

# Oxidative Stability of Sunflower Oil Bodies

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1 ABSTRACT

2 This study investigates the oxidative stability of sunflower oil body suspensions (10% w/w lipid). Two  
3 washed suspensions of oil bodies were evaluated over 8 days at three temperatures (5°C, 25°C and  
4 45°C) against three comparable sunflower oil emulsions stabilized with dodecyltrimethylammonium  
5 bromide (DTAB), polyoxyethylene-sorbitan monolaurate (tween 20) and sodium dodecyl sulphate  
6 (SDS) (17mM). The development of oxidation was monitored by the production of lipid hydroperoxides  
7 and hexanal. Oil body suspensions were significantly more stable to oxidation than the three emulsions  
8 at each temperature. The reasons and biological demands for oxidative stability in-vivo are clear  
9 although they have never been previously demonstrated ex-vivo.

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

11

## 12 INTRODUCTION

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

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

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

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

37

39 **Materials**

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

42 **Methods**

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

48 *Lipid Extraction.* Moisture content of oil body and seed samples (5g) was determined gravimetrically  
49 by drying to constant weight (vacuum oven, 60°C). Lipid content of both oil body and seed samples was  
50 calculated by repeated extraction with diethyl ether after freeze drying and determined gravimetrically  
51 (10).

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

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

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

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

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

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

78 *Hexanal Headspace Concentration.* Hexanal concentration was calculated from the headspace  
79 concentration using solid phase micro-extraction gas chromatography mass spectroscopy (SPME-  
80 GCMS) (14). This was achieved by adding 1,2 dichlorobenzene (100ppmv) as an internal standard  
81 (10µl) to the emulsion or oil body suspension (1mL) and capping in a 20mL vial. Headspace  
82 concentration was determined using a CTS Analytics PAL system autosampler and a DSQ and Trace  
83 GC Ultra (Thermo Electron Corporation). Samples were agitated (50°C for 3 min) prior to extraction  
84 (20 min at 50°C), desorption was achieved in 5 min (250°C). Compounds were separated with  
85 30mL.min<sup>-1</sup> nitrogen, a ZB-5 Phenomenex gas chromatography column (Macclesfield, UK) and oven  
86 temperatures controlled at 40°C (1 min) then ramped (3°C.min<sup>-1</sup>) to 140°C, ramped (15°C.min<sup>-1</sup>) to  
87 210°C and held at 210°C for 1min. Volatiles were quantified with authentic standards.

88 *Oxidation Conditions.* Samples were stored at 5°C, 25 °C and 45 °C with restricted lighting in closed  
89 20mL glass vials.

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



93 RESULTS AND DISCUSSION

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

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

116 **Hydroperoxide Formation.** Oxidation did not occur in either of the oil body isolates at most  
117 temperatures, although lipid hydroperoxide concentration did increase slightly in the 25°C salt washed  
118 suspension and more significantly at 45°C in the water washed oil body suspension (**Figure 1**). The  
119 concentration of hydroperoxides in all samples, apart from the 45°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<sup>-1</sup> oil as the limit of acceptability for polyunsaturated vegetable oils, this would equate  
122 to a value of 5.5 mmol hydroperoxides.kg<sup>-1</sup> 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 peroxy 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°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<sup>-1</sup> 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°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°C>25°C>5°C), samples  
144 stored at higher temperatures oxidized more rapidly than those stored at 5°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°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  $2.96 \pm 0.31$ ,  $2.66 \pm 0.18$  and  
169  $1.77 \pm 0.27$  mmol hydroperoxides.kg<sup>-1</sup> 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  $-27\text{mV} \pm 1.4\text{mV}$  and  $-30\text{mV} \pm 3.1\text{mV}$  respectively) but despite this

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

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

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- 231

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

<sup>a</sup>numerical mean diameter.

	<b>D(v,0.5) <math>\mu\text{m}</math></b>			
	<b>Day 0</b>	<b>Day 8 (5°C)</b>	<b>Day 8 (25°C)</b>	<b>Day 8 (45°C)</b>
Water Washed	0.91	4.95 $\pm$ 1.75	17.42 $\pm$ 4.97	9.72 $\pm$ 2.00
Salt Washed	0.90	6.15 $\pm$ 0.26	9.72 $\pm$ 2.00	16.06 $\pm$ 13.52
SDS	0.78	0.31 $\pm$ 0.01	0.32 $\pm$ 0.01	0.31 $\pm$ 0.01
Tween-20	0.32	0.33 $\pm$ 0.01	0.33 $\pm$ 0.02	0.32 $\pm$ 0.01
DTAB	0.29	0.29 $\pm$ 0.01	0.32 $\pm$ 0.02	0.35 $\pm$ 0.02

