Journal of Food Engineering 117 (2013) 505–512

Contents lists available at SciVerse ScienceDirect

Journal of Food Engineering

journal homepage: www.elsevier.com/locate/jfoodeng



# Co-encapsulation of fish oil with phytosterol esters and limonene by milk proteins

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ARTICLE INFO

*Article history:*
Available online 10 February 2013

*Keywords:*
Encapsulation
Fish oil
Phytosterol
Limonene
Milk protein
Polyunsaturated fatty acid

ABSTRACT

Co-encapsulated microcapsules containing three lipophilic bioactive components (LBCs) including fish oil, phytosterols and limonene were studied and compared with those containing only fish oil. Milk proteins (whey protein isolate and sodium caseinate in a ratio of 4:1) were used as wall materials. Results show that good quality microcapsules can be obtained at inlet temperature of 170 °C and outlet temperature of 70 °C, with the wall to core ratio of 4:1. There was no significant different ($p > 0.05$) in the microencapsulation efficiency and the oxidation indicators (PV and AV) after the accelerated storage for both types of microcapsules. However, the retention of EPA and DHA in the LBCs-microcapsules was significantly higher ($p < 0.05$) than the fish oil microcapsules. The phytosterols content was unchanged but loss of limonene occurred after storage. The LBCs-microcapsules had better flavor/odour profile than the fish oil microcapsules after drying and storage.



## 1. Introduction

Lipophilic bioactive components (LBCs), such as fish oil, phystosterols and limomene, have received increasing attention in research because of their reported health benefits. Fish oil is known to be a rich source of omega-3 long chain polyunsaturated fatty acids, containing both docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Several health benefits, such as ameliorative effects on brain and nervous systems, and prevention of certain diseases including diabetes, inflammatory and autoimmune disorders, coronary artery disease, thrombosis, cardiac arrhythmias and hypertension, have been associated with regular consumption of omega-3 fatty acids (Newton, 2001). Phytosterols have been shown to decrease the serum cholesterol levels and the ratio of the low-density lipoprotein (LDL) to high-density lipoprotein (HDL) bound cholesterol in serum (Mattson and Grundy, 1982). Limonene has various health benefits including chemo-preventive activity against cancers, gastric acid neutralizing effect, supporting normal peristalsis and dissolving cholesterol-containing gallstones (Sun, 2007). Nevertheless, the highly lipophilic nature of these compounds results in poor absorption and palatability, in addition to deterioration by oxidation (McClements et al., 2007), and these factors limit their use in functional foods.

Microencapsulation is a technology that allows food ingredients or bioactive components to be enveloped as a "core" material with a polymer matrix or "wall". Thus, the encapsulated materials are protected against adverse reactions, such as lipid oxidation, nutritional deterioration (Hogan et al., 2003; Kagami et al., 2003), or volatile loss (Bangs and Reineccius, 1988; Kim and Morr, 1996) during production, storage and handling. The encapsulated materials can be subsequently released under desired conditions. Spray drying is a suitable method to encapsulate these valuable components in a protective matrix as it is a well-established technology, and large scale production with relatively economic cost can be achieved for food application. To obtain spray-dried microcapsules, the wall material with emulsifying characteristics is needed, since it is crucial to form a stable emulsion of fine droplets consisted of the core material and wall solution before drying (Risch and Reineccius, 1988). Surface active materials such as whey proteins, sodium caseinate, gelatin, and gum arabic have been used as wall materials to protect fish oil or volatile compounds (Sheu and Rosenberg, 1993; Kim and Morr, 1996).

Co-encapsulation of several core materials in an encapsulation system may enhance the bioactivity of individual components (Halwanil et al., 2008). This technique has been widely used in pharmaceutical delivery systems, either in liposome or in capsule form. However, using this concept to encapsulate more than one bioactive component for food application, as we report here, has been limited in literature. The co-encapsulation of fish oil, phytosterols and limonene has certainly not been attempted before, and there are rationales behind the selection of these LBCs as core materials. Firstly, the intake of plant sterols together with fish oil

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http://dx.doi.org/10.1016/j.jfoodeng.2013.01.011

has been shown to have synergistic effects on cardiovascular health, due to the reduction of serum cholesterol and the ratio of LDL/HDL (Khandelwal et al., 2009; Micallef and Garg, 2009). The intake of both the abovementioned LBCs has also been shown to reduce systemic inflammation in hyperlipidemic individuals (Micallef and Garg, 2009). In addition, phytosterols has been found to increase the oxidation stability of lipids (Yasukazu and Etsuo, 2003). Limonene is a natural flavor which may mask fishy odour, and deliver health benefits as mentioned. Therefore, co-encapsulation of fish oil with phytosterols and limonene may exhibit promising health benefits.

