Oxidative Stability of Sunflower Oil Bodies

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ABSTRACT

This study investigates the oxidative stability of sunflower oil body suspensions (10% w/w lipid). Two washed suspensions of oil bodies were evaluated over 8 days at three temperatures (5°C, 25°C and 45°C) against three comparable sunflower oil emulsions stabilized with dodecyltrimethylammonium bromide (DTAB), polyoxyethylene-sorbitan monolaurate (tween 20) and sodium dodecyl sulphate (SDS) (17mM). The development of oxidation was monitored by the production of lipid hydroperoxides and hexanal. Oil body suspensions were significantly more stable to oxidation than the three emulsions at each temperature. The reasons and biological demands for oxidative stability in-vivo are clear although they have never been previously demonstrated ex-vivo.

Hexanal, lipid hydroperoxides, oil body, oxidation, sunflower, surfactants.

INTRODUCTION

Oxidation of lipids in food can result in the production of anti-nutritional factors and undesirable flavour and aroma compounds, the process of oxidation may also generate genotoxic and cytotoxic oxygenated aldehydes (*1*). Knowledge and control of lipid oxidation is therefore fundamental to the preservation of a food's nutritional and sensory quality, and the ability to limit or delay the onset of oxidation is important to the food industry.

Tocopherol (vitamin E) can confer increased oxidative stability to lipid emulsions through an extended induction period due to scavenging of lipid peroxyl radicals (*2*). Recent work has shown that seed tocopherol is an intrinsic component of both oilseed and cereal grain oil bodies (*3-5*). Oil bodies are discrete plant organelles found principally within desiccation resistant oilseeds; their function is to store neutral lipids during seed dormancy. They are formed of a neutral lipid core surrounded by a phospholipid monolayer, associated phytochemicals and a coat of strongly amphiphilic proteins (*6, 7*). Oil bodies therefore offer oxidative protection to seed oil in-vivo. It is not known however, if oil bodies recovered from oilseeds are stable against oxidation in-vitro.

In formulated emulsions, surfactant chemistry has the potential to significantly effect the development of oxidation through the promotion of metal catalyzed oxidation (*8*) or the presence of residual surfactant hydroperoxides (*9*). A study by Mancuso et al. (*8*) used a range of surfactants at equimolar concentrations of cationic, anionic and non-ionic surface charge to illustrate their potential to effect oxidative stability.

Oil bodies from sunflower (*Helianthus annus)* were extracted and washed at two purification levels (water washed and salt washed) to remove extraneous proteins (*4*); these natural suspensions were then assessed for oxidative stability at different temperatures in comparison to sunflower oil emulsions stabilized with a range of synthetic surfactants (cationic dodecyltrimethylammonium bromide (DTAB), nonionic tween 20 and anionic sodium dodecyl sulphate (SDS)) to validate their potential as natural food emulsions.

MATERIALS AND METHODS

Materials

All Chemicals are analytical grade or higher and were sourced from Fisher UK (Loughborough, UK) unless otherwise stated. Surfactants were sourced from Sigma-Aldrich Co. (St Lewis, MO, USA)

Methods

Oil Body Recovery. Water washed and salt washed oil bodies were prepared as described previously (*4*). In brief, dehulled sunflower seeds were wet milled, the resulting slurry filtered and the filtrate centrifuged to isolate the buoyant oil body fraction. Oil bodies were water washed by resuspension in buffer (10mM sodium phosphate buffer, pH 7.5) and recentrifugation. This process was repeated in 1M sodium chloride buffered solution and again in buffer to yield salt washed oil bodies.

Lipid Extraction. Moisture content of oil body and seed samples (5g) was determined gravimetrically by drying to constant weight (vacuum oven, 60^{o}C). Lipid content of both oil body and seed samples was calculated by repeated extraction with diethyl ether after freeze drying and determined gravimetrically (*10*).

Emulsion Formulation. Oil body suspensions at 10% (w/w) lipid were formulated with sodium azide as a preservative (0.02mM) and stored at 5^{o}C under nitrogen prior to use. Emulsions were formulated from sunflower oil extracted from the same batch of dehulled sunflower seeds by diethyl ether extraction, excess solvent was removed by drying under nitrogen. Extracted lipid was added slowly to surfactant solutions (dodecyltrimethylammonium bromide, tween 20 and sodium dodecyl sulphate predissolved for 24 hours at 17mM in deionised water) using an Ultraturax homogenizer (IKA-WERKE, Staufer, Germany) at 10,000 rpm, the emulsion was further homogenized by three passes through an Emulsiflex-C5 (Glan Creston, Stanmore, UK) at an emulsification pressure of 10,000kPa.