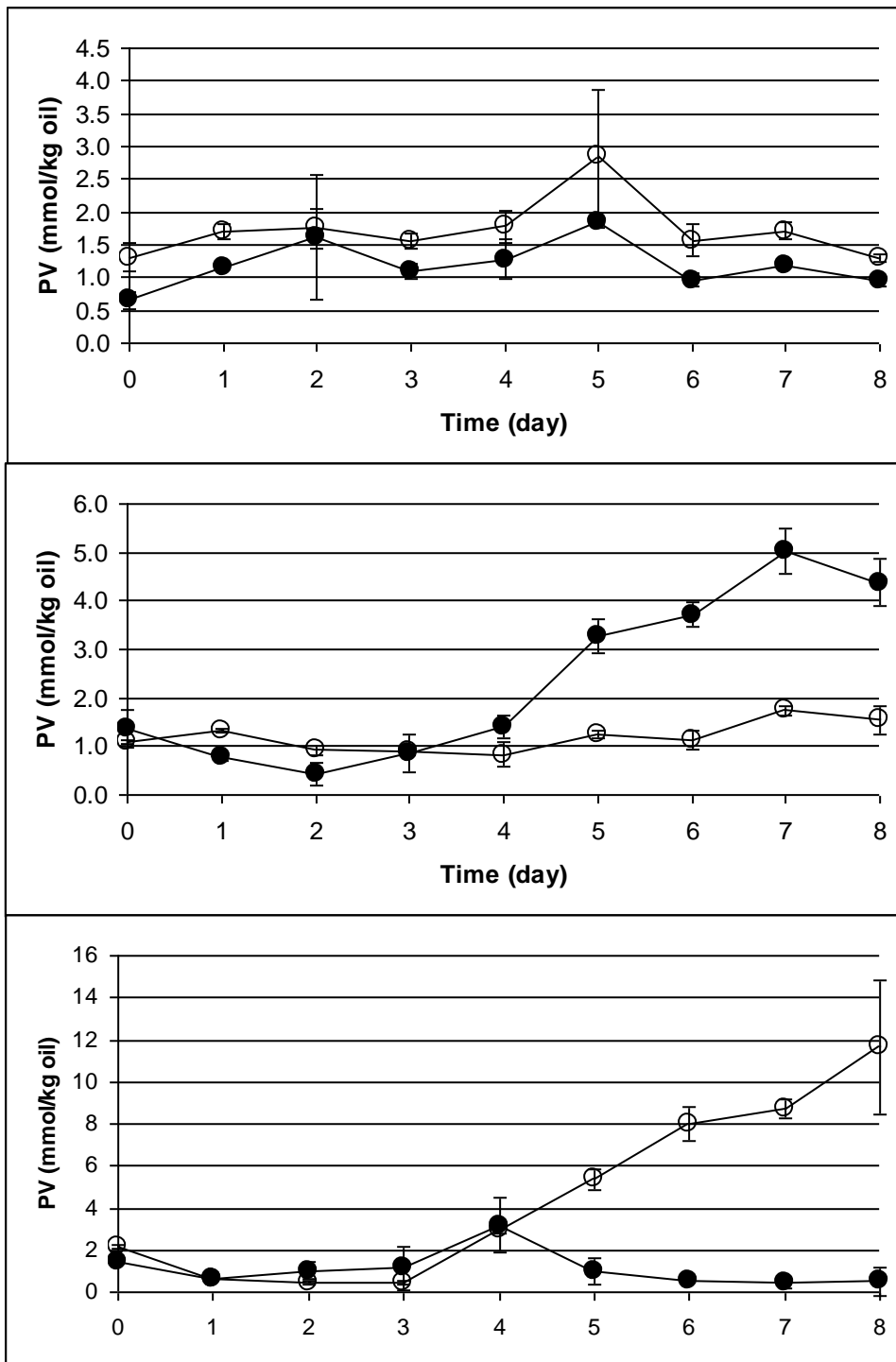


Figure 1: Peroxide Values mmol.kg<sup>-1</sup>oil for water washed (empty circles) and salt washed oil bodies (filled circles). Samples were analysed at (a) 5°C (b) 25°C and (c) 45°C. All data points represent means (n=3) ± Standard Deviation.