The objective of this work is to study the feasibility of applying co-encapsulation using spray drying technique, to protect fish oil from oxidation and to mask the fishy odour by incorporating phytosterol esters (PE) and limonene as core materials together with fish oil. Whey protein isolate (WPI) and sodium caseinate (NaCA) were used as wall materials. The effects of inlet and outlet temperatures as well as the wall to core ratio had on the properties of powder were studied. The oxidative stability of core material, retention of the individual lipophilic components, and the flavour profiles of both the co-encapsulated and the fish oil-microcapsules were evaluated.

## 2. Materials and methods

### 2.1. Materials

Whey protein isolate (WPI, ALACEN™ 895, protein content 92.0%) and sodium caseinate (NaCA, ALANATE™ 180, protein content 92.7%) were provided by Fonterra Ltd., New Zealand (NZ). Fish oil (Croda Incromega™ TG3322) was given by Nutura (Chemcolour Industries Ltd., NZ) Phytosterol esters (CoroWise™, product code: SE-C100) were from Cargill Incorporated, USA while limonene (VALENCIA sweet orange oil) was given by H & K Flavour, Shanghai, China. All the standards for gas chromatography (37 FAMES standards, internal standards including tridecanoic acid (13:0) and tricosanoic acid (23:0), limonene standard ((R)-(+)-limonene, (+)-limonene oxide (mixture of *cis* and *trans*) and (+)-carvone) and individual phytosterols (5α-cholestane, β-sitosterol, campesterol and stigmasterol) were obtained from Sigma (Sigma–Aldrich, Chemicals Co., St. Louis, MO, USA). Deionised water was used in all experiments. All chemicals used were of analytical grade.

### 2.2. Microencapsulation by spray drying

Oil-in-water emulsions were prepared prior to spray drying according to the modified method of Khwaldia et al. (2004) and Hogan et al. (2001a). Aqueous phase was prepared by dissolving powdered milk proteins (WPI/NaCA in ratio of 4:1) in deionised water at 55 °C while stirring for 60 min, after which the solution was left standing at room temperature for 12 h to ensure complete dispersion. The resulting mixture was adjusted to pH 6.7 using NaOH (1 M). The oil phase, containing 100% fish oil, or fish oil, physterols and limonene in a ratio of 6:1:1, was then blended with the above aqueous solution using an Ultra-Turrax T25 homogeniser (IKA labortechnik) at 13,500 rpm for 1 min. Fine emulsions were produced by a high pressure homogenizer (APV, model 1000, Denmark) at 80 MPa with 4 passes. The emulsion samples contained 30% solid content, which was composed of milk protein and fish oil in the ratios of 1:1, 2:1 and 4:1 and milk protein and LBCs at ratio of 4:1.

The emulsions were spray dried using a spray drier (Sunshine, model GZ-5 with 850 mm diameter of chamber, Wuxi, China) at inlet temperatures of 160, 170 and 180 °C, and outlet temperature of 70 and 80 °C, at flow rates of 15–20 ml/min.

### 2.3. Oil recovery and microencapsulation efficiency (ME)

The solvent extractable total non-volatile fat content of the samples was measured using Röse-Gottlieb method (Richardson, 1985) while the total volatile oil (limonene) retained during drying was determined by Clevenger distillation (Jafari et al., 2007). The recovery of non-volatile and volatile oil was defined as follows:

$$\text{Recovery of non-volatile fat } (\%) = (\text{Total non-volatile oil extracted from powders} / \text{Total non-volatile oil added in formulation}) \times 100 \quad (1)$$

$$\text{Recovery of volatile oil } (\%) = (\text{Total volatile oil extracted from powders} / \text{Total volatile oil added in formulation}) \times 100 \quad (2)$$

The recovery of total oil (%) was calculated as below:

$$\text{Recovery of total oil } (\%) = \{\text{Total extractable oil (the sum of volatile and non} - \text{volatile oils) from powders/Total oil added in formulation}\} \times 100 \quad (3)$$

Microencapsulation efficiency (ME) was calculated as:

$$\text{ME } (\%) = \{(\text{Total extractable oil from powders-Extractable surface oil}) / \text{Total extractable oil from powders}\} \times 100 \quad (4)$$

The extractable surface oil was quantified according to Hogan et al. (2003). The experiments were carried out in duplicates. Limonene is not taken into account when calculating the extractable surface oil as it is volatile in nature and it would be evaporated at high temperature during spray drying.

### 2.4. Reconstitution properties

The reconstitution speed of microcapsule in water was assessed by mixing 0.5 g spray-dried sample with 150 mL Milli-Q water at room temperature (25 ± 2 °C) at a constant stirring rate of 400 rpm using a magnetic stirrer. The time to achieve complete dispersion of microcapsule was counted by a timer. The droplet size distributions of the resulting dispersions were determined by the Nano Zetasizer (Malvern Instruments Ltd., Worcestershire, UK). The dispersions were also observed by optical microscopy (Olympus BX51/52, Japan) at 400× magnification. Duplicate experiments were performed.

