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Article

ANIMAL FOOD, PET FOOD, AND PLANT NUTRIENT METHODS

The Analysis of Docosahexaenoic Acid (DHA) in Dried Dog Food Enriched with an *Aurantiochytrium limacinum* Biomass: Matrix Extension Validation and Verification of AOAC Method 996.06

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Abstract

Background: Docosahexaenoic acid (DHA) plays an important role in brain and retinal development in dogs. However, supranutritional dietary supplementation can result in health issues, including gastrointestinal bleeding, making the accurate analysis of DHA in dog food important for nutritional and welfare regulatory compliance.
Objective: The aim of this study was to conduct a validation and verification of the AOAC **996.06** method, and hence establish its fitness for purpose, for the analysis of DHA in dried dog food supplemented with a heterotrophically grown unextracted DHA-rich *Aurantiochytrium limacinum* biomass.
Methods: The AOAC **996.06** method, which involves the use of gas chromatography coupled to flame ionization detection (GC-FID), was used to conduct a validation of the analysis of DHA in dried dog food and the results were verified in a second laboratory.
Results: The method was found to be linear over the ranges analyzed and results were found to be within the acceptance criteria for precision and accuracy, verifying the applicability for this matrix. The selectivity and sensitivity of the method were also determined.
Conclusions: The AOAC **996.06** method is fit for purpose for the analysis of DHA in dry dog food kibble.
Highlights: The method can be applied to various dog food samples, supplemented with an unextracted *Aurantiochytrium limacinum* biomass, using alternative manufacturing methods, i.e. pelleted and extruded with no significant matrix effects being observed.

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The omega-3 fatty acids (n-3 FA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are frequently formulated into companion animal diets. The United States National Research Council recommends a dietary intake of preformed DHA + EPA of 0.4 g/kg DM (dry matter) as the maintenance requirement for dogs (1). The consumption of preformed DHA during early development has been found to be particularly beneficial, with a higher intake associated with improved retinal, cognitive, psychomotor, and immunologic functions while deficiencies of these nutrients can result in permanent functional abnormalities (2, 3). Furthermore, nutritionists and veterinarians recommend omega-3 dietary supplements in the prevention and management of a variety of canine diseases (4). Increasing a dog's dietary intake of EPA and DHA has been found to alleviate symptoms associated with canine atopic dermatitis (5), cognitive dysfunction syndrome (6), and osteoarthritis (7). Aggressive behavior has been associated with low n-3 FA status in German Shepard dogs (8), while supplementation has been found to reduce the severity of various other behavioral disorders, such as fearfulness, destructiveness, and inappropriate elimination (9). EPA and DHA can deliver an anti-inflammatory effect and they have also been shown to have additional benefits, such as the reduction of blood lipid concentration (10) and anti-tumor effects (11).

However, there are also some adverse effects associated with supranutritional n-3 FA intake in dogs (4). Gastrointestinal issues can arise when dogs are treated for illnesses with high levels of n-3 FA, and have been cause for stopping supplementation in some studies (12). Potential detrimental effects on wound healing, lipid peroxidation, and weight gain have been observed due to a high intake of n-3 FA (4). As a result, a safe upper limit of combined EPA + DHA has been established as 2800 mg/1000 kcal of diet, which is equivalent to 2080 mg for a 10 kg dog (1, 4).

Fish oils are a rich source of EPA and DHA and are used to supplement canine diets with n-3 FA. However, the use of fish oil as a long-term source of dietary n-3 FA is not considered sustainable due to declining fish stocks (13). Plant-based ingredients such as linseed or canola are also used to increase the n-3 FA content of pet foods; however, these ingredients contain alpha-linolenic acid (ALA), which has not been shown to provide similar benefits as supplementation with EPA and DHA (4). Mammals have the capacity to convert ALA to EPA and DHA, but this process is inefficient, leading nutritionists to recommend the direct consumption of the preformed long chain n-3 fatty acids (1). Oily fish are a rich source of these long chain n-3 FA as a result of their diets. Various unicellular organisms such as algae and other protists are primary producers of EPA and DHA in the aquatic environment. The *Thraustochytrids,* a group of protists commonly misclassified as microalgae but with fungus-like properties, which includes *Schizochytrium* and *Aurantiochytrium sp*., can contain high concentrations of DHA (14, 15). Some of these organisms can be grown sustainably on a large scale, through heterotrophic fermentation on simple carbon sources, and subsequently their biomass fed to food animals to enrich the n-3 FA concentrations of milk, pork, chicken meat, and eggs (16–23).

Given the benefits of the effects of an increased dietary intake of n-3 FA, but also considering the existence of the safe upper limit for EPA + DHA consumption, the necessity to accurately measure these fatty acids in dog food is of clear importance. In addition, large variations between commercial dog food brands have been observed in terms of their DHA contents and thus a validated method is desirable in order to make reliable comparisons (24).

