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ORIGINAL PAPER

The Determination of n-3 Fatty Acid Levels in Food Products Containing Microencapsulated Fish Oil Using the One-Step Extraction Method. Part 1: Measurement in the Raw Ingredient and in Dry Powdered Foods

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Abstract An optimized one-step extraction and methylation method (OSE) for the determination of n-3 fatty acid levels in microencapsulated fish oil products has been described. Validation of the method for the food ingredient powder MEG-3® (a commercial microencapsulated fish oil product manufactured by Ocean Nutrition Canada Ltd, with a gelatin shell matrix) has been demonstrated and compared to a method that combines enzymatic digestion of the shell material, gravimetric determination of released oil payload and an official method of fish oil analysis. Excellent agreement between the two methods was also achieved using a microencapsulated standard of pure ethyl palmitate. The OSE method was applied to an infant formula containing microencapsulated fish oil powder and is shown to be a valid method for such dry powdered foods.

Keywords One-step extraction · n-3 Fatty acids · Microencapsulation · Fish oil · Infant formula · Functional foods · Method validation

Introduction

The availability of the fatty acid docosahexaenoic acid (DHA) in the diet of early humans is believed to have played a major role in the evolution of the human brain [1]. In modern times, the inadequate supply of long chain n-3 fatty acids in diets typical of many countries is believed to result in sub-optimal brain and retinal development [2, 3], lower intelligence [4] and a variety of neurodevelopmental [2, 5] and psychiatric [6] disorders. There is a wide spectrum of neurological disorders which have been shown to improve on supplementation with n-3 PUFA including attention deficit hyperactivity disorder (ADHD), bipolar affective disorder [7, 8], major depression and Alzheimer's disease. However, perhaps the most widely recognized benefits of n-3 PUFA are in the prevention and management of cardiovascular disease [9, 10] as seen in some large-scale trials [11].

The demonstrated importance of n-3 fatty acids in human health has led to a recent surge in interest in increasing consumption of these fats in a wide range of dietary sources [12, 13], given the practical limitations in widespread fish or fish-oil consumption as well as cultural dietary prejudices against fish in some regions. Thus, n-3 fatty acids largely from fish oil but also from oleaginous marine microorganisms, have been incorporated into a diverse range of commercial food products including for example, orange juice, bread, milk, yogurt, cereal, margarine and prepared frozen meals. By far the greatest challenge in producing successful functional foods containing fish oils is in the avoidance of any traces of fishy odours and flavours, which are characteristic of even minor amounts of PUFA oxidation [14, 15]. These flavours may be present in oils of apparently high quality as judged by typical methods of oil quality testing such as peroxide or *p*-anisidine values [16]. One important strategy to limit the sensory impact of fishy off-notes on the final food product is to keep the amount of fish oil included per serving of food to a reasonable level, typically in the range of 20–150 mg n-3 PUFA per serving. For comparison, a serving of two typical 1 g fish oil capsules contain ~0.6 g n-3

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PUFA, two fish oil concentrate capsules ~1.2 g n-3 PUFA and a 100 g portion of farmed Atlantic salmon contains ~2 g n-3 PUFA [17]. Minimum recommended daily intakes are around 0.5 g/day (e.g. American Heart Association, International Society for the Study of Fats and Lipids [18, 19]), which can be provided by two oily fish meals per week, or 1–2 g/day for therapeutic doses. Hence, functional foods can provide useful increases in omega-3 intakes for people not regularly consuming fish, but multiple servings are required because of the limited amounts that can be incorporated without affecting the taste of the food product. In some cases, processed fish oils have the advantage over fish consumption that contaminants often associated with fish (e.g. heavy metals, PCBs, etc.) can be effectively removed.

Considering the diversity of food products into which manufacturers have attempted to incorporate fish oils, it is not surprising that a number of technologies have emerged [12, 20] to convert such oils into stable ingredients which are suitable for large-scale food manufacturing. The combination of ingredient and packaging technology can result in a longer shelf-life and better characteristics for handling in a food processing facility where fishy off-odours and potential cross-contamination can be a major concern. The most widely used delivery system is microencapsulation [20] which converts fish oil into a powder, ideally with better oxidative stability and sensory properties than the starting fish oil. Generally, the process of microencapsulation begins with the emulsification of highly refined fish oil containing antioxidants in a solution of a food-grade encapsulant. Various fish oil emulsions have been used directly in food and beverage applications but this generally requires specialized aseptic packaging and is only suitable for relatively short shelf-life products.