### 2.5. Water activity

Water activity ($a_w$) of the spray-dried microcapsules was measured using a water activity analyser (HydroLab 3, Rotronic, USA) at a temperature of 25 °C. Duplicate measurements were performed.

### 2.6. Glass transition temperature (Tg)

Samples were conditioned at 30% relative humidity (with saturated magnesium chloride solution) for 24 h after drying. $T_g$ was measured using a differential scanning calorimeter (DSC-7, Perkin–Elmer, USA) under nitrogen (flow rate of 20 mL/min) with the temperature ramped from 20 to 120 °C at a rate of 10 °C/min, as modified from Enrione et al. (2010). $T_g$ was taken as the onset point of the change on the heat flow curve. Experiment was conducted in duplicate.

### 2.7. Evaluation of oil phase oxidation

The oxidative stability of the microcapsules was evaluated in an accelerated storage trial at 45 °C and 30% relative humidity (RH) under saturated oxygen for 7 days, using desiccators. A sample of 30 g for each type of microcapsules was used for the experiment and the degree of lipid oxidation was periodically analysed at day 0, 1, 4 and 7. Oil phases were extracted using the modified Bligh and Dyer method (Katvi, 2005) and stored at −20 °C under nitrogen less than 24 h before further analysis. Peroxide value (PV), anisidine value (AV), fatty acid content and oxidation of limonene were determined to indicate the oxidative stability of microcapsules.

A rapid spectrophotometric method (Hornero-MéNdez et al., 2001) was applied for the PV measurement. The PV was calculated as follows:

$$\text{PV (mequiv peroxide/kg of sample)} = (A_{sm} - A_{b1})/(55.84 \times 2 \times m \times W_{sm}) \quad (5)$$

where $A_{sm}$ is the absorbance of the sample at 470 nm; $A_{b1}$ is the absorbance of the blank at 470 nm (both absorbances were baseline-corrected by subtracting absorbance at 670 nm); m is the slope of the calibration curve; $W_{sm}$ is the sample weight in grams. The AV was determined according to the AOAC official method 365.93 (AOAC, 1990). The data was reported as mean value of triplicate samples.

The changes of fatty acid content, especially EPA and DHA, after spray drying and during accelerated storage, were determined using a GC–FID (Hewlett Packard 5890 Series II, Paolo Alto, CA). Fatty acid methyl esters were prepared according to the modified method of Hartman and Lago (1973). The temperature of the GC oven was programmed at an initial value of 50 °C for 1 min followed by ramping to 175 °C at 25 °C per min, then to 225 °C at a rate of 4 °C/min, where it was held for 5 min. The 37-fatty acid methyl esters standards were used to identify the individual fatty acid in the samples using the peak areas of the fatty acids and the relative response factor. Duplicate experiments were conducted.

For determination of limonene oxidation and retention, sample preparation was conducted according to the modified method of Miller et al. (2008). The analysis of limonene and its degradation products was conducted by GC–FID following the temperature programme as below: heating from 80 °C to 110 °C at a rate of 3 °C/min, then to 200 °C at a rate of 20 °C/min. The temperature was ramped to 250 °C in 2 min and held for 5 min in order to prevent excess long chain fatty acids from accumulating on the GC column. The external standard method was used to determine the limonene, limonene oxide and carvone content. Duplicate experiments were performed.

Separation of phytosterols was performed by saponification followed by silylation according to the modified method of Ramadan et al. (2006). The silylated sterols were analysed by GC–FID using 5α-cholestane as internal standard. The oven temperature was set at 200 °C for 2 min after injection, followed by ramping to 290 °C at a rate of 20 °C/min. External standards including campesterol, stigmasterol and β-sitosterol were used for identification of individual sterols in the sample. Duplicate experiments were conducted.

### 2.8. Sensory evaluation

A sensory evaluation was conducted for the spray-dried powders and the reconstituted emulsions immediately after the drying process and at the end of the 7-day accelerated storage. A trained panel consisting of nine healthy assessors (4 male, 5 female, age ranged from 21 to 31 year old) was employed for this study. The sensory panel was trained according to ISO6658, ISO8586, and Kolanowski et al. (2007). Milk proteins (WPI and NaCA), fish oil, limonene and oxidised materials including oxidised fish oil, carvone and limonene oxide with different intensities were used as standard during training.

Samples (powder 1.0 g and reconstituted emulsion 20.0 g) were presented in 30 mL glass bottles with glass tops and coded with three digit random numbers. A thin layer of cotton was used to hide the contents and the bottles were covered with aluminium foil to mask any possible colour differences between the samples. Thirty minutes after sample preparation, the sensory panelists were asked to evaluate the intensity of the following attributes by sniffing the samples: dairy flavour, fishy odour, off-flavour (oxidized fish oil odour), limonene flavour, carvone and limonene oxide odour. The intensity of the attributes was marked on a linear scale (0–9), anchored "none" to "very strong". Two sensory sessions were conducted and each panel evaluated two samples in a session in random order.

