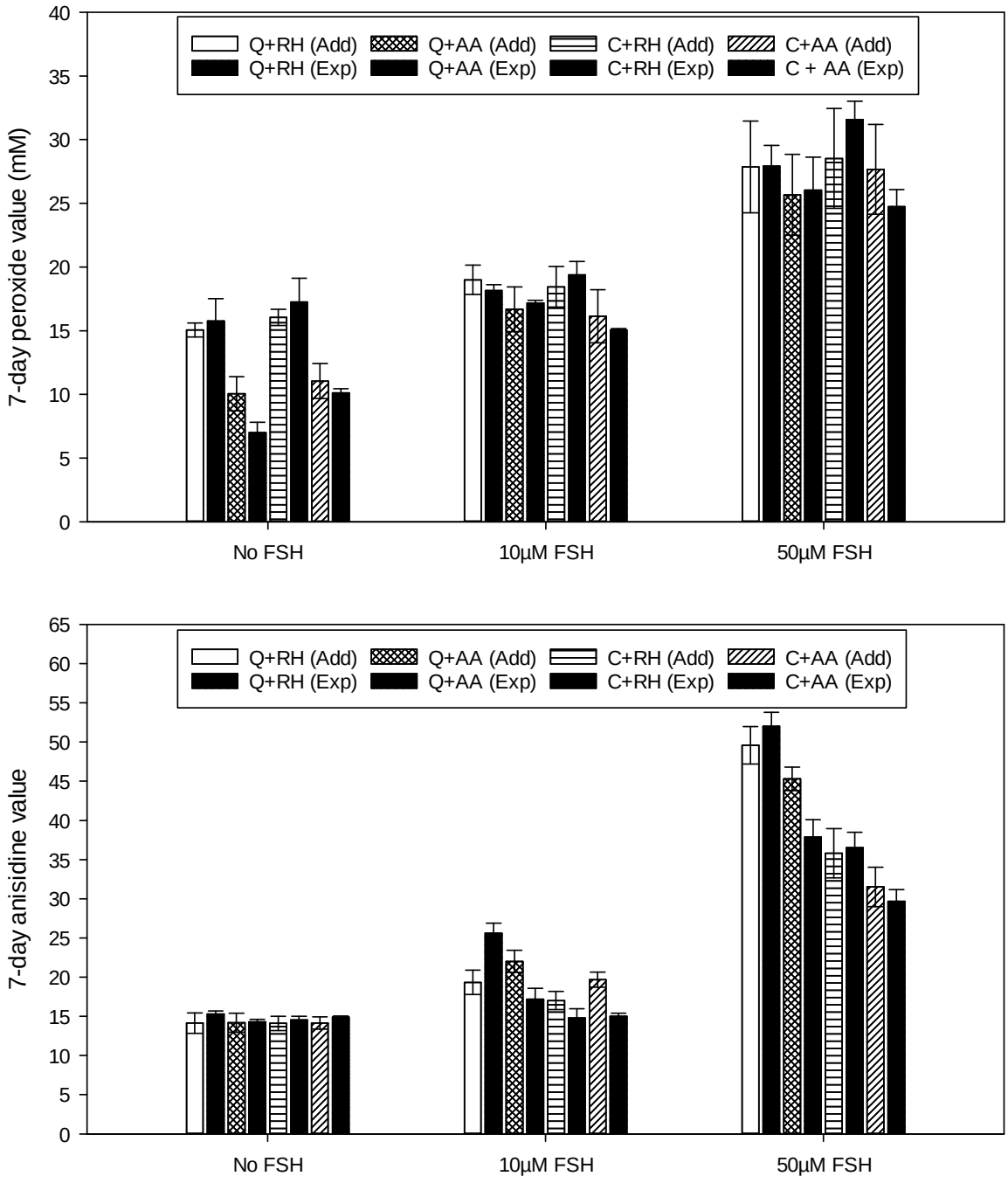

Figure 8- Effect of antioxidant combinations on oxidative stability. Experimental values given to the right of predicted values as solid black bars. Where Q= quercetin, C=curcumin, RH=rutin hydrate, and AA= ascorbic acid.

- Antioxidant behaviour of quercetin, curcumin, rutin hydrate and ascorbic acid explained within different O/W emulsion environments.

- Generally, antioxidants with greatest DPPH and Ferrozine inhibition combatted lipid oxidation most effectively.

- Pro-oxidant activity of antioxidants in presence of iron far more important to oxidative stability than their antioxidant iron chelating activity.

- Non-polar compounds more effective than polar compounds in low oil phase volume emulsions; providing more evidence of the ‘polar paradox’.

Author contributions

1. **John Noon-** Formulated initial hypothesis, designed and carried out all experimental work and analysis. Wrote first draft of the manuscript. Corresponding author.

2. **Tom Mills-** Provided input on experimental design, analysis of data and edited first draft of manuscript. Secondary PhD supervisor to John Noon.

3. **Ian Norton-** Provided input on experimental designs, analysis of data and edited second draft of manuscript. Primary PhD supervisor to John Noon.

Conflicts of Interest

The authors have no conflicts of interest to disclose.