The method for the analysis of fatty acids by the extraction from their relevant matrices and the preparation of fatty acid methyl esters (FAME) was first developed by Folch et al. (25). This method was further applied to the analysis of fatty acids in other matrices such as meat and feeding stuffs and subsequently detection and quantification was developed using gas chromatography (GC) (26, 27). The AOAC method **996.06** is now routinely used to determine the fatty acid content of various substances and uses GC coupled with flame ionization detection (FID) (28). While other techniques do exist, such as nuclear magnetic resonance (NMR) infrared spectroscopy (IR) and mass spectrometry interfaced with gas and liquid chromatography (GC/MS and LC/MS), GC-FID methods are considered to be the most robust methods, and are overwhelmingly popular, for the analysis of fatty acids; their applications have been thoroughly reviewed (29). Methods involving esterification are generally preferred as the resulting esters are highly volatile in the GC conditions and easy to measure (30). As government agencies and regulatory authorities often require that laboratories or researchers use AOAC methods for regulatory studies (30, 31), the AOAC **996.06** method had previously been selected by the present authors to validate methodologies for the analysis of DHA and fatty acids in the *Thraustochytrid* feed ingredient, unextracted *Aurantiochytrium limacinum* biomass (32), which is rich in DHA and also to the analysis of edible chicken tissues enriched with DHA (33). Therefore, the current study aimed, for the first time, at validating the AOAC **996.06** method to accurately quantify DHA in dog food when enriched with an unextracted *A. limacinum* biomass as a matrix extension, and then to verify these results in a second laboratory. The method was also used to assess the recovery of DHA in a large batch of dog food produced for consumption. In performing the analytical determination using different canine diets, we wanted to investigate if DHA in dog food fortified with unextracted *A. limacinum* biomass could be recovered to comparable extent with DHA-free control dog food spiked with standard DHA from a commercial supplier. This would help identify if any matrix effects might be encountered in the analysis of DHA in dog food where unextracted *A. limacinum* biomass is used as the source of DHA.

Experimental

Instrumentation and Chemicals

The validation study was performed at Eurofins Nutrition Analysis Centre (Des Moines, USA). All experiments were carried out using an Agilent 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA) with a flame ionization detector and Agilent 7683 autosampler (Agilent Technologies, Santa Clara, CA). The following chemicals were acquired from Fisher Scientific (Iowa, USA): hydrochloric acid, ammonium hydroxide, chloroform, ethanol diethyl ether, petroleum ether, methanol, toluene, hexane, sodium sulphate, and boron fluoride. Pyrogallic acid and methyl undecanoate were purchased from Sigma-Aldrich (Iowa, USA). GLC-85 FAME mix, methyl 4,7,10,13,16,19-docosahexaenoate (C22:6 FAME), C13:0 triglyceride (internal standard), and DHA were purchased from Nu-Chek Prep (Minnesota, USA).

The verification analysis was carried out at Mérieux NutriSciences (Burnaby, Canada). Analytical experiments were conducted using an Agilent 6890 N gas chromatograph (Agilent, Ontario, Canada) with a FID and an Agilent 7683 autosampler

(Agilent, Ontario, Canada). All appropriate chemicals for the experimentation were obtained from Fisher Scientific (Ontario, Canada). Solvents included: ethanol, methanol, chloroform, toluene, hexane, diethyl ether, and petroleum ether. Chemicals and reagents included sodium sulphate, boron fluoride, ammonium hydroxide and hydrochloric acid. Pyrogallic acid was sourced from TCI America (Portland, USA). The following standards were acquired from Nu-Chek-Prep Inc. (Minnesota, USA): GLC-85 FAME mix, methyl 4,7,10,13,16,19-docosahexaenoate, 1,2,3-triundecanoylglycerol C11:0 (internal standard for sample extraction), methyl undecanoate, and DHA.

Preparation of Stock Solutions, Internal Standards, and Calibration Standards

Similar sample preparation procedures were followed for the validation and verification studies. A mixed FAME standard solution (GLC 85) for establishing retention times and calculating relative response factors was prepared by breaking open a standard vial and transferring contents to a 3-dram glass vial. The original vial was washed with hexane, added to the 3-dram vial and then the solution was made up to approximately 3 mL with hexane. A methyl undecanoate internal standard solution was prepared in hexane by dissolving 500 mg of methyl undecanoate in a final volume of 10 mL using a volumetric flask yielding a concentration of 50 mg/mL. A 5 mg/mL internal standard solution of C13:0 triglyceride (for the validation) and 1,2,3-triundecanoylglycerol (for the verification) was prepared in a volumetric flask with chloroform. Standard DHA stock solutions were prepared in hexane for spiking into samples of control (blank) dog food matrix as described in the precision and accuracy experiments.

Dog Food Matrices

Three different dog food diets were used during the validation and verification. These included: (i) a control (blank) dog food matrix which contained no DHA. This matrix was used for the spiked precision and accuracy experiments: (ii) pelleted dog food test samples which were formulated to include specific amounts of unextracted *A. limacinum* biomass; and (iii) an extruded dog food containing 0.5% of unextracted *A. limacinum* biomass which was prepared for a recovery experiment.