Particle Size Distribution. Mean droplet diameter was measured by use of a low angle light scattering detector (Malvern Mastersizer, Malvern Instruments, England) and represented as the numerical mean droplet diameter, D(v,0.5).

Protein Characterization. Isolated oil bodies (0.1g) were suspended in distilled water (1mL) and vortexed (2min); to which 100µL of 100% TCA (w/v) was added. The tube was chilled on ice for 30

min to promote the precipitation of proteins and centrifuged (13,000rpm for 5min). The supernatant was aspirated and discarded and the pellet vortexed with 200µL SDS solution (10% w/v SDS, 10mM β-mercapto-ethanol, 20% v/v glycerol, 0.2M tris-HCl, pH 6.8, 0.05% bromophenol blue.) Proteins were resolved by SDS-PAGE using 15% and 4.0% polyacrylamide gels in the separating and stacking gel respectively. After electrophoresis the gel was stained with BioRad coomassie blue (R-250) and destained with excess methanol:water:acetic acid (9:9:2 v/v).

Zeta Potential. Zeta potential measurements were carried out using a Malvern Zetasizer IV (Malvern instruments, UK) (*11, 12*). Oil bodies (20mg dry weight basis) were suspended in 10mM sodium phosphate buffer, pH 7.5 (10ml, 0.2µm filtered) and sonicated (10 min). The suspension was then further diluted to achieve a final concentration of 30µg.mL^{-1}. Suspension buffer was adjusted prior to the addition of sample to pH values ranging between pH 2.0 and pH 12.0.

Lipid Hydroperoxides. Lipid hydroperoxide concentration was measured using the method of Shantha and Decker (*13*) as modified by Nuchi et al.(*9*).

Hexanal Headspace Concentration. Hexanal concentration was calculated from the headspace concentration using solid phase micro-extraction gas chromatography mass spectroscopy (SPME-GCMS) (*14*). This was achieved by adding 1,2 dichlorobenzene (100ppmv) as an internal standard (10µl) to the emulsion or oil body suspension (1mL) and capping in a 20mL vial. Headspace concentration was determined using a CTS Analytics PAL system autosampler and a DSQ and Trace GC Ultra (Thermo Electron Corporation). Samples were agitated (50^{o}C for 3 min) prior to extraction (20 min at 50^{o}C), desorption was achieved in 5 min (250^{o}C). Compounds were separated with 30mL.min^{-1} nitrogen, a ZB-5 Phenomenex gas chromatography column (Macclesfield, UK) and oven temperatures controlled at 40^{o}C (1 min) then ramped (3^{o}C.min^{-1}) to 140^{o}C, ramped (15^{o}C.min^{-1}) to 210^{o}C and held at 210^{o}C for 1min. Volatiles were quantified with authentic standards.

Oxidation Conditions. Samples were stored at 5^{o}C, 25 ^{o}C and 45 ^{o}C with restricted lighting in closed 20mL glass vials.

Statistics. The experiment was carried out in a fully balanced, randomized experimental design using triplicate samples for each data point. Statistical analysis was performed by one-way ANOVA ($P<0.05$).

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RESULTS AND DISCUSSION

The development of oxidation in two oil body suspensions was assessed by comparison with oil-in-water emulsions stabilized by SDS, tween 20 and DTAB. Droplet size, lipid content and initial hydroperoxide concentration were measured and controlled to minimize inter-sample variation. The presence of hydroperoxides and production of hexanal were used as markers for the state of oxidation over the time course of the experiment. Oil bodies were prepared by wet-milling, centrifugation and subsequent water washing, this "water washed" preparation contained residual proteins not directly associated with oil bodies; the isolate was then salt washed to clean up the preparation and water washed again producing a "salt washed" preparation with fewer contaminating proteins.