In microencapsulation, the encapsulant(s), which eventually becomes the microcapsule shell material, is chosen depending on the process to be followed and the desired properties of the final powder. Materials such as proteins (gelatins, whey protein, caseinate, vegetable proteins) starches (maltodextrins, modified starches) gums (alginates, carrageenan, gum arabic, pectin), cellulose derivatives, chitosans, cyclodextrins, waxes and fats have all been used [20, 21], sometimes in combination with other modifiers. Often, the emulsified oil is then spray-dried to produce a powder which can then be further coated if required. Typical oil payloads (i.e. the weight percentage oil in the powder) for spray-dried powders are in the 20–50% range. Another method of microencapsulation now commercialized for production of fish oil powder (MEG-3®, Ocean Nutrition Canada Ltd) uses a process known as complex coacervation [22] in which oil is emulsified in the presence of two food grade polymers (primarily a gelatin). Careful adjustment of solution pH results in the two polymers bearing opposite charges; this causes them to combine to form a coacervate with accompanying phase change. The coacervate forms on the surface of oil droplets and with careful manipulation of production conditions (agitation, sequential addition of shell materials, temperature, pH changes, etc.) the morphology of the microcapsule can be changed from a single oil droplet with a single outer shell to a tight cluster of small oil droplets (~1 μm diameter) each individually coated with shell material, surrounded by a thick (~several micrometer) outer shell forming a particle of 20–80 μm in diameter. The oil payloads can be high in this process, 50–70% is typical but even higher is possible with single shell particles. The shell material can be stabilized by the use of enzymes (*transglutaminases*) which cross-link proteins. Such particles are simultaneously insoluble in water or boiling water but can be broken down by prolonged acid hydrolysis or by proteases. Finally, the suspension of finished microcapsules can be spray-dried to produce a free-flowing powder. However, incorporation of these stabilized microcapsules formed by complex coacervation into functional foods results in a particular challenge of food analysis—the stability imparted by encapsulation also means that typical procedures for fat extraction such as the well-known *Bligh and Dyer method* [23] are ineffective in extracting the oil from the core of the powder even without the food matrix present. Clearly for trade in functional foods containing this type of ingredient it is necessary to develop and validate analytical methods which can accurately and reproducibly determine modest n-3 PUFA levels in a wide range of functional foods. Here, we describe the use of an optimized version of a previously published method for "one-step" extraction and methylation procedure (OSE) [24–31] employing the use of methanolic HCl. Results from the validation of the method for determining the n-3 PUFA content of microencapsulated fish oil powder and n-3 PUFA levels in dry foods are presented and discussed. The extension of this work to other food types will be presented in a further paper in this series.

Experimental Procedures

OSE Method for Fish Oil Powder

A 0.05-g sample (M_S) of MEG-3 powder (Ocean Nutrition Canada, Dartmouth, NS, Canada) was accurately weighed into a screw-top 50-mL centrifuge tube. To this was added a weighed 1-mL aliquot of the recovery standard (M_{C19}) solution (accurately known concentration C_{C19} mg/g) which is a 1 mg/g solution of trinonadecanoin (Nu-Chek Prep Inc., Elysian, MN, USA) in toluene. A weighed 2 mL

of toluene was added (M_{T1}) plus 6 mL of a freshly prepared solution of 10% acetyl chloride in methanol (Sigma–Aldrich, Inc., St Louis, MO, USA). The tube was capped tightly with a Teflon-lined top, mixed using a vortex mixer and placed in an agitating hot water bath for 2 h at 80 °C. At the end of this time the centrifuge tube was cooled to room temperature and the contents mixed thoroughly on a vortex mixer. 10 mL of 6% aqueous sodium carbonate solution was added and a further 2 mL of toluene (weight M_{T2}). The tube was centrifuged at 1,100 rpm for 15 min and the upper layer (toluene containing FAME) was decanted into a clean vial along with about 1 g of anhydrous sodium sulfate. A 1 mL aliquot of the dried toluene extract was then accurately weighed (M_{TE}) into a 2-mL GC vial. To this was added 0.2 mL (weight M_{C23}) of the GC quantification standard solution of 0.7 mg/g methyl tricosanoate (Nu-Chek Prep Inc., Elysian, MN, USA) in toluene (exact concentration C_{C23} mg/g). The GC vial was capped and finally analyzed by GC for EPA and DHA using an official method which uses experimentally determined response factors for EPA and DHA (e.g. Ph. Eur. [32] or AOCS [33]). However, unlike these quoted methods the GC response factors for EPA and DHA should be determined from a reference solution containing EPA and DHA in their methyl ester forms (Nu-Chek Prep Inc., Elysian, MN, USA, purity >99% verified by GC-FID), along with methyl tricosanoate (at concentrations of $C_{EPA\text{-}R}$, $C_{DHA\text{-}R}$, and $C_{C23\text{-}R}$ mg/g) and not derivatised since derivatisation losses are accounted for by the recovery standard. This was achieved by weighing accurately pure methyl esters of DHA (24 mg), EPA (36 mg) and C23:0 (28 mg) and dissolving in 10 mL of 0.05 g/L BHT in iso-octane, recording the final weight and vortexing to ensure homogeneity.