Control (Blank) Dog Food Matrix

A formulation of a control (blank) dog food matrix was specifically prepared to include no DHA-rich ingredient in the formulation (Purina, TestDiet, St. Louis, MO, USA). This specifically excluded fishmeal and fish oil ingredients. The diet consisted of: rice flour, porcine meat meal, corn gluten meal, ground wheat, wheat middlings, corn oil, dehulled soybean meal, dried beet pulp, porcine red blood cells, dried whey, salt, dicalcium phosphate, brewers dried yeast, wheat germ, calcium carbonate, ferrous sulfate, choline chloride, pyroxidine hydrochloride, vitamin A acetate, cholecalciferol (vitamin D3), sodium selenite, menadione dimethylpryimidinol bisulfite (vitamin K), calcium iodate, folic acid, calcium pantothenate, DL-alpha tocopherol acetate, cobalt carbonate, manganese oxide, thiamine mononitrate, nicotinic acid, vitamin B12, copper sulfate, riboflavin, and biotin. The ingredients were mixed, and subjected to the TestDiet proprietary standard methodology for preparing a dry dog food on a lab-scale pelletizer, including conditioning with steam and concomitantly pelleting through an 8 mm pellet die.

Pelleted Dog Food Diets Supplemented with Unextracted *A. limacinum* Biomass

Aurantiochytrium limacinum powder (Alltech Inc., Nicholasville, KY, USA) contained 69 g crude fat/100 g DM biomass and 16 g DHA/100 g DM biomass. A commercial diet, LabDiet® 5006, (Purina, TestDiet, St. Louis, MO, USA), was used as the basal formulation for the dog food and consisted of the following ingredients: Ground corn, porcine meat and bone meal, dehulled soybean meal, corn gluten meal, porcine animal fat preserved with BHA and citric acid, wheat middlings, whole wheat, dried beet pulp, spray dried animal blood cells, whey, salt, wheat germ, fish meal, brewers dried yeast, calcium carbonate, choline chloride, pyridoxine hydrochloride, vitamin A acetate, cholecaliferol, ferrous sulfate, menadine dimethyl pyrimidinol bisulfate, zinc oxide, folic acid, calcium iodate, DL-alpha tocopheryl acetate (vitamin E), calcium pantothenate, manganous oxide, thiamine mononitrate, nicotinic acid, copper sulfate, vitamin B12 supplement, riboflavin, biotin, cobalt carbonate, and sodium selenite.

Customized isonitrogenous and isocaloric preparations of the basal diet were formulated to include 0.5, 1, 3, and 5% DHA-rich unextracted *A. limacinum* biomass powder (ALL-G-RICH®, CCAP 4087/2; Alltech Inc. Nicholasville, KY) with minimal reformulation to minimize nutritional variation between the diets. On a lab-scale apparatus, diets were conditioned and pelleted into 8 mm chunks, before packaging into 10 kg bags and stored at room temperature in a dry dark area. The nutritional profile of each diet is provided in Table 1.

Recovery of DHA in an Extruded Dog Food Diet

A commercial scale batch of an extruded standard dog food was prepared by Perfection Pet Foods (Visalia, CA, USA) under Good Manufacturing Practice. The dietary formulation included corn ground, chicken meal, brown rice, corn gluten meal, soybean meal, wheat millrun, poultry fat, dicalcium phosphate 21%, AFB optimizer liquid, beet pulp, whole flaxseed, AFB Dog Digest, potassium chloride, salt, vit premix dog/cat, TM premix dog/cat, Petox Plus Dry, Rendox AT (BHA/BHT), and 0.5% unextracted *A. limacinum* biomass. The *Aurantiochytrium* biomass powder used in this experiment contained 68.9 g crude fat and 19.3 g DHA/100 g DM biomass, respectively, and resulted in 965 g DHA/tonne dog food based on the dietary inclusion level. All primary ingredients were mixed prior to water and steam addition in the pre-conditioner at approximately 90°C and then cooked until the food reached 90°C minimum through-out. The food was passed through a number of critical processing steps including the extruder (Extru-Tech Inc., Sabitha, KS), drier at 85–100°C for approx. 25 min, conveyed to the enrobing system for final liquid ingredient addition and the finished kibble was finally passed through a cooling system and into a bin storage unit.

Preparation of Test Samples

(a) Extraction of fat

As per the extraction procedure outlined in AOAC 996.06 (28), approximately 1 g of a thoroughly homogenized dog food sample was weighed into a Mojonnier flask. Pyrogallic acid (100 mg) was added to the flask with 2 mL each of the internal standard and ethanol. To this solution was added 10 mL of 8.3 M hydrochloric acid and the flask was placed in a 70–80°C water bath for 40 min while mixing the contents every ten minutes. The flask was cooled to room temperature, and subsequently vortex-

Table 1. Analytical composition (%) of the dog food diets supplemented at a rate of 0, 0.5, 1, 3, or 5% with unextracted *Aurantiochytrium limacinum* (AURA) biomass

		% AURA inclusion				
Analyzed nutrient value, %	Basal diet[a]	0%	0.5%	1%	3%	5%
Protein, %	25.3	25	24.9	24.9	24.6	24.4
Fat (ether extract), %	7	8.4	8.7	9	10.2	11.4
Fat (acid hydrolysis), %	8.0	9.6	9.6	9.5	9.3	9.1
Fibre (max), %	2.4	3	3	3	2.9	2.8
Nitrogen-free extract (by difference), %	47.2	45.9	45.7	45.5	44.8	44
Ash, %	8.1	7.7	7.7	7.6	7.5	7.3
Energy, kcal/g	3.53	3.59	3.61	3.63	3.7	3.77

[a] DHA free.

mixed for 15 s. Enough ethanol was added to fill the bulb of the Mojonnier flask. Twenty-five milliliters of diethyl ether was added to the flask, which was stoppered and shaken gently. Petroleum ether (25 mL) was added and the flask was again shaken and vortex-mixed for two minutes. The flask was centrifuged for 5 min at 600 rpm to yield a clear supernatant, which was decanted into a round bottomed flask and evaporated using a rotary evaporator and nitrogen to remove any residual solvent.