Physical Stability. Emulsions formulated from sunflower oil and surfactant were stable over the experimental time course and did not phase separate. DTAB stabilized emulsions did increase in droplet diameter slightly (21% increase over 8 days) when exposed to elevated temperatures (**Table 1**). Measured oil body droplet diameter also increased; this was due to aggregation as light microscopy proved that although the oil bodies were clumping they were still intact entities (data not shown). Increased emulsion droplet size is theorized by McClements and Decker (*15*) to reduce oxidation rate, as the surface area on which oxidation chemistry can occur is reduced, a more recent paper by Osborn and Akah (*16*) found no significant effect of surface area on systems with low hydroperoxide concentrations. These results supported McClements' and Decker's (*15*) theory that droplet size is only influential when reactant concentration (hydroperoxides) at the surface is high. In systems of a high hydroperoxide concentration a doubling of the surface area would double the number of available reactants, in a low concentration system this would not be true as all reactants would be available on the droplet surface. In our experimental design we can make the assumption that droplet size will not influence oxidation as samples have a low hydroperoxide concentration.

Hydroperoxide Formation. Oxidation did not occur in either of the oil body isolates at most temperatures, although lipid hydroperoxide concentration did increase slightly in the 25^{o}C salt washed suspension and more significantly at 45^{o}C in the water washed oil body suspension (**Figure 1**). The concentration of hydroperoxides in all samples, apart from the 45^{o}C water washed samples, are below

120 the level usually associated with poor oil quality. Frankel (*2*) states a lipid hydroperoxide concentration
121 of 10 mequiv.kg^{-1} oil as the limit of acceptability for polyunsaturated vegetable oils, this would equate
122 to a value of 5.5 mmol hydroperoxides.kg^{-1} oil.

123 Water washed oil bodies contain a number of residual proteins originating from the seed cell debris,
124 upon salt washing this number is selectively reduced, and only the more strongly associated proteins
125 remain (**Figure 2**). Protein oxidation is known to be concurrent with lipid oxidation in many foods and
126 is proposed to initially retard lipid oxidation by preferentially oxidizing over lipid species or through the
127 chelation of free metal ions (*17, 18*). Proteins may also initiate lipid oxidation through the formation of
128 peroxyl radicals or reactive carbonyls, this balance between pro-oxidant and antioxidant effects is both
129 concentration and matrix dependant (*19*). In the water washed oil body suspension at 45^{o}C residual
130 contaminating proteins may have contributed to the increased hydroperoxide concentration relative to
131 the salt washed suspension through this pro-oxidant effect (**Figure 1**); although it should be noted that
132 the difference in concentration is minor when compared to the surfactant stabilized emulsions and it
133 could be hypothesized that the strongly associated proteins contribute an overall protection against
134 oxidation through free radical scavenging or the chelation of pro-oxidant metal ions.

135 The artificial emulsions were formulated from sunflower seed oil extracted from the same seed batch
136 as the oil bodies, and in all cases the starting lipid hydroperoxide concentrations were similar (1.9±0.75
137 mmol hydroperoxides.kg^{-1} lipid). **Figure 1** and **Figure 3** allow a comparison of the oxidative stability of
138 oil bodies and the artificial emulsions. In all cases, except tween 20 at 25^{o}C, natural oil bodies were
139 significantly more stable against oxidation than the artificial emulsions made from the same oil base.
140 The extent of oxidation was dependant on the time and the nature of the emulsifier in the order DTAB >
141 SDS > tween 20 (based on the maximum hydroperoxide concentration over the experimental time
142 course) (**Figure 3**).

143 Development of oxidation was dependant upon temperature in the order (45^{o}C>25^{o}C>5^{o}C), samples
144 stored at higher temperatures oxidized more rapidly than those stored at 5^{o}C. Thermal dependence
145 concurs with classical models of oxidation and the concentration of hydroperoxides is in line with
146 literature values during early stages of oxidation (*13*).

147 **Secondary Product Formation.** No increase in hexanal concentration could be detected in any of the
148 oil body samples (**Figure 4**), indicating that hydroperoxide break down was minimal during the time
149 course of the experiment. It is interesting to note that oil body samples did contain endogenous hexanal
150 at the start of the experiment at relatively high levels; alternative volatile secondary products of linoleic
151 acid oxidation were also followed but did not indicate oxidation (data not shown). The presence of
152 endogenous hexanal has been noted previously in the hydrophobic subcompartments of carnation petals
153 by Hudak and Thompson (*20*), who suggest that these structures resemble oil bodies and contain a
154 number of volatile compounds including hexanal that originated as byproducts of the lipoxygenase
155 pathway.