In the present work, an Agilent 6890 GC with an FID detector (Agilent Technologies, Wilmington, DE, USA) and a 30 m × 0.32 mm i.d × 0.25 μm film thickness Restek Famewax column (Restek, Bellefonte, PA, USA) was used. Typical run conditions were—injector temperature 250 °C, detector 275 °C, hydrogen carrier gas at 2.5 mL/min, split ratio of 100:1, make-up flow of 45 mL/min. The initial oven temperature was 190 °C held for 1 min then ramped at 5 °C/min to reach the final temperature of 235 °C and held for 2 min. This basic method was optimized to ensure that the measured n-3 fatty acids were all properly resolved and in particular that DHA was resolved from 24:0 and from 24:1. The GC FID peak areas determined from the sample analysis are A_{EPA}, A_{DHA}, A_{C23} and $A_{n\text{-}3}$ where $A_{n\text{-}3}$ is the summed peak areas for all of the n-3 fatty acid methyl esters (i.e. 18:3, 18:4, 20:4, 20:5, 21:5, 22:5 and 22:6). The GC FID peak areas determined from the reference solution analysis are $A_{EPA\text{-}R}$, $A_{DHA\text{-}R}$, and $A_{C23\text{-}R}$.

A freeze-dried reference bread material containing MEG-3 fish oil powder was analysed using the same procedure except that a 1 g sample (M_S) was used. All weights in the procedure were recorded in grams measured to the nearest 0.1 mg and concentrations were recorded in mg/g.

Calculations:

The GC response factor for EPA or DHA ($RF_{EPA\text{ or }DHA}$) determined from the reference solution:

$$RF_{EPA} = (A_{C23-R} \times C_{EPA-R})/(A_{EPA-R} \times C_{C23-R})$$

The mass (mg) of EPA, DHA or 19:0 ($X_{EPA,\text{ DHA or C19}}$) in the GC vial:

$$X_{EPA} = (A_{EPA} \times C_{C23} \times RF_{EPA} \times M_{C23})/A_{C23}$$

In the entire sample added to the reaction tube, the mass (mg) of EPA, DHA or C19 ($Y_{EPA,\text{ DHA or C19}}$):

$$Y_{EPA} = (X_{EPA}/M_{TE}) \times (M_{T1} + M_{T2} + M_{C19})$$

The recovery factor, F_{REC}:

$$F_{REC} = (M_{C19} \times C_{C19})/Y_{C19}$$

The mass (mg) of EPA or DHA per gram MEG-3 powder, ($Z_{EPA\text{ or }DHA}$):

$$Z_{EPA} = (1/M_S) \times Y_{EPA} \times F_{REC}$$

The above calculations express the final result as milligram lipid in the methyl ester form per gram of powder since the weights relate to the weight of the methyl tricosanoate internal standard (with response and recovery corrections). To express the final result in triglyceride or free fatty acid form multiply by 1.0 or 0.96, respectively as described in other methods [32, 33].

Enzymatic Digestion of the Microcapsule Shell to Determine Oil Payload

All weights in this procedure were recorded to ±0.1 mg on a calibrated balance. A sodium phosphate buffer was prepared by mixing 80 mL of Solution A (2.8 g NaH_2PO_4 in 100 mL deionized water) with 420 mL of Solution B (14.2 g Na_2HPO_4 in 500 mL deionized water) in a 1-L bottle. Porcine pancreatin (30 mg, Sigma–Aldrich, Inc., St Louis, MO, USA) was weighed into a clean 30-mL vial and microcapsules (250 mg) were added and weighed (W_{mc}). To this was added 10 mL of the sodium phosphate buffer (W_{buff}). The vial was vortexed and placed in a shaking water bath at 37 °C at 60 rpm for 1 h. The vial was then cooled to room temperature and 10 mL of ethyl acetate (W_{sol}) added. The vial was shaken twice for 1 min by hand and then centrifuged at 1,000 rpm for 10 min; 1–2 mL of the organic layer (the top layer) was removed and added to

a tared vial and the weight (W_{ext}) recorded. This layer was then dried under nitrogen and placed uncapped on a heating block at 40 °C for 1 h to ensure that all solvent had evaporated, giving a final weight of extracted oil (W_{oil}). From this was calculated the total oil extracted and hence the exact percentage oil in the microcapsules:

Total oil extracted, $W_{tot} = (W_{sol}/W_{ext}) \times W_{oil}$

Percentage oil in the microcapsules (payload) $= (W_{tot}/W_{mc}) \times 100$

Infant Formula OSE

A 0.23-g sample (M_S) of dry commercial infant formula was accurately weighed into a screw-top 50-mL centrifuge tube. To this was added an accurately weighed 0.1 g aliquot of the recovery standard (M_{C19}) solution (accurately known concentration C_{C19} mg/g) which is a 1 mg/g solution of trinonadecanoin (Nu-Chek Prep Inc., Elysian, MN, USA). A weighed 4 mL of toluene was added (M_{T1}) plus 6 mL of 10% acetyl chloride in methanol. The tube was capped tightly with a Teflon-lined top, mixed using a vortex mixer and placed in an agitating hot water bath for 2 h at 80 °C. At the end of this time the centrifuge tube was cooled to room temperature and the contents mixed thoroughly on a vortex mixer. 10 mL of 6% sodium carbonate solution was added along with 2 mL of toluene (weight M_{T2}). The tube was centrifuged at 1,100 rpm for 15 min and the upper layer (toluene containing FAME) decanted into a clean vial along with about 1 g of anhydrous sodium sulfate. A 1-mL aliquot of the toluene extract was then accurately weighed (M_{T3}) into a 2-mL GC vial. A silica cartridge (Waters Sep-Pak® Vac 6 cc 500 mg) was conditioned by passing 4 mL of toluene through using a vacuum manifold and discarding the filtrate. The weighed extract was cleaned-up by passing through the cartridge with vacuum applied followed by two 1-mL aliquots of toluene used to rinse the GC vial and cartridge. The filtrate was collected into a pre-weighed test tube and weighed to determine amount of toluene collected (M_{T4}). A 1 mL of the toluene extract (M_{TE}) was accurately weighed into a 2-mL GC vial and 0.2 mL (weight M_{C23}) of the GC quantification standard solution added (0.7 mg/g methyl tricosanoate in toluene, exact concentration C_{C23} mg/g). The GC vial was capped and the extract analyzed by GC as described for powder above. The GC FID peak areas determined from the sample analysis are A_{EPA}, A_{DHA}, A_{C23} and $A_{n\text{-}3}$ where $A_{n\text{-}3}$ is the summed peak areas for all of the seven n-3 fatty acid methyl esters given earlier. The GC FID peak areas determined from the reference solution analysis are $A_{EPA\text{-}R}$, $A_{DHA\text{-}R}$, and $A_{C23\text{-}R}$.

Calculations:

Calculations are as described for powder above except that in this case in the entire sample added to the reaction tube.

The mass (mg) of EPA, DHA or C19 ($Y_{EPA,\ DHA\ or\ C19}$):

$$Y_{EPA} = X_{EPA} \times (M_{T4}/M_{TE}) \times [(M_{T1} + M_{T2} + M_{C19})/M_{T3}]$$

The mass (mg) of EPA (or DHA) in methyl ester or triacylglyceride form per gram of infant formula (Z_{EPA} or Z_{DHA}):

$$ZEPA = (1/M_s) \times Y_{EPA} \times F_{REC}$$

Results and Discussion

Analysis of Microencapsulated Fish Oil Powder

Prior to discussing measurements of microencapsulated oil in foods it is important to demonstrate the effectiveness of the OSE method for the microencapsulated powder ingredient itself. The exact oil payload must be determined experimentally since there are small losses during the process, mainly as a result of fines (which may be enriched in shell material) being vented during spray-drying.

A number of available direct solvent extraction methods have been attempted by us including soxhlet extraction with a variety of solvents commonly used for lipid extractions, pressurized fluid extraction and supercritical fluid extraction. However, we have consistently found in our laboratory that prior disruption of the shell material is essential for quantitative fat recovery. This could be achieved via acid digestion, enzymatic digestion of the shell material, or mechanical disruption. We have achieved the latter by means of a planetary ball mill (Pulverisette 6, Fritsch GmbH, Idar-Oberstein, Germany), using a stainless steel enclosure and grinding balls which can grind the powder to sub-micron sizes and hence release the encapsulated oil. This has the advantage that relatively large amounts of oil can be readily extracted as required for testing oil quality parameters. However it is not practical as a routine quantitative method since it is slow, only one sample at a time can be extracted and specialized grinding equipment is required. In contrast, enzymatic digestion using readily available proteases provides a simple method to completely liberate encapsulated oil. Solvent extraction then allows quantitative recovery of encapsulated oil which can be measured gravimetrically. This measurement of oil payload in the microcapsules can then be combined with FAME determination of the starting oil which we have performed following a reliable official method [32, 33] to give an overall weight percentage of the n-3 components of