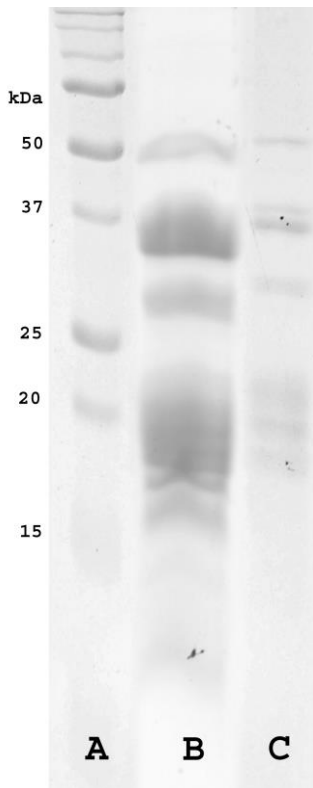


Figure 2: SDS-PAGE of proteins from sunflower oil bodies. A. Protein standards of Mw as indicated; B. Water washed oil body preparation; C. Salt washed oil body preparation. Lane B illustrates a number of proteins associated to water washed oil bodies, these residual proteins are selectively removed 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.

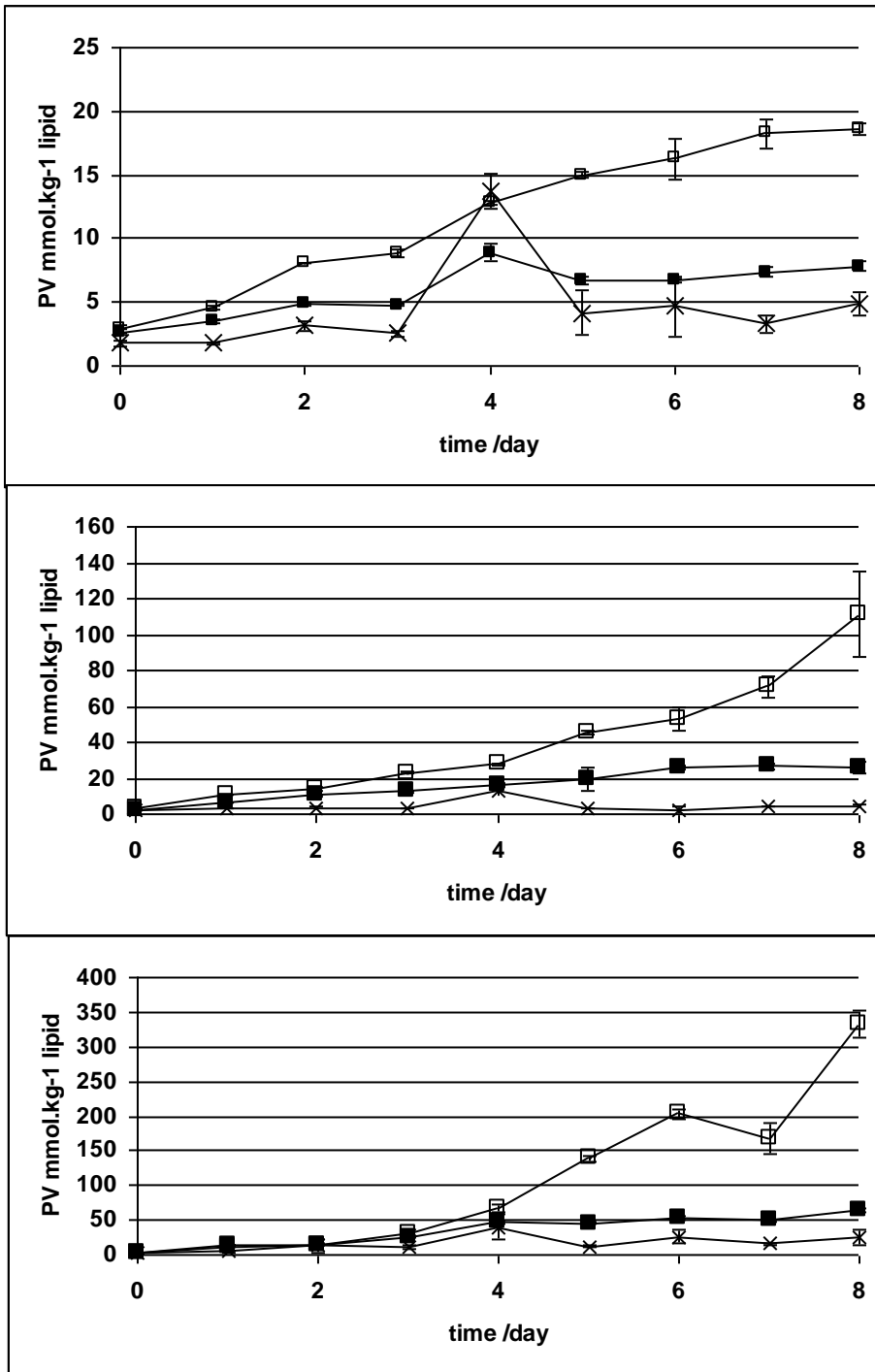


Figure 3: Peroxide Values mmol.kg<sup>-1</sup> oil for 10% lipid emulsions stabilised by SDS (filled