156 Hydroperoxides were formed in the surfactant stabilized emulsions increasing to levels associated
157 with oil just prior to the development of oxidative rancidity. Hexanal, 2-Heptanal (data not shown) and
158 1-octen-3-one (data not shown) headspace data supported this and their concentrations increased in
159 DTAB samples at all temperatures; this was also found in the SDS stabilized emulsions at 45^{o}C (**Figure**
160 **3 and Figure 5**).

161 Three surfactants were chosen to test the effect that surfactant chemistry has on the development of
162 oxidation. It would be expected that the most negatively charged emulsion (SDS) would oxidize more
163 rapidly than the emulsion formed from the non-ionic surfactant (tween 20) as the SDS emulsion would
164 attract cationic ions that may facilitate the initiation of lipid oxidation (*2*), this can be seen when we
165 compare the SDS and the tween 20 stabilized emulsions (**Figure 3**). Surprisingly, hydroperoxides were
166 formed most rapidly in emulsions stabilized by cationic DTAB. DTAB stabilized emulsions had the
167 highest hydroperoxide concentration at the start of the experiment (hydroperoxide concentration of
168 DTAB, SDS and tween 20 stabilized emulsions prior to storage were 2.96±0.31, 2.66±0.18 and
169 1.77±0.27 mmol hydroperoxides.kg^{-1} lipid respectively). This may indicate DTAB naturally contained
170 hydroperoxides that could have promoted the development of oxidation resulting in the rapid production
171 of further hydroperoxides. Oil bodies are negatively charged (zeta potential of water washed and salt
172 washed oil bodies at pH 7.5 are -27mV ± 1.4mV and -30mV ± 3.1mV respectively) but despite this

potential to attract oxidation-promoting cations they are significantly more resistant to oxidation than emulsions formulated from synthetic emulsifiers.

In conclusion, oil bodies are stable against oxidation at 5^{o}C, and when stored at elevated temperatures hydroperoxide formation is significantly less than within emulsions formulated with synthetic surfactants. The level of purity of oil bodies also effects oxidation rate, the more purified preparations showing a further reduction in hydroperoxide formation. The oxidative stability of sunflower oil bodies ex-vivo demonstrates protection of the naturally encapsulated polyunsaturated oil; oil bodies therefore merit further study in specific industrial applications.

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Table 1: Variation in droplet diameter of sunflower oil bodies over 8 days stored at 5°C, 25°C and 45°C.

[a]numerical mean diameter.

	D(v,0.5) µm			
	Day 0	**Day 8 (5°C)**	**Day 8 (25°C)**	**Day 8 (45°C)**
Water Washed	0.91	4.95 ± 1.75	17.42 ± 4.97	9.72 ± 2.00
Salt Washed	0.90	6.15 ± 0.26	9.72 ± 2.00	16.06 ± 13.52
SDS	0.78	0.31 ± 0.01	0.32 ± 0.01	0.31 ± 0.01
Tween-20	0.32	0.33 ± 0.01	0.33 ± 0.02	0.32 ± 0.01
DTAB	0.29	0.29 ± 0.01	0.32 ± 0.02	0.35 ± 0.02