### 2.9. Statistical analysis

Mean values ± standard deviations are reported for each sample. The experimental data were subjected to a one-way ANOVA using SAS 9.1. Significance level was determined at 95% confident limit ($p < 0.05$) and *post hoc* test (Duncan's new multiple range test) was conducted if significance were found in the ANOVA.

## 3. Results and discussion

### 3.1. Drying temperature, wall to core ratio and the properties of fish oil-microcapsules

The inlet temperatures of 160, 170 and 180 °C and outlet temperatures of 70 and 80 °C were investigated as they were commonly used in commercial production of spray-dried milk products, such as whole milk and skim milk (Okos et al., 1992). Results show that there are significant differences ($p < 0.05$) in the properties of microcapsules when dried under different inlet and outlet temperatures (Table 1). The microcapsules obtained at higher inlet and outlet temperatures had significantly lower $a_w$ and higher $T_g$. The results of $a_w$ is consistent with those of Maa et al. (1997), who studied spray-dried recombinant human deoxyribonuclease and lactose. Both $a_w$ and $T_g$ can be used to define a relationship between moisture and chemical reaction rate during storage because the moisture content in dried dairy products is critical to the stability of protein (Williams et al., 1955). An increase in moisture can result in a lower $T_g$, which will increase the mobility of the protein, thus affecting the kinetics of degradation reactions. Overall, a low $a_w$ and high $T_g$ will give samples a more promising stability during storage.

The reconstitution property of the microcapsules in water is another important aspect. As the reconstituted emulsions showed bimodal distributions (Fig. 1a), the polydispersity index (PDI), instead of the average droplet size, was used to compare the reconstitution properties. A lower PDI indicates narrower particle size distribution and *vice versa*. Results show that the microcapsules dried at the outlet temperature of 80 °C had significantly higher PDI ($p < 0.05$) than those dried at 70 °C for most inlet temperatures. These results reflect that drying at a lower outlet temperature (70 °C) can produce reconstituted emulsions with narrower droplet size distribution which is generally more stabile. Previous literature reported that the degree of heat denaturation (Millqvist-Fureby et al., 2001) and aggregation (Bernard et al., 2011) of WPI during spray drying could influence the solubility of microcapsules, and hence, the droplet size after reconstitution. While our results

**Table 1**
Properties of the spray-dried microcapsules containing fish oil as core material and WPI/NaCA as wall materials: effect of drying temperature and wall to core ratio.

| Ratio of wall to core | Outlet temp. (°C) | Inlet temp. (°C) | Water activity ($a_w$) | Glass transition temperature ($T_g$, °C) | Droplet size distribution after reconstitution (PDI) | Microencapsulation efficiency (ME, %) |
|---|---|---|---|---|---|---|
| 1:1 | 80 | 160 | $0.20 \pm 0.00^{b}$ | $51.9 \pm 0.7^{ab}$ | $1.00 \pm 0.00^{a}$ | $38.0 \pm 8.7^{de}$ |
| | | 170 | $0.15 \pm 0.00^{c}$ | $54.4 \pm 0.3^{a}$ | $0.34 \pm 0.01^{cd}$ | $38.5 \pm 1.1^{d}$ |
| | | 180 | $0.16 \pm 0.01^{c}$ | $53.3 \pm 0.2^{a}$ | $0.65 \pm 0.13^{b}$ | $21.9 \pm 2.9^{f}$ |
| | 70 | 160 | $0.24 \pm 0.00^{a}$ | $42.4 \pm 0.3^{c}$ | $0.39 \pm 0.06^{c}$ | $47.8 \pm 0.4^{c}$ |
| | | 170 | $0.20 \pm 0.00^{b}$ | $49.0 \pm 0.4^{b}$ | $0.38 \pm 0.02^{c}$ | $48.9 \pm 1.9^{c}$ |
| | | 180 | $0.19 \pm 0.00^{b}$ | $49.5 \pm 0.4^{b}$ | $0.32 \pm 0.01^{d}$ | $29.1 \pm 1.0^{e}$ |
| 2:1 | 70 | 170 | $0.19 \pm 0.00^{b}$ | $50.4 \pm 0.3^{b}$ | $0.15 \pm 0.02^{f}$ | $71.6 \pm 0.3^{b}$ |
| 4:1 | | | $0.19 \pm 0.00^{b}$ | $51.9 \pm 0.6^{ab}$ | $0.17 \pm 0.01^{e}$ | $95.1 \pm 0.7^{a}$ |

Superscripts with a different letter in the same column are significantly different ($p < 0.05$) from each other.