the powder. This method of enzymatic digestion of the shell combined with methylation of the oil (referred to hereafter as ECM) is used here as an independent method to verify the accuracy of the direct OSE approach. Whilst it would be ideal to have used a formally validated method instead for this purpose, our experience has been that available methods (such as the widely used AOAC 996.06 [34]) employing separate acid digestion, extraction and methylation result in low recoveries of microencapsulated oil in food systems and is therefore not considered suitable for determination of accuracy in this application.

In order to reduce the error associated with fatty acid measurements in fish oil (which is a complex mixture of triacylglycerides with minor amounts of other lipids, antioxidants, sterols, etc.) a standard of ethyl palmitate was microencapsulated for the determination of method accuracy. GC FID indicated that the purity of this standard was 99.8%. Since a pure standard was used, the ECM method can be performed without the need for methylation and GC analysis—the gravimetric oil determination can be simply corrected for the measured purity. The results are given in Table 1 below and show remarkably good agreement between the two methods (1.1% difference) demonstrating that the accuracy of the OSE method (combined extraction, methylation and GC analysis) is comparable to the gravimetric determination of oil. Note that each experiment was performed on multiple days with resulting relative standard deviations of 3.3% for OSE ($n = 9$) and 1.3% for ECM ($n = 6$).

Table 1 Comparison of powder fatty acid content using OSE and gravimetric payload determination following enzymatic digestion of the shells of microcapsules containing only ethyl palmitate

	OSE	ECM
Number of determinations	9	6
Conditions	2 analysts, 3 days	2 days, 2 enzyme lots
Payload (%)		45.2
Average mg (ethyl palmitate)/g (powder)[a]	458	453
SD	15	6

[a] Corrected for ethyl palmitate purity

As a further test of accuracy and method robustness, powder measurements for OSE and ECM were compared using microencapsulated powder prepared with a range of payloads, nominally between 55 and 70%. In this case fish oil (from *anchovy*) was encapsulated and results for (EPA + DHA) and total n-3 (the sum of the n-3 fatty acids 18:3, 18:4, 20:4, 20:5, 21:5, 22:5, 22:6) were compared as shown in Table 2. Each of the OSE results is the average of duplicate injections on each of three separate one-step extractions (i.e. six GC runs). The gravimetric payload determination was also performed in triplicate. The maximum deviation between methods was about 2% for both (EPA + DHA) and total n-3 fatty acids, indicating excellent accuracy and robustness of the method towards payload variations.

It would be highly desirable to produce microencapsulated food ingredients from oils containing the highest possible concentrations of n-3 fatty acids. This would allow delivery of a desired *per serving* amount of n-3 fatty acids using the minimum amount of microencapsulated ingredient, thus minimizing its effect on the food product and potentially the cost. The processes used in producing concentrates of n-3 fatty acids often involve conversion of the natural oil triacylglycerides to ethyl esters, distillation and finally reesterification to form n-3 triacylglyceride concentrates. In order to test the robustness of the OSE method to variations in omega-3 concentration and oil type, OSE and ECM results were compared for both microencapsulated triglyceride concentrates (which may also contain small amounts of mono- and di-acylglycerides [35]) and microencapsulated ethyl ester concentrates. These concentrates have a total n-3 content of approximately 70% compared to 35% for the natural anchovy oil and were made in lab-scale batches into powders with payloads of 50–55%. The results given in Table 3 indicate that there is similar agreement between the two methods for natural oil and n-3 concentrate in either ethyl ester or triacylglyceride form.