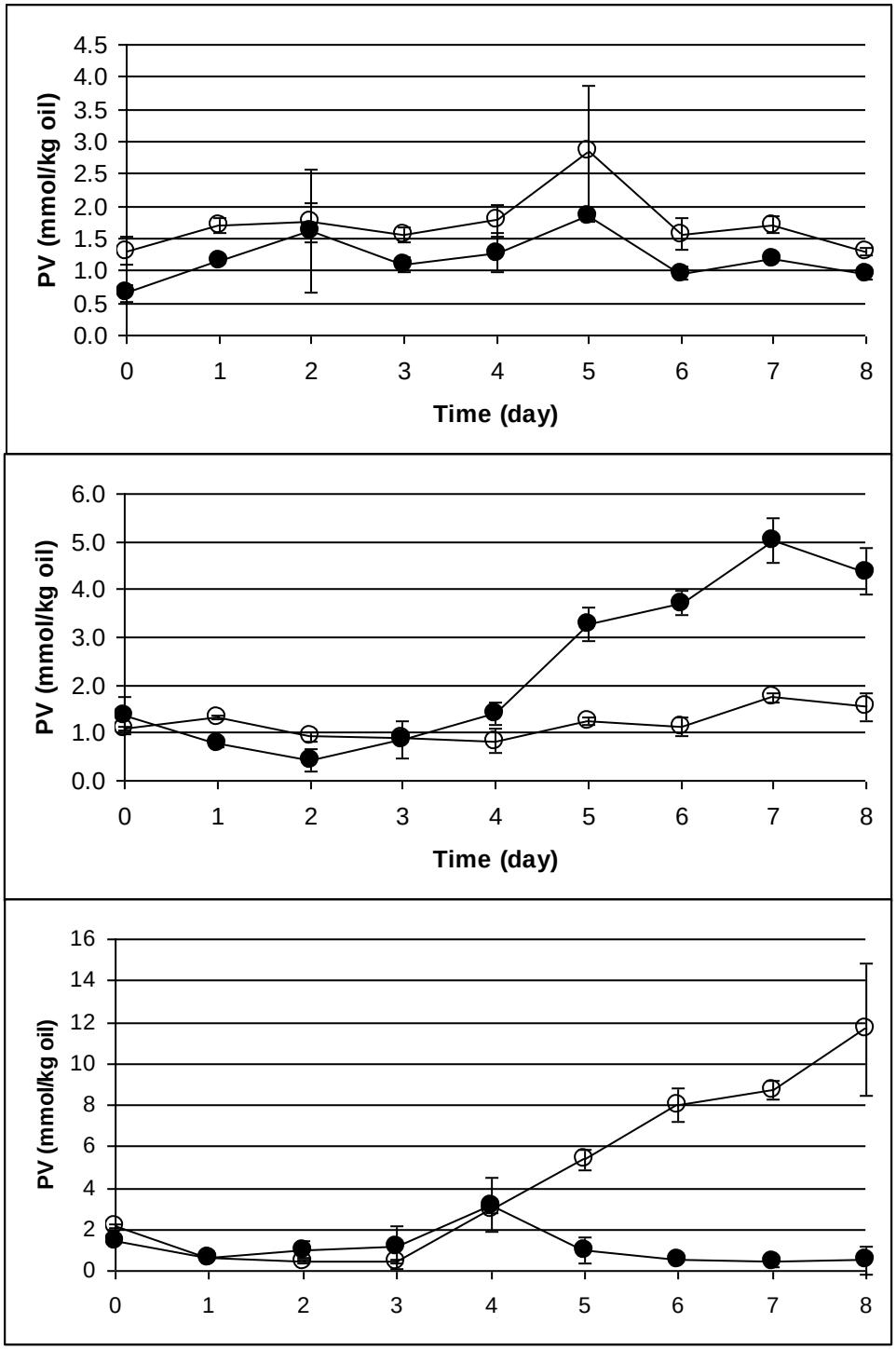

Figure 1: Peroxide Values mmol.kg^{-1}oil for water washed (empty circles) and salt washed oil bodies (filled circles). Samples were analysed at (a) 5^{o}C (b) 25^{o}C and (c) 45^{o}C. All data points represent means (n=3) ± Standard Deviation.

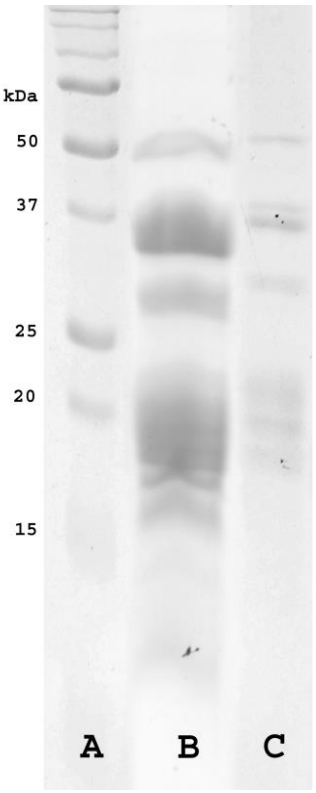

Figure 2: SDS-PAGE of proteins from sunflower oil bodies. A. Protein standards of Mw as indicated; B. Water washed oil body preparation; C. Salt washed oil body preparation. Lane B illustrates a number of proteins associated to water washed oil bodies, these residual proteins are selectively removed by salt washing. Which can be clearly observed in lane C, in which a salt washed preparation is shown and the major remaining proteins are oleosin isoforms which range from 18kDa to 22 kDa.