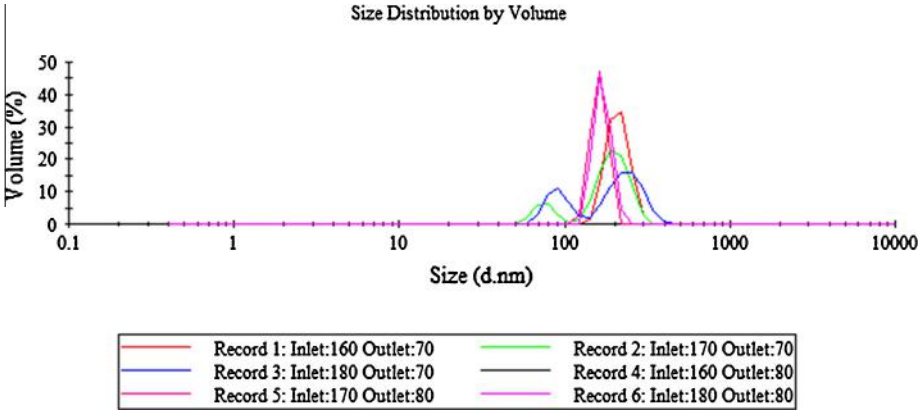


**Fig. 1a.** Size distribution of the reconstituted emulsion showing bimodal distribution.


**Fig. 1b.** Optical microscopy of the reconstituted emulsions with different wall to core ratios (200× magnification). (A) 4:1; (B) 2:1; (C) 1:1.

showed PDI values were only significantly larger at higher outlet temperature (80 °C), this suggests that the extent of protein denaturation and aggregation was mostly influenced by the higher outlet temperature, making drying at lower outlet temperature (70 °C) a better choice.

Hogan et al. (2001b) suggested that reconstitution properties of microcapsules could be affected by the presence of surface fat, which could be related to microencapsulation efficiency (ME). The presence of higher surface fat could cause poorer reconstitution properties and lower ME. However, current result reveals a low correlation coefficient (-0.235) between PDI and ME, indicating that the droplet size distribution of the reconstituted emulsions did not fully dependent on the microencapsulation efficiency in this case.

Table 1 shows that both outlet and inlet temperatures played an important impact on the ME. At the same outlet temperature, a higher inlet temperature decreased the ME of microcapsules. Similarly, at the same inlet temperature, drying the emulsions at higher outlet temperature reduced the ME. These results are consistent with the finding of Drusch and Berg (2008) and Paramita et al. (2010). At higher air inlet temperature, excessive evaporation could occur, causing cracks in the microcapsule membrane (refer Fig. 2), consequently inducing premature release of the encapsulated ingredients (Zakarian and King, 1982). The non-encapsulated core materials may not be necessarily correlated with reduced shelf life of the products as demonstrated by orange oil (Finney et al., 2002) and methyl linoleate (Minemoto et al., 1997), but they may strongly impair the acceptability of product containing core materials rich in polyunsaturated fatty acids (Drusch and Berg, 2008). In current research, lower non-encapsulated oil is crucial to ensure longer shelf life of the microcapsules since 75% of the core materials was consisted of fish oil with high level of polyunsaturated fatty acids.

Table 1 also shows the results of microcapsule properties as influenced by ratio of wall to core materials at inlet and outlet temperatures of 170 °C and 70 °C, respectively. It was found that the $a_w$ and $T_g$ of the dried samples were not affected by the ratio of wall to core material, consistent with the result of Hogan et al. (2001b,c). However, a higher oil load (1:1) led to larger droplet size (Figs. 1a and 1b) and broader size distribution in the reconstituted emul-

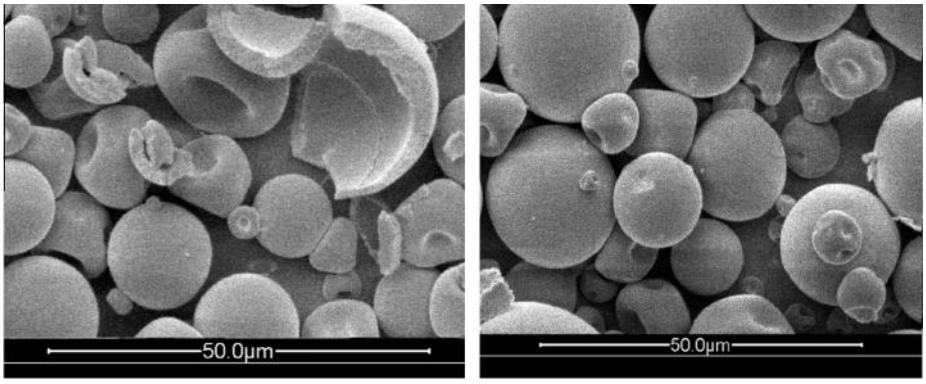


**Fig. 2.** Scanning electron micrographs for microcapsules dried at similar outlet temperature (70 °C) but different inlet temperatures. (A) Inlet temperature at 180 °C; (B) inlet temperature at 170 °C.

sions. This may be explained by the presence of surface oil after drying, related to the ME. The high oil-loading microcapsules (1:1) have low ME, indicating the occurrence of large scale emulsion destabilization during drying process, causing higher surface oil on the particles, and consequently leading to poor dispersion of dried microcapsules. The finding is in agreement with that of Hogan et al. (2001c).