Another variable encountered in production of microencapsulated oils, is in the type of shell protein used—in

Table 2 Comparison of powder fatty acid content using OSE and ECM for microcapsules containing fish oil at various payloads

Stated payloads are nominal values only but experimental payloads are used in determining ECM results (see text)

Nominal payload (%)	mg (EPA + DHA)/g (powder)			mg (total n-3)/g (powder)		
	ECM	OSE	Δ (mg/g)	ECM	OSE	Δ (mg/g)
55	141	138	3	173	173	0
60	160	161	−1	196	193	3
65	170	173	−3	209	207	2
70	183	183	0	224	220	4
	Average Δ (mg/g)		−0.3	Average Δ (mg/g)		2.3
	Average absolute Δ (mg/g)		1.8	Average absolute Δ (mg/g)		2.3

Table 3 Comparison of powder fatty acid content using OSE and ECM for microcapsules containing fish oil and fish oil concentrates

Oil type	mg (EPA + DHA)/g (powder)				mg (total n-3)/g (powder)			
	ECM	OSE	Δ (mg/g)	Δ (%)	ECM	OSE	Δ (mg/g)	Δ (%)
Ethyl ester concentrate 70% n-3	356	363	−7	−2.0	396	394	2	0.5
Triacylglyceride concentrate 70% n-3	315	311	4	1.3	353	338	15	4.2
Anchovy oil (*triacylglyceride*) 35% n-3	173	174	−1	−0.6	209	205	4	1.9
	Average Δ (mg/g)		−1.3	−0.4	Average Δ (mg/g)		7.0	2.2
	Average absolute Δ (mg/g)		4.0	1.3	Average absolute Δ (mg/g)		7.0	2.2

the present case gelatins which can vary in animal origin, bloom strength and method of manufacture. The effect of gelatin type was tested using fish oil microencapsulated using two types of fish gelatin (bloom strength 220–240), and one type of porcine gelatin (bloom strength 270), as well as from production on two manufacturing sites. The results (each triplicate OSE measurements) are given graphically in Fig. 1a compared to ECM values and it can be seen that a similar level of agreement is achieved to other results above. These data were also evaluated to see whether the number of replicates could be satisfactorily reduced from 3 to 2 which would be more suitable for a Quality Control laboratory environment. Based on this set of seven samples it was found that the maximum deviation between the average value for each possible duplicate (i.e. all three possible combinations of two results for each triplicate) and the triplicate average is 0.9% (1.6 mg/g) and the overall average difference between duplicate and triplicate determinations is 0.4% (0.7 mg/g). Hence, it can be concluded that under the conditions used for analysis of the microencapsulated ingredient alone, duplicate determination would be adequate.

Figure 1b indicates the level of precision that is obtained with the OSE method for nine independent measurements on a single lot of fish oil powder. Overall, a mean of 175 mg/g with relative standard deviation of 1.0% ($n = 9$) was achieved for measurement of (EPA + DHA) and 186 mg/g with relative standard deviation of 1.4% ($n = 9$) for the measurement of total n-3. The slightly higher variance for total n-3 is a reflection of the greater uncertainty associated with quantifying seven FAME rather than two in the case of (EPA + DHA) and similar trends are observed in the above tables. However, this degree of precision is similar to that expected for GC analysis alone, indicating the excellent precision from the OSE procedure itself.

A final test of reproducibility between three analysts was performed using two types of fish oil powder (fish gelatin and porcine gelatin shell material) each of which was analysed by OSE in triplicate. A summary of the results are given in Fig. 1c which indicates that in the worst case, a deviation of 4% from the average was achieved and it may be significant that this was from an analyst with no previous experience in using the method. For comparison, the ECM result is included which differs from the mean of the three triplicate OSE results by +2.8% in one case and