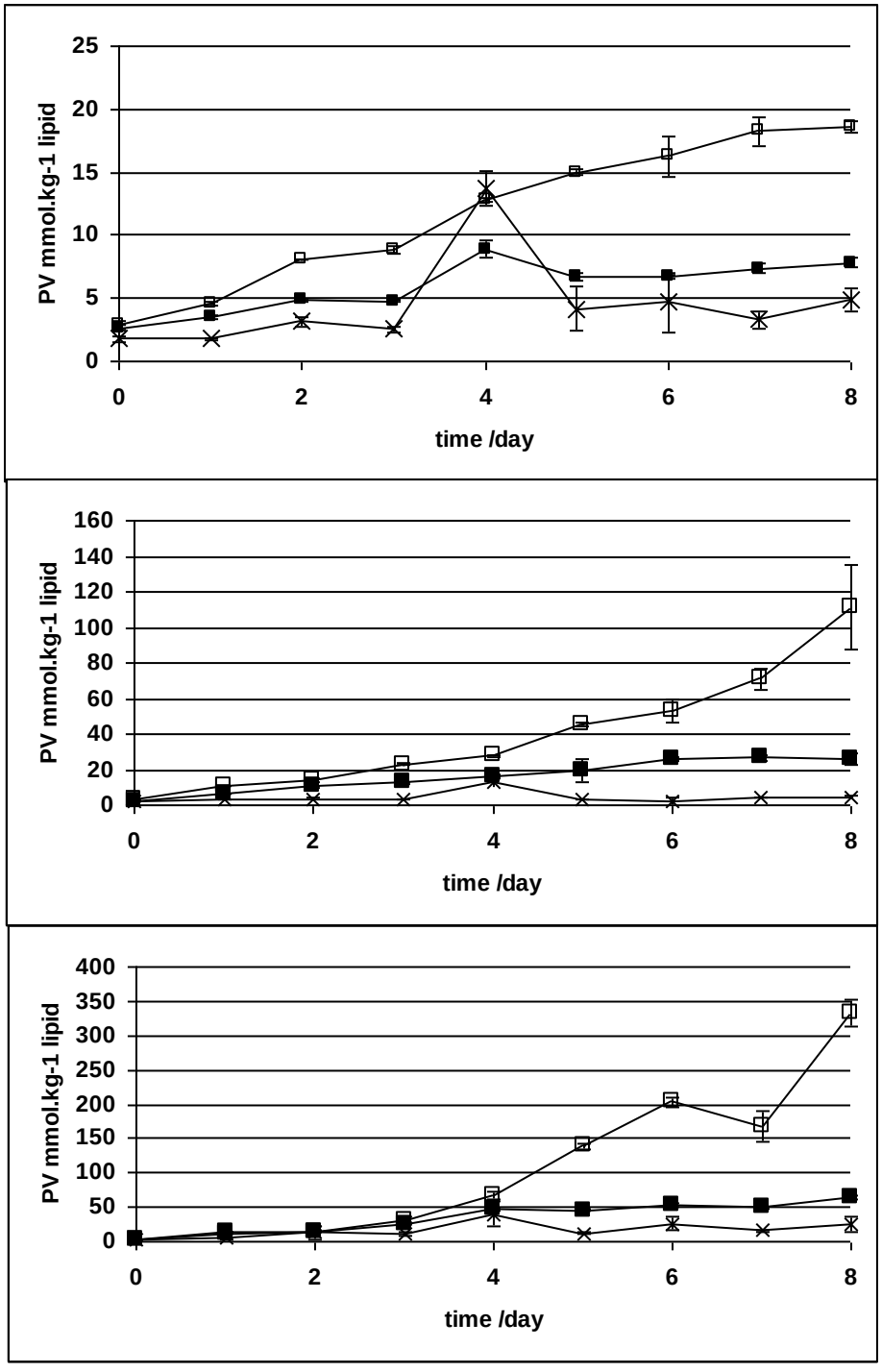

Figure 3: Peroxide Values mmol.kg^{-1} oil for 10% lipid emulsions stabilised by SDS (filled square), Tween 20 (cross) and DTAB (empty square). Extraction by solid phase microextraction and quantification by GC-MS. Emulsions were analysed at (a) 5ºC (b) 25ºC and (c) 45ºC. All data points represent means (n=3) ± Standard Deviation