### 3.2. Oil recovery and ME

Fig. 3 shows the recovery (%) of the non-volatile, volatile and total extractable oil, and ME of the optimum system (wall to core ratio = 4:1, inlet and outlet temperatures of 170 and 70 °C respectively) after spray drying. The recovery of total oil was reasonably high (>90%) in both the fish oil- and co-encapsulated microcapsules. However, the total oil recovered from the co-encapsulated microcapsules was lower than that of the fish oil-microcapsules ($p < 0.05$), most probably due to the lower retention of volatile compound (limonene) in the co-encapsulated microcapsules. The ME was not influenced by the type of core material used in the system.

The recovery of non-volatile oil, including fish oil and phytosterol esters, was significantly higher than the volatile component (limonene) because of their non-volatile nature. The much lower interfacial tension at the triglyceride–water interface than the hydrocarbon–water interface could be another possible reason accounted for the above differences (Fisher et al., 1985). The protein-based emulsifiers, especially β-casein, tend to adsorb at the hydrocarbon–aqueous interface than the triglyceride–aqueous interface to lower the interfacial tension, as revealed in our study (Chen, 2012). This meant that limonene would be concentrated at the oil–water interface, making it easily lost at the first instant during the drying process, by diffusion through any damaged wall matrices.

### 3.3. Oxidative stability of microcapsules

Generally, the primary oxidation (PV, Fig. 4) and secondary oxidation (AV, Fig. 5) of all samples increased during the accelerated storage trial. The encapsulated samples showed good protection from oxidation compared to the bulk fish oil ($p < 0.05$). There was no significant difference ($p > 0.05$) in the PV and AV between the encapsulated fish oil and the co-encapsulated LBCs.

The changes in EPA and DHA were evaluated as they were the important essential fatty acids contributed to the health benefit of fish oil. These fatty acids are highly unsaturated and prone to oxidation, so it is crucial to study their stability in the encapsulated system. Results (Fig. 6) showed that the encapsulated oils were highly protected in the wall matrices compared to the bulk fish oil, which had the low retention of 13.8% EPA and 10.8% DHA after storage. The loss of EPA and DHA during storage was significantly lower ($p < 0.05$) in the LBCs microcapsules than those contained only fish oil. The protection of EPA and DHA in the co-encapsulated microcapsules may be contributed by the antioxidant property of PE. Yasukazu and Etsuo (2003) studied the antioxidant effects of PE and its components on the oxidation of methyl linoleate and found that PE effectively suppressed the oxidation of methyl linoleate solution. We conducted a DPPH assay and found that PE had a good radical scavenging capacity (1609.8 ± 113.1 μmol TE/mg sample), indicating a high antioxidant property.

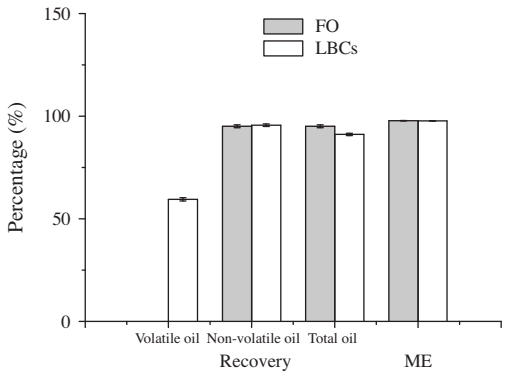


**Fig. 3.** Oil recovery and microencapsulation efficiency of microcapsules with fish oil and LBCs (wall to core = 4:1). FO = microcapsules with FO; LBC = microcapsules with BLCs. Error bars represent standard deviation.

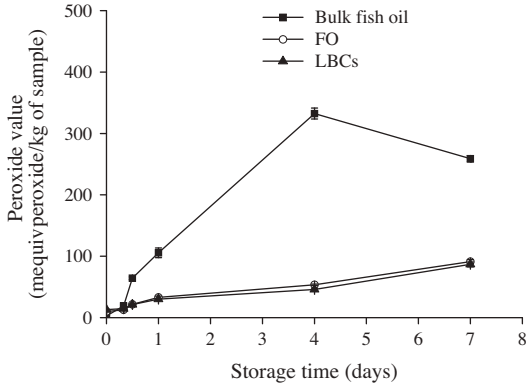


**Fig. 4.** Changes in the PV of bulk fish oil, encapsulated fish oil and LBCs from microcapsules during storage (45 °C, 30% RH with saturated oxygen). FO = microcapsules with FO; LBC = microcapsules with LBCs. Error bars represent standard deviation.