square), Tween 20 (cross) and DTAB (empty square). Extraction by solid phase microextraction and quantification by GC-MS. Emulsions were analysed at (a) 5°C (b) 25°C and (c) 45°C. All data points represent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



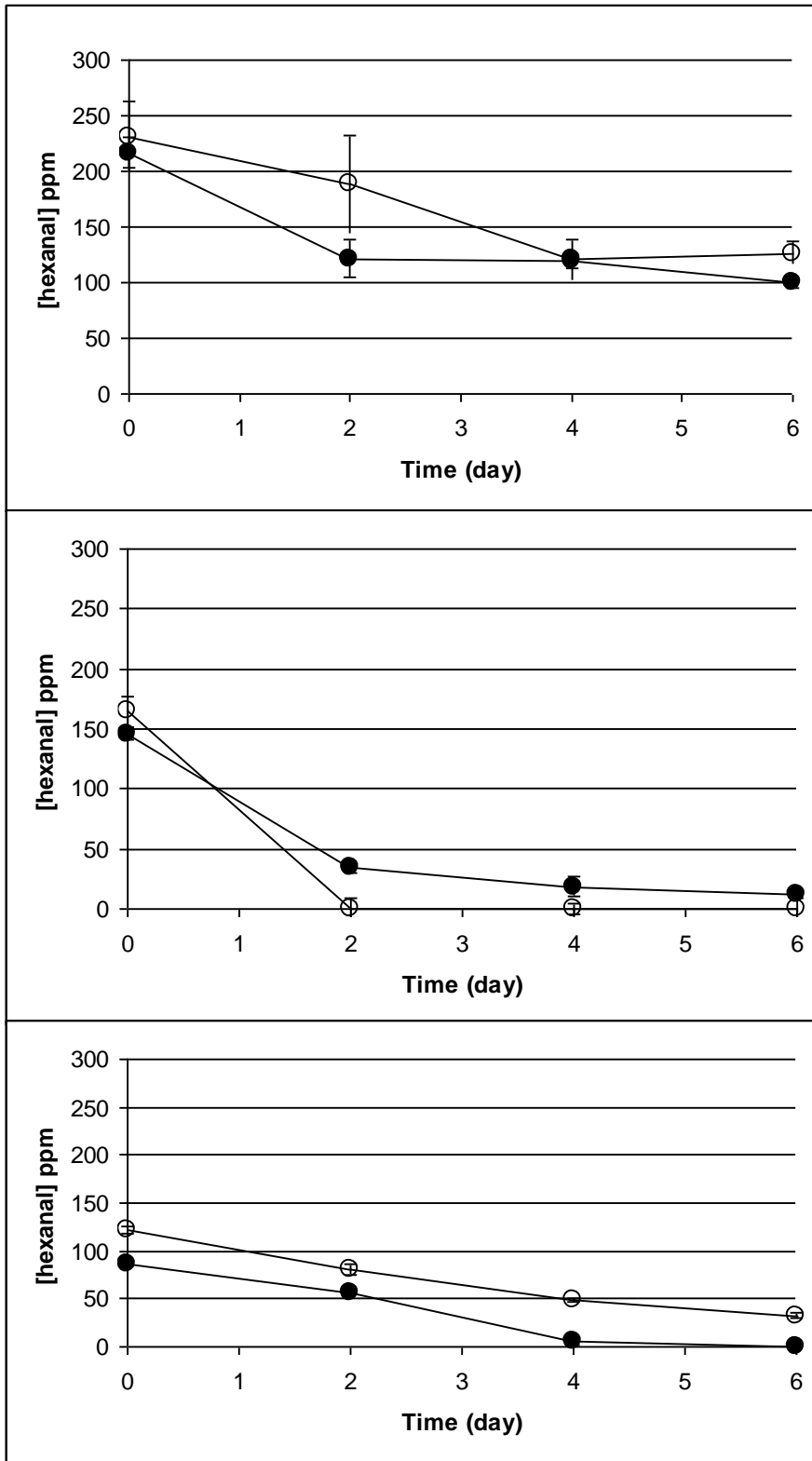


Figure 4: Influence of temperature on hexanal production in water washed (empty circles) and salt washed oil bodies (filled circles). Samples were analysed at (a) 5°C (b) 25°C and (c) 45°C. All data points represent means (n=3) ± Standard Deviation.