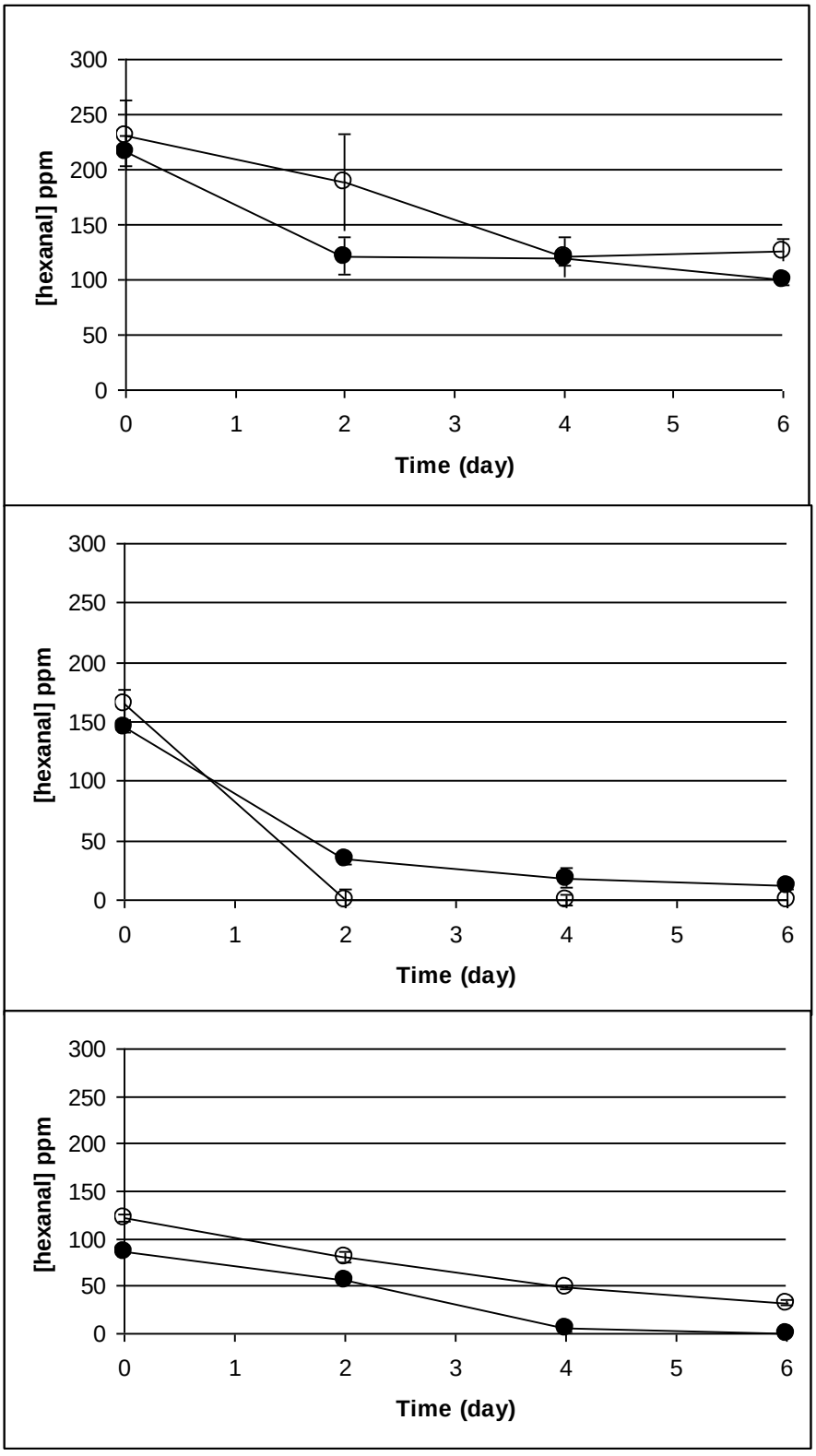

Figure 4: Influence of temperature on hexanal production in water washed (empty circles) and salt washed oil bodies (filled circles). Samples were analysed at (a) 5°C (b) 25°C and (c) 45°C. All data points represent means (n=3) ± Standard Deviation.