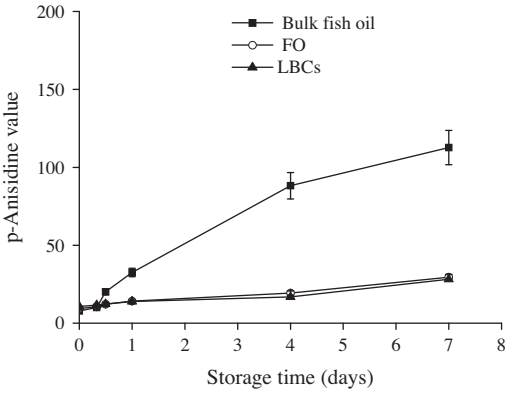


**Fig. 5.** Changes in the AV of bulk fish oil, encapsulated fish oil and LBCs from microcapsules during storage (45 °C, 30% RH with saturated oxygen). FO = microcapsules with FO; LBC = microcapsules with LBCs. Error bars represent standard deviation.

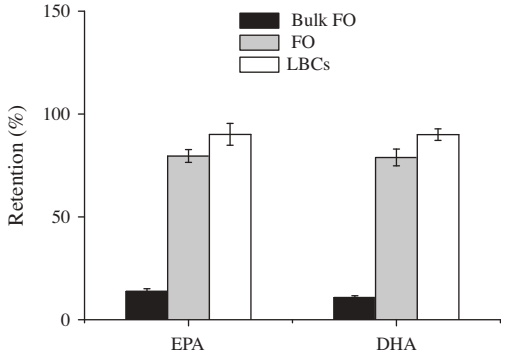


**Fig. 6.** Changes of EPA and DHA in fish oil, encapsulated fish oil and LBCs from microcapsules during storage (45 °C, 30% RH with saturated oxygen). FO = microcapsules with FO; LBC = microcapsules with LBCs. Error bars represent standard deviation.

In contrast to the retention of EPA and DHA, current results show that the differences in PV and AV were not significant between the fish oil-microcapsules and the LBCs-microcapsules during storage. This suggests that other factors, apart from the oxidation of polyunsaturated fatty acids, might contribute to the PV and AV of the LBCs-microcapsules. Since the LBCs-microcapsules contained three components, namely fish oil, PE and limonene as core material, the other two materials would probably contribute to the PV and AV. To investigate further, we evaluated the individual PE and the limonene contents of the LBCs-microcapsules, as well as assessed the PV of those components after storage. We found that the individual PE content has no significant change after storage but there was a change in the limonene (Table 2). In addition, although PE did not contribute to the increase of PV, limonene showed a dramatic increase in PV (data not shown). This suggests that the increase of PV in the LBCs-microcapsules could be contributed by the primary oxidation of limonene as there was 12.5% limonene present in the core. Oxidation of limonene could happen more easily due to its higher surface tension at the interface than that of the triglycerides (fish oil and phytosterol esters), at which protein prefers to adsorb to reduce the interfacial tension (as explained before). This results in the concentration of limonene at the interface (in this case the lipid-air interface) where oxidative stress is higher. The oxidation of limonene can lead to formation of hydroperoxides at the initial oxidation stage; and formation of derivatives, especially carvone, in line with the oxidation of unsaturated fatty acids (Djordjevic et al., 2008). Table 2 shows oxidation products were detected after storage.

**Table 2**
The limonene, individual phytosterol, and limonene oxide contents in the LBCs-microcapsules at day 0 and after 7-day accelerated storage.

| Components | Day 0 | Day 7 |
|---|---|---|
| Limonene (mg/g core materials) | 74.4 ± 2.3[a] | 65.6 ± 3.0[b] |
| Limonene oxides (mg/g core materials) | n/d | 0.117 ± 0.008 |
| β-Sitosterol | 414.4 ± 3.8[a] | 415.8 ± 2.9[a] |
| Campesterol | 218.7 ± 5.7[a] | 217.9 ± 6.1[a] |
| Stigmasterol | 216.3 ± 6.9[a] | 214.6 ± 10.2[a] |

n/d = not detected;
Superscripts with different letter in the same row are significantly different ($p < 0.05$) from each other.

There were about 74% limonene remained in the co-encapsulated microcapsules after drying and (Table 2) and this reflected the overall loss of volatiles due to evaporation (diffusion of volatiles through wall materials) during drying. The content of limonene was reduced to 65% after accelerated storage due to either oxidation to its derivatives as explained above or evaporation. Since the amount oxidation products detected was low, evaporation was thought to be the main factor contributing to the loss of limonene during storage.