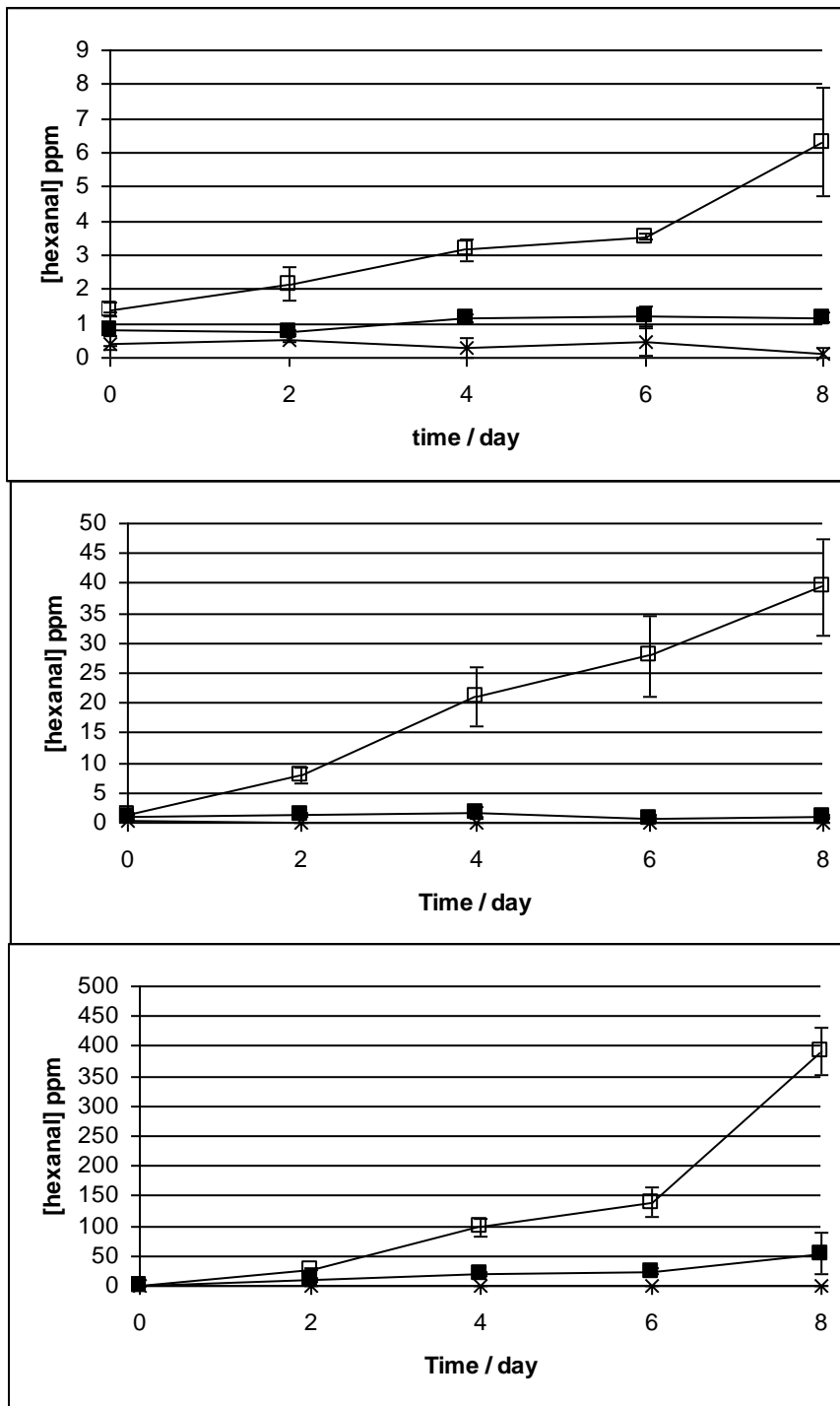


Figure 5: Influence of temperature on hexanal production in 10% lipid emulsions stabilised by SDS (filled square), Tween 20 (cross) and DTAB (empty square). Extraction by solid phase microextraction and quantification by GC-MS. Emulsions were analysed at (a) 5°C (b) 25°C and (c) 45°C. All data points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