(b) Methylation

Methylation was carried out in line with the procedure in AOAC **996.06** (28). The extracted fat residue was dissolved in 2–3 mL of chloroform and 3 mL volume of diethyl ether was added. The solution was added into 10 mL glass tube. The solution was evaporated to dryness under nitrogen at 40˚C and 2 mL of 7% BF_3 and 1 mL of toluene was added. The solution was vortex-mixed for 30 sec to suspend the residue in solution and sealed with a screw-cap top with a Teflon/silicone septum. The tube was heated in a forced air oven for one hour at 100˚C. The tube was cooled to room temperature. A 5 mL aliquot of HPLC-grade water, 1 mL hexane, and about 1 g sodium sulfate were added and the tube was capped and vortex-mixed for one minute. The mixture was centrifuged at 4000 rpm and the clear supernatant was dried again with sodium sulfate and injected into the GC-FID.

(c) GC-FID conditions

The GC system utilized a SP2560 100 m long capillary column, 0.25 mm internal diameter; 0.20 μm film thickness (Supelco, Pennsylvania, USA). The system used helium as a carrier gas with an initial flow of 0.75 mL/min and an average velocity of 18 cm/sec at a pressure of 30 psi. Air and hydrogen were used for the FID with pressures of 60 and 40 psi, respectively. The initial oven temperature was 100 ˚C which was ramped up to 240 ˚C at a rate of 3 ˚C/min and was held for 15 min. The injector was employed in the split mode at a ratio of 200:1 and at a temperature of 225˚C. The detector temperature was set to 285˚C. System suitability was evaluated using the calibration GLC-85 reference FAME standards injected in five replicates.

Experiments

(a) Linearity

To demonstrate the linearity of the GC-FID system, a standard curve that covered a range of concentrations of C22:6 FAME in the samples was prepared. For the validation study six calibration standards were made covering the range 0.3 to 15 mg/mL. The range employed for the verification study was 0.01 to 5 mg/mL. Calibration standards were prepared fresh for respective linearity experiments. Linear regression, forced through the origin and with equal weighting, was applied to the peak area ratios plot for the construction of calibration curves and to provide information on the slope, coefficient of determination and intercept. The acceptance limit was set at $\geq$ 0.995 to demonstrate the linearity of the system. This limit is within that as described by Peris-Vicente et al. (34)

(b) Sensitivity

For the validation study, to determine the limit of detection (LOD) and limit of quantification (LOQ), the control (blank) dog food matrix was spiked with 0.01% DHA. The DHA concentration was established in 10 replicates in order to calculate the LOD and LOQ. In the verification study the lowest standard on the calibration line was accepted as the lower limit of quantification.

(c) Selectivity

For both the validation and the verification, selectivity was assessed qualitatively by examining chromatograms for the presence of potentially interfering peaks. The control (blank) dog food matrix with no internal standard was examined for interference at the retention times for both DHA and the internal standard. A zero sample (i.e., control (blank) dog food matrix plus internal standard) was also assessed, prior to the sample sets being analyzed, for possible interference with DHA from the internal standard. Any interference would be corrected prior to performing calculations.

(d) Precision and accuracy

Precision and accuracy were tested by spiking control (blank) dog food matrix at three concentrations of DHA and performing replicate analyses. For the validation study three replicate determinations were made at spike concentrations of DHA at approximately 1000, 6000, and 9500 μg/g. The analysis was also performed singly by a second analyst. For the verification study, four replicates were performed on two separate batches at three different target concentrations of 100, 2000, and 8000 μg/g.

The precision of the analysis was determined by evaluating the repeatability (%RSD) of the replicates of the respective spiked samples at each concentration. %RSD is calculated as (standard deviation (n replicates) * 100)/mean calculated concentration (n replicates).

The accuracy of the method was determined by measuring the concentrations in respective samples and comparing the mean measured concentration with the nominal concentration.

The accuracy of measurement is expressed as relative error (%RE). The acceptance criteria for precision (%RSD) was ≤ 10% while the acceptance criteria for accuracy (%RE) was ± 10% i.e., yielding recoveries between 90 and 110%. %RE is calculated as ((mean calculated concentration−nominal concentration) * 100)/nominal concentration. Acceptance criteria are within those presented in scientific literature (34).

To demonstrate inter-analyst repeatability in the validation study, a second analyst repeated the accuracy study by spiking the control (blank) dog food matrix sample once at each of the three levels and determining the quantity of DHA.

(e) Analysis of dog food diets supplemented with an unextracted A. limacinum *biomass*

For the validation study, each dog food sample (i.e., basal diet including 0%, 0.5%, 1%, 3%, or 5% *A. limacinum* biomass) was analyzed by a first analyst in triplicate on two separate occasions. A second analyst performed the analysis on each of the dog food samples in triplicate. For the verification study, six replicate samples of each of the five dog food samples were analyzed over two days, with a second set of the dog food samples also being analyzed in duplicate over two days.