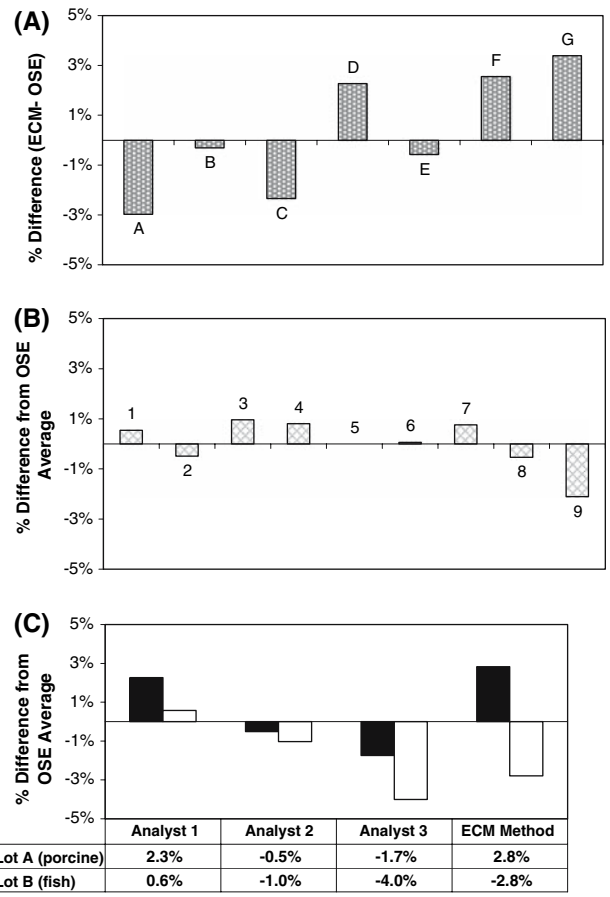

Fig. 1 Precision and reproducibility results: **a** percentage difference between mg (EPA + DHA)/g powder results using ECM and OSE methods for seven production powders containing lots *A–G* where *A–D* contain fish gelatin and tuna oil, *E* contains porcine gelatin and anchovy oil, *F* contains fish gelatin and anchovy oil from a second production facility and *G* is a kosher version of *F*; **b** percentage difference between nine OSE mg (EPA + DHA)/g powder determinations for a single lot compared to the average as a measure of precision; **c** percentage difference between OSE mg (EPA + DHA)/g powder results for triplicate analysis by three independent analysts and the average value for each powder type; the difference from the ECM result is also given

−2.8% in the other. Overall, considering all of the above data, the OSE method is shown to be a satisfactory method for the determination of n-3 content of various oils microencapsulated inside shells which are primarily various types of gelatins.

It would be desirable to prove the validity of this method for microencapsulates containing other oils, shell materials, and originating from different processes. However, since the results presented here are for microcapsules which contain highly unsaturated, readily oxidisable oil along with cross-linked protein shells which are resistant to solvent extraction, bases and mild acid conditions, this represents a demanding test case for the method. Furthermore, the general principle of using acid digestion in the analysis of fat from broad range of foods is widely used (e.g. AOAC 996.06 [34]). Hence, it is highly likely that using the OSE procedure similar results could be obtained with a variety of microencapsulated oil products and it would be desirable that future work could extend this validation to include these.

OSE Analysis of an Infant Formula Containing Microencapsulated Fish Oil

A commercial infant formula not containing DHA was dissolved in water and spiked with MEG-3 powder to give four DHA levels of between 0 and 80 mg per 100 g serving. The resulting milk was then spray-dried on a Niro Mobile Minor spray-drier (Niro Inc., Columbia, MD, USA) and all four levels were analysed by the OSE method. The results are presented in Fig. 2 which shows that the observed DHA levels were considerably below the expected values when spray-drying was used prior to OSE. The experiment was then repeated with new milk preparations, but using freeze-drying in enclosed flasks rather than spray-drying. It can be seen from Fig. 2 that the latter procedure resulted in close agreement between the spike amounts and measured results. Hence, it was concluded that the poor recovery of DHA seen when spray-drying was used prior to OSE was due to the spray-drying process itself. A possible explanation for this is the selective loss of fine particles to the spray-drier exhaust. Nonetheless, using freeze-drying the experiment demonstrated that good accuracy can be achieved by the method. The level of accuracy achieved is shown in Fig. 2 where it can be seen that the maximum deviation between the actual and measured DHA amount is 2 mg/serving (for the lowest level spike); the SD of triplicate measurements is also 2–3 mg/serving. Overall, the average deviation from expected DHA level was 3.8% with the deviation for the two highest levels being below 2%.

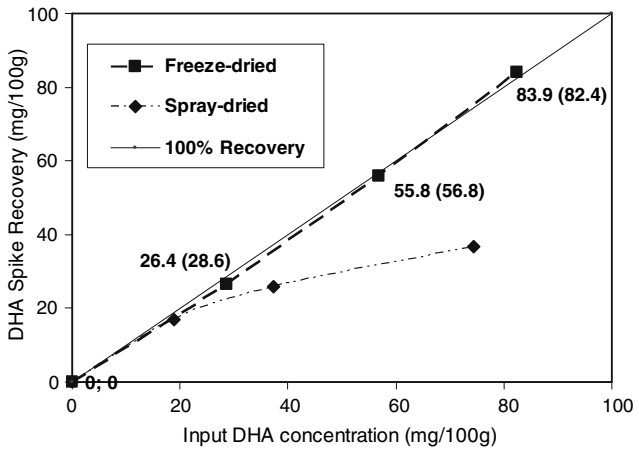

Fig. 2 Recovery of DHA measured by the OSE method in infant formula spiked with MEG-3 powder and dried by freeze-drying and spray-drying. Data labels given for the freeze-dried results indicate measured DHA level (mg DHA/100 g) with input level in *parenthesis*