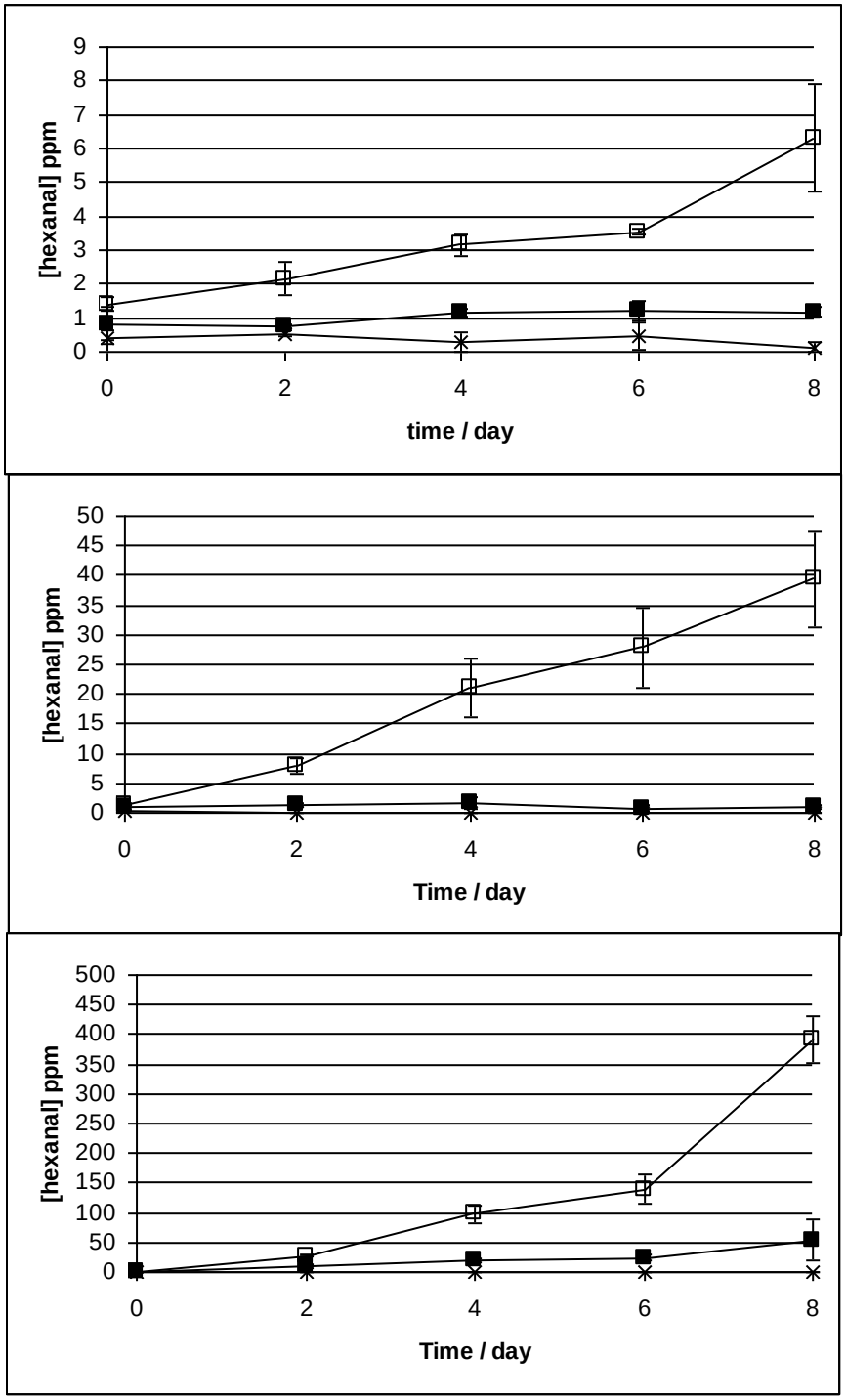

Figure 5: Influence of temperature on hexanal production in 10% lipid emulsions stabilised by SDS (filled square), Tween 20 (cross) and DTAB (empty square). Extraction by solid phase microextraction and quantification by GC-MS. Emulsions were analysed at (a) 5ºC (b) 25ºC and (c) 45ºC. All data points represent means (n=3) ± Standard Deviation.