(f) Recovery of DHA in an extruded dog food diet

The recovery of DHA in a batch of commercially produced dog food was also tested using the above methodology (Mérieux NutriSciences, Burnaby, BC). The total number of bags required for the analysis was calculated by taking the square root of the estimated total number of bags expected from the batch, plus one (i.e., $\sqrt{413}$ bags + 1 = 21 bags). To implement a systematic sampling approach, the total number of bags expected was divided by the number of samples required (413/21) resulting in every 20th bag being tested for DHA concentration. The mass of each laboratory sample was ∼200 g of dog food kibble. The data were checked for outliers and summary statistics, recovery and the coefficient of variation were calculated.

Results

Validation Study Results

(a) Linearity

The correlation coefficients for C22:6 FAME ranged between 0.99981 and 0.999942 and hence met the requirement of ≥ 0.995 to demonstrate the linearity of the system.

(b) Sensitivity

The LOD was established as the standard deviation of the 10 replicates x 3 while the LOQ was established as the standard deviation of the 10 spiked replicates x 10. The experimentally determined LOQ of 0.012% agreed with the expected method LOQ of 0.01%.

(c) Selectivity

The criterion for selectivity was met with no peaks detected near the retention time C22:6 FAME for any of the control (blank) dog food matrix samples. Figures 1 and 2 show chromatograms for control (blank) dog food matrix and a spiked sample of control (blank) dog food matrix containing C22:6 FAME and internal standard.

(d) Precision and accuracy

The results of the precision and accuracy assessment of the validation are shown in Table 2. The precision (%RSD) was within acceptance limits while the recovery was between 90% and 110% and hence met the criteria for the accuracy of the system. The second analyst's results were within three standard deviations of the mean established by the first analyst. All spike recoveries were within 90% to 110% of the expected levels and no peaks were detected in the control (blank) dog food matrix analysis.

(e) Analysis of dog food diets supplemented with unextracted A. limacinum *biomass*

The concentrations of DHA detected in the five dog food diets supplemented with unextracted *A. limacinum* biomass are summarized in Table 3. The mean and standard deviation of the results for each experimental diet were calculated and the variability was calculated for each batch analyzed by the first and second analysts. The %RSD calculated for was ≤ 10% in each case except in the analysis of the second analyst on the batch of basal diet that included 0% *A. limacinum*. This outlier was due to the low level of DHA present in the test samples (i.e., less than 100 μg/g), which was less than the LOQ determined in the sensitivity experiment.

Verification Study Results

(a) Linearity

The correlation coefficient was established as 1.000 for DHA which met the acceptability criterion of R ≥ 0.995.

(b) Sensitivity

The lower limit of quantification is accepted as the lowest standard on the calibration line which is 10 μg/mL. The analyte response at the LLOQ was at least five times the average response due in the control (blank) matrix.

(c) Precision and accuracy

Results were found to be within the acceptance criteria of ≤ 10% for precision (%RSD), and ± 10% for intra- and inter-assay accuracy (%RE). Results for precision and accuracy are shown in Table 4.

(d) Selectivity

There were no interfering peaks observed at the retention times of interest for the internal standard and DHA (C11:0, 12.2 min; DHA, 26.8 min) in the control (blank) dog food matrix.

(e) Analysis of dog food diets supplemented with unextracted A. limacinum *biomass*

Results of the analysis of the dog food samples supplemented with unextracted *A. limacinum* biomass are summarized in Table 3. The analysis was carried out in five experimental batches with the following concentrations of basal diet including 0%, 0.5%, 1%, 3%, or 5% *A. limacinum* biomass equating to DHA in the range of 0 to approximately 8000 μg/g. Each dog food sample was analyzed in six replicates on the instrument over a single day. The concentration of DHA was calculated in μg/g for each sample and the mean, standard deviation and precision (%RSD) were calculated for each sample set. The %RSD calculated for each sample ranged from 1.70 to 6.49, within the criteria of ≤ 10%. In addition, a second analysis was carried out on each of the five dog food samples (four replicates) over two days. The %RSD calculated for the second analysis ranged from 0.76 to 6.31, also within the criteria of ≤ 10%.