### 3.4. Sensory evaluation

Sensory evaluation was conducted for the spray-dried powder and the reconstituted emulsions. After drying, off-flavour (oxidized fish oil odour) and fishy odour were not detected in the LBCs-microcapsules but they were perceptible in the fish oil-microcapsules (Table 3). The limonene flavour was scored as slightly perceptible in the co-encapsulated microcapsules. The odour attributes of the fish oil- and the LBCs-reconstituted emulsions follow similar trends to those of the dried samples. Comparing the powder and the reconstituted emulsion samples, the intensity of fishy odour was significantly higher ($p < 0.05$) in the reconstituted emulsion than the fish oil-microcapsules. However, there was no significant difference ($p > 0.05$) in the LBCs-microcapsules and its reconstituted emulsions for fishy odour. Feedback from the sensory panel revealed that the limonene flavour present in the emulsions has masked the fishy odour.

After the accelerated storage, fishy odour could be noticeably detected in both types of microcapsules. The intensity of off-flavour was perceived as significantly lower ($p < 0.05$) in the LBCs-microcapsules than that of the fish oil-microcapsules. Little change was detected in the attributes of limonene and limonene oxides in the co-encapsulated samples after the storage. For the reconstituted emulsion, fishy odour was more noticeable than the off-flavour after storage. The intensity of limonene flavour increased and limonene oxide was perceived in the reconstituted emulsion of the co-encapsulated samples. Comparing the fish oil microcapsules and their reconstituted emulsion forms, the perceived fishy odour and off-flavour was significantly stronger ($p < 0.05$) in the emulsion form. On the other hand, for the co-encapsulated samples, the fishy odour was the same in the powder and the reconstituted emulsion; but off-flavour was much lighter while limonene intensity was higher in the emulsion form. The stronger limonene intensity is probably contributed to the lower off-flavour perceived in the reconstituted emulsion. This can be supported by the finding of Serfert et al. (2010) who studied the influence of flavour compounds (including orange flavour) on the odour quality of reconstituted fish oil microcapsules. Their finding indicated that the increase of orange flavour to 0.2% (wt/wt total emulsion) could mask the odour of fish oil emulsions.

The sensory evaluation results are in agreement with the chemical analysis of EPA and DHA retention, where the LBCs-microcapsules had significant lower oxidized fishy odour and higher

**Table 3**
The intensity of odour attributes of the microencapsulated fish oil and the LBCs at day 0 and after 7-day accelerated storage.

| Day of storage | Odour attributes | Powder sample | | Reconstituted emulsion | |
|---|---|---|---|---|---|
| | | FO | LBC | FO | LBC |
| 0 | Fishy | $1.2 \pm 0.4^{b}$ | $0.8 \pm 0.6^{c}$ | $2.6 \pm 0.7^{a}$ | $0.8 \pm 0.4^{c}$ |
| | Off-flavor (oxidised FO) | Trace | n/d | Trace | n/d |
| | Limonene | – | Trace | – | $1.0 \pm 0.6^{a}$ |
| | Carvone and limonene oxide | – | n/d | – | n/d |
| 7 | Fishy | $2.0 \pm 0.6^{b}$ | $1.9 \pm 0.9^{b}$ | $4.5 \pm 0.8^{a}$ | $1.7 \pm 0.5^{b}$ |
| | Off-flavor (oxidised FO) | $2.8 \pm 0.7^{b}$ | $1.8 \pm 0.6^{c}$ | $3.3 \pm 0.5^{a}$ | Trace |
| | Limonene | – | Trace | – | $1.7 \pm 0.5^{a}$ |
| | Carvone and limonene oxide | – | n/d | – | Trace |

FO = microcapsules with FO; LBC = microcapsules with BLCs; n/d = not detected;
"Trace" means very subtle odour was detected with mean value equal or less than 0.5.
Values with different superscript letters in the same row are significantly different ($p < 0.05$) from each other.

essential fatty acid retention after storage than those containing fish oil only.

## 4. Conclusions

This study has provided some useful insight into the application of the co-encapsulation concept to protect spray-dried fish oil microcapsules from oxidation by introducing other lipophilic bioactive components, namely phytosterol esters and limonene as core material. Good quality microcapsules could be produced with spray drying performed at inlet and outlet temperatures of 170 and 70 °C respectively, using a ratio of wall to core of 4:1. Current finding show that co-encapsulation of fish oil with PE could effectively prevent polyunsaturated fatty acids from oxidation, and the incorporation of limonene showed good ability to mask the undesirable fishy odour.

## Acknowledgements

We wish to thank the University of Auckland for providing a doctoral scholarship to the first author and a staff research grant to make this research possible. We would also like to acknowledge the New Zealand Postgraduate Study Abroad Award to the first author.

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