A further test of accuracy and precision was performed on infant formula powder prepared to have about 30 mg DHA per 100 g formula. Firstly, the OSE procedure was performed five times by one analyst and a further two times by a second; the results are given in Table 4. This illustrates the precision of the OSE method for this matrix with a relative SD of 4.2% achieved overall. For comparison, a single extract was injected into the GC five times and the level of instrumental precision resulted in a relative SD of 2.2% for DHA. Good agreement was also achieved between the two analysts. To confirm the accuracy of these results, identical tubes were prepared for OSE containing the dry infant formula and internal standard. These were then spiked with accurately known amounts of DHA ethyl ester solution in a standard

Table 4 OSE measurements on infant formula containing MEG-3 powder and OSE results from standard additions experiments using spiking solutions of DHA ethyl ester

	DHA mg/serving	Deviation from average (mg/serving)
Unspiked OSE		
1st Analyst	30.2	2.2
	34.2	−1.8
	31.8	0.6
	33.1	−0.7
	32.6	−0.1
2nd Analyst	31.4	1.0
	33.6	−1.2
Average	32.4	
% RSD ($n = 7$)	4.2	
Standard additions		
1st Analyst	29.7	−2.7
2nd Analyst	36.8	4.4
Average	33.3	0.8

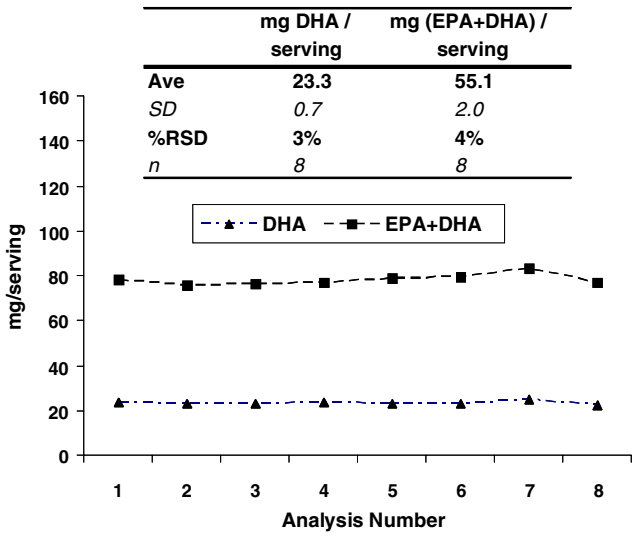

Fig. 3 Summary of results obtained throughout the validation exercise for the bread reference material

additions experiment which was performed separately by two analysts. The standard additions data showed some scatter ($R^2 = 0.99,\ 0.95$) and this is reflected in the deviation of the extrapolated results (−2.7 (−8.3%) and +4.4 (+13.6%) mg DHA/serving) from the mean result (32.4 mg/serving) of the unspiked data (Table 4). This may be a due to inaccuracies associated with additions of the spiking solution into the reaction tubes. However, the overall standard addition result (33.3 mg/serving), obtained through additions of accurately known DHA ethyl ester solution, is in agreement with the average result from the OSE obtained with reference to the microencapsulated recovery standard.

An additional test of accuracy and method repeatability was performed by means of an in-house food reference material run alongside samples on each day of the validation experiments. The reference material is a homogeneous powder of freeze-dried bread containing the MEG-3 microencapsulated fish oil powder. The ongoing use of this material alongside all types of food analysis provides a valuable check of data quality. The results summarized in Fig.3 indicate good method precision with a relative SD of 3% for DHA measurement and 4% for the combined (EPA + DHA) measurement. Furthermore, the results are in good agreement with the averages of 23 ± 3 mg DHA/serving and 54 ± 5 mg (EPA + DHA)/serving taken from a control chart of the last 90 measurements. Thus, good precision and reproducibility, similar to that seen for infant formula, was also obtained for an altogether different food type during the validation. It is worth noting as well that the usual range of recoveries for the trinonadecanoin internal standard is different for different food matrices. With microencapsulated fish oil ingredient alone, a recovery of 90–100% is typical, for the freeze-dried reference bread recovery is in the 80–90% range and for the infant formula more variability is seen with recoveries between 65 and 85%. There are a variety of experimental factors and matrix effects which contribute to these recoveries. In particular, the EPA and/or DHA concentration in the food is significant which highlights the importance of adjusting the trinonadecanoin concentration to approximately match the analyte concentration and hence achieve a similar recovery. Overall, the level of accuracy and precision in the data presented here clearly indicates that with recoveries in the 65–100% range, the trinonadecanoin internal standard properly corrects for n-3 concentrations. Furthermore, the OSE method is shown to be suitable for the analysis of dried powders as exemplified by infant formula.

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