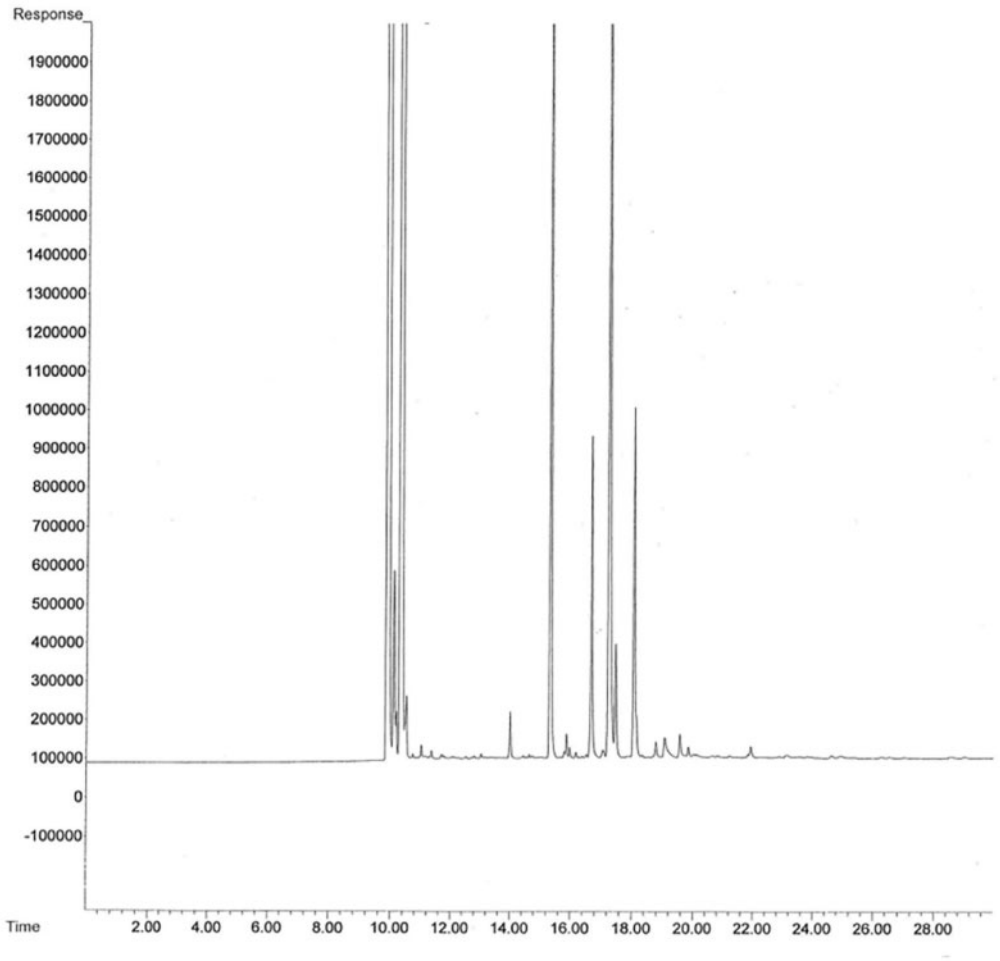

Figure 1. Chromatogram of control (blank) dog food matrix.

(f) Recovery of DHA in an extruded dog food diet

The summary statistics detailing the concentration of DHA detected in the dog food recovery experiment are presented in Table 5. The mean recovery of DHA detected was 85.1% while the coefficient of variation was 7.66%.

Discussion

An increasing public awareness of the importance of DHA in human nutrition has led to the exploitation of various aquatic sources of DHA. Historically, fish have been this primary source, with fishmeal commonly used as a source of DHA in animal nutrition (35). Shortfalls in the sourcing of DHA encouraged the feed additive industry to look at more sustainable ways at obtaining DHA, and especially to explore the potential of microorganisms, as the one used herein, *A. limacinum*, in their ability at sustaining global dietary requirements (36). While the sourcing of DHA is evolving, it consequently implies re-optimization or re-inspecting analytical methods for the correct determination of such analytes, especially when coming from novel ingredient sources amended into animal feed and pet food matrices. Matrix effects as well as extractability limitations represent the main challenges between cross matrix method transfer, and the research herein represents an essential step in evaluating the attribute of new DHA-rich ingredient, within expected inclusion levels, and validating its correct absolute quantification.

The validation results presented in this study indicate that the method is suitable for the determination of C22:6 FAME over the range 300–15 000 μg/mL. The verification study extended the linearity to a lower threshold to 10 μg/mL. The upper limit analyzed was 5000 μg/mL, which was within the range analyzed during the validation. The results for precision and accuracy met the acceptance criteria in both the validation and verification studies. Selectivity experiment demonstrated that method was specific for the analyte. Inter-analyst repeatability was examined in the validation study by virtue of a second analyst performing parts of the validation. The verification was a further demonstration of the repeatability of the method as it was conducted in an independent laboratory and with different analysts. Taken together the results of the validation and verification experiments demonstrate the fitness for purpose of the method in determining DHA in dog food which was the central objective of the studies undertaken. In addition, the method was applied to the analysis, in a practical sense, of determining DHA in pelleted and extruded dog food.

The recoveries of the analysis of pelleted dog food samples supplemented with unextracted *A. limacinum* biomass were within acceptable limits as were the recoveries of the control (blank) dog food matrix spiked with commercially available DHA. These results agree with the 89–95% recovery of DHA in pelleted layer hen diets (temperature set to 65–70°C for between 10–15 sec and pelleted through a 3 mm pellet die) as observed

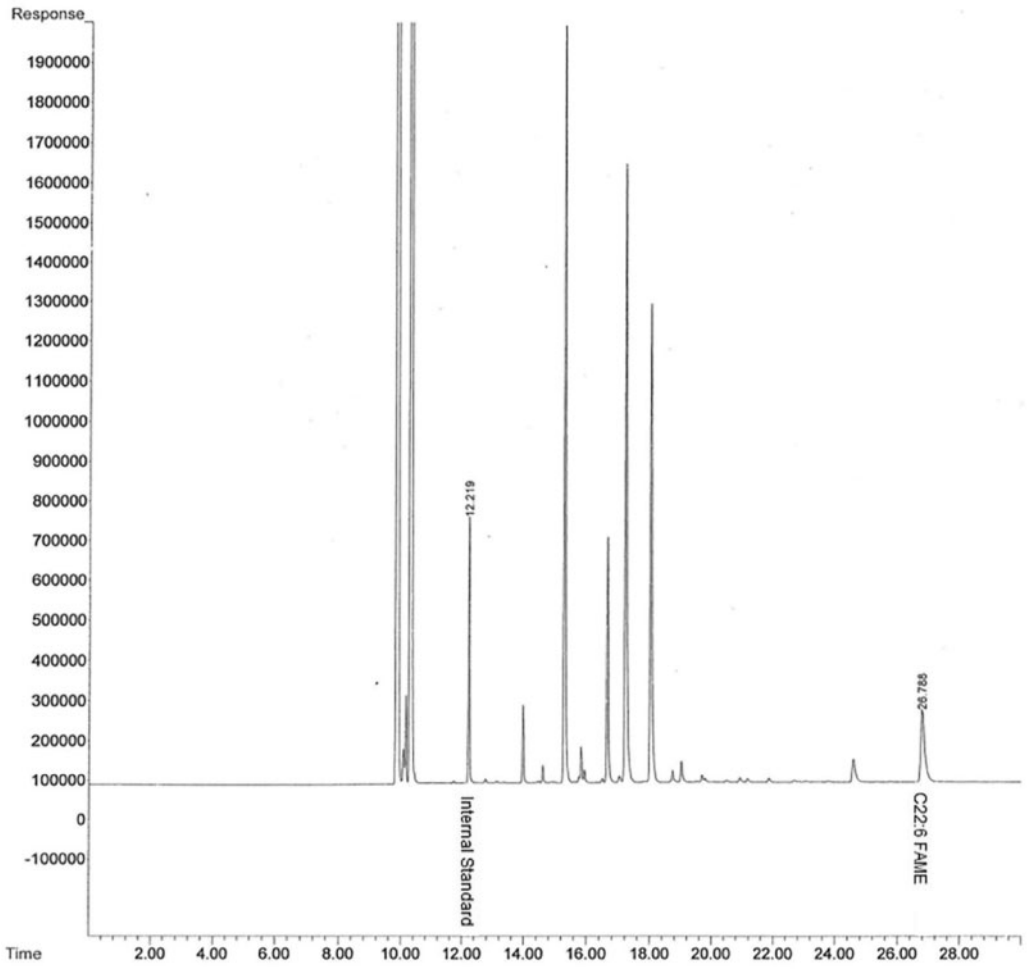

Figure 2. Chromatogram of a spiked sample of control (blank) dog food matrix containing C22:6 FAME and internal standard.

Table 2. Precision and accuracy of DHA detected in control (blank) dog food matrix spiked with three concentration levels (approx. 1000, 6000, and 9500 μg/g) of DHA in the validation study

	1000 μg DHA/g spike			6000 μg DHA/g spike			9500 μg DHA/g spike		
Replicate no.	Expected result (μg/g)[a]	Sample result (μg/g)	% recovery	Expected result (μg/g)	Sample result (μg/g)	% recovery	Expected result (μg/g)	Sample result (μg/g)	% recovery
# 1	970	890	91.7	5660	5390	95.2	9470	8990	95
# 2	940	850	90.6	5830	5570	95.6	9330	9030	96.7
# 3	960	890	92.6	5830	5500	94.4	9700	9210	94.9
Mean		877	91.6		5487	95.1		9077	95.53
S.D.		23.09			90.74			117.19	
%RSD		2.63			1.65			1.29	
%RE			8.37			4.93			4.47
Second Analyst	950	860	90.8	4240	4380	103.2	9110	8750	96.1

[a] Expected Result (μg/g) = (concentration of DHA in spike solution [mg/ml]*vol of spike solution [ml]/weight of control (blank) dog food matrix [g])*1000.
Spike recoveries must be between 90% and 110%.

$$\%RSD = \frac{\text{standard deviation (n replicates)} * 100}{\text{mean calculated concentration (n replicates)}}$$

$$\%RE = \frac{(\text{mean calculated concentration} - \text{nominal concentration}) * 100}{\text{nominal concentration}}$$

by Keegan et al. (37). However, Moran and co-workers (16) found a lower DHA recovery (82.3%) in a dairy protein concentrate pellet, which was produced under a higher conditioning and pelleting temperature (70–75°C). In the commercial production, dry dog food is not pelleted but 95% is processed through an extruder, a high-temperature short-time bioreactor (38). The extrusion process imparts several advantages on the nutritional and physical quality of the food including improving starch and

Table 3. DHA concentrations detected in the dog food diets supplemented with 0, 0.5, 1, 3, and 5% *Aurantiochytrium limacinum* (AURA) as part of the validation and verification studies

	Validation			Verification		
Sample	Mean DHA μg/g[a]	SD	%RSD	Mean DHA μg/g[a]	SD	%RSD
Analyst 1	(n = 6)			(n = 6)		
Basal diet with 0% AURA	80	4	5.67	77	4	5.62
Basal diet with 0.5% AURA	870	10	1.70	901	15	1.7
Basal diet with 1% AURA	1570	20	1.22	1747	113	6.49
Basal diet with 3% AURA	4810	140	2.81	5172	84	1.63
Basal diet with 5% AURA	7920	280	3.53	8028	266	3.31
Analyst 2[b]/Analysis 2[c]	(n = 3)			(n = 4)		
Basal diet with 0% AURA	70	10	15.75[d]	76	5	6.31
Basal diet with 0.5% AURA	870	10	1.15	898	21	2.23
Basal diet with 1% AURA	1580	30	1.93	1752	13	0.76
Basal diet with 3% AURA	4960	40	0.76	5102	104	2.03
Basal diet with 5% AURA	7790	260	3.29	8176	102	1.25

[a]Weight of FA in Fatty Acid Form (μg)) = [((Response FAME * Weight of IS * Response Factor IS)/Response IS * Response Factor FAME) * Conversion factor for FAME form to FA form].
[b]For the validation study, a second person analyzed the experimental canine diets for inter-analyst repeatability.
[c]For the verification study, a repeat analysis by the same analyst was completed over two days (n = 4) for repeatability.
[d]This %RSD does not meet the acceptance criteria, however as the Limit of Detection was established as 100 μg/g, this sample would be reported in practice as <100 μg/g.

Table 4. Precision and accuracy of DHA detected in control (blank) dog food spiked with three concentration levels (approx. 100, 2000, and 8000 μg/g) of DHA in verification study

Batch No.	100 μg/g spiked sample		2000 μg/g spiked sample		8000 μg/g spiked sample	
	Sample result (μg/g)	% recovery	Sample result (μg/g)	% recovery	Sample result (μg/g)	% recovery
Batch 1-1	107	107	1943	97	7840	98
Batch 1-2	90	90	1903	95	7805	98
Batch 1-3	96	96	1942	97	7735	97
Batch 1-4	94	94	1929	96	7802	98
Mean	97	97	1929	96	7795	97
S.D.	7.07		18.91		43.64	
%RSD	7.32		0.98		0.56	
%RE		3.45		3.54		2.56
Batch 2-1	99	99	1919	96	7833	98
Batch 2-2	97	97	1943	97	7882	99
Batch 2-3	98	98	1946	97	7588	95
Batch 2-4	97	97	1909	95	7899	99
Mean	98	98	1929	96	7801	98
S.D.	1.14		18.12		144.68	
%RSD	1.17		0.94		1.85	
%RE		2.48		3.56		2.49
Mean (n = 8)	97	97	1930	96	7798	97
S.D.	4.72		17.15		98.97	
%RSD	4.86		0.89		1.27	
%RE		2.97		3.55		2.53

protein digestibility, toxin inactivation, and pasteurization (39). Some heat labile ingredients, including DHA with numerous methylene-interrupted ethylenic double bonds (40), may undergo oxidation during extrusion thereby decreasing in the dog food palatability, stability and nutritional value (41). In our investigation of the recovery of DHA in extruded dog food we found on average an 85.1% recovery (0.821 g recovered vs 0.965 g added DHA/kg dog food; with Standard Error = 0.0137 g and CV = 7.66% over 21 samples). This suggests that the DHA present in the unextracted *Aurantiochytrium limacinum* biomass has an acceptable recovery from the extruded dry dog food kibble using the extraction process of the assay proposed. The recovery was lower than that observed in the lab-scale pellets produced at TestDiet, but could be expected from the lower temperatures and duration of the heat treatment and lower exposure to shear stress. The results were more in agreement with the commercial layer and dairy pellet studies (16, 37). Moran et al. (16) were unable to explain the lower recovery in the dairy protein concentrate, as the short-term conditioning and pelleting should not have resulted in DHA degradation. The higher protein content may have increased the shear stress at the pellet die and resulted in greater friction and biomass cell structure destruction leading to lipid leakage and potential oxidation (39).

Table 5. Summary statistics for the recovery of DHA in 21 samples taken from a commercial batch of extruded dog food supplemented with 0.5% unextracted *Aurantiochytrium limacinum* biomass

Sample number	DHA concentration mg/kg
1	925
2	652
3	762
4	748
5	918
6	799
7	867
8	793
9	742
10	832
11	779
12	835
13	859
14	890
15	814
16	844
17	856
18	860
19	840
20	822
21	818
Mean DHA concentration	821.7
Mean DHA recovery[a]	85.1%
Standard deviation	62.9
Standard Error	13.7
Coefficient of variation	7.66%

[a]Mean recovery was based on an inclusion of 0.965 g DHA/kg in the extruded dog food.

As unextracted *A. limacinum* biomass has been approved for use in Canada and is also the subject of a novel food additive application in the U.S., it is likely to be used in a much greater commercial extent into the future in the manufacture of premium dog foods due to its high DHA content. It can therefore be a welcome finding that the method is adaptable enough to handle the extraction and analysis of DHA in various dog food matrices. The findings highlight the utility and versatility of this method for the routine quantification of DHA and hence can be reliably employed when conducting regulatory studies where it is necessary to establish the quantity of DHA over the course of a given study.

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Conflict of Interest

The funders had no role in the design of the study; in the collection, analyses, or the interpretation of data. GD, JK, AY, and CM work for Alltech, who sponsored the research, contributed to the writing of the manuscript, and was involved in the decision to publish the results. The authors declare that there is no conflict of interest regarding the publication of this paper.

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