An Accurate Assessment of Docosahexaenoic Acid in Laying Hen Serum for Regulatory Studies

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ABSTRACT: Diets rich in omega-3 fatty acids (n-3 FA) have been associated with several health benefits. With the increased interest in n-3 FA both scientifically and societally, the accurate detection of such analytes has become increasingly important. Recently, tandem mass spectrometry (MS/MS) with electrospray ionization interface (ESI), hyphenated to both gas chromatography (GC) and liquid chromatography (LC), has become a valuable tool in the detection of docosahexaenoic acid (DHA). Liquid chromatography-electrospray ionization interface-tandem mass spectrometry methods have been developed for the determination of DHA in canine and poultry species. The objective of this article is to investigate whether LC-ESI-MS/MS is fit for purpose for the determination of DHA in laying hen serum. The disclosure of this work will be beneficial for researchers investigating poultry enrichment for regulatory and toxicological studies. The method was found to be linear over the range. Precision and accuracy results met acceptance criteria and the Limit of Quantitation (LOQ) was established as 1 µg/mL. Recoveries of DHA were obtained for quality control samples and stability studies were performed. The results of the verification study complimented those of the validation study. In summation, the method was established as fit for purpose for measuring total DHA in laying hen serum.

KEYWORDS: DHA, enrichment, LC/MS, serum, regulatory studies

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Introduction

The consumption of diets rich in omega-3 fatty acids (n-3 FA) has been associated with a variety of health benefits, such as a reduced risk of cardiovascular disease and improved cognitive development.[1] The essential n-3 FA, α-linolenic acid (ALA), cannot be synthesized in the body and must be acquired through the diet.[2] The n-3 FA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) can be converted from ALA in the body; however, this conversion is limited, and as a result, the consumption of foods or supplements with sufficient quantities of these n-3 FA is necessary.[3] The recommended intake for healthy adults is 250 mg each of EPA and DHA per day, with higher amounts required to reduce the risk of cardiovascular and other illnesses.[4] The consumption of n-3 FA falls below these recommended levels in many modern diets.[5] This deficiency in dietary n-3 FA has been attributed to a change in animal production, with livestock feed having higher levels of saturated fats and fewer n-3 fatty acids than they had in the past or when compared with animals living in the wild.[6] To address the lack of n-3 fatty acids present in the food chain, the supplementation of livestock diets with n-3 FA-rich oils has been successfully employed to enrich the meat and milk of a variety of animals.[7-11]

With the increased scientific interest in n-3 FA, and in particular DHA, the accurate detection in various sources, including animal feeds, meat and dairy products, and blood, has become increasingly important. Conventionally fatty acids are analyzed by first extracting them from their relevant matrix using the method developed by Folch et al.[12] Methods allowing for the synthesis of fatty acid methyl esters (FAME) directly from fresh tissue, oil, and feed samples have been employed in recent years.[13] Once the FAME have been obtained, they are then usually analyzed by gas chromatography (GC).[14] Variations of GC methodology to include ionic liquids have been reported and provide a more rapid analysis with improved separation and resolution.[15,16] High performance liquid chromatography (HPLC) methods incorporating ultraviolet (UV) detection have also been used for the analysis of fatty acids.[17-19] Alternative HPLC methodologies employing refractive index detection[20] and light-scattering detection[21] have also used.

Tandem mass spectrometry (MS/MS) with electrospray ionization interface (ESI), hyphenated to both GC and LC (liquid chromatography), has become a valuable tool in the detection of DHA. The addition of ultra-performance functionality to the LC system (UPLC) in combination with atmospheric pressure ionization (API-MS/MS) detection enables simpler sample preparation procedures. This is due to better selectivity and increased sensitivity, which are of specific importance for blood biological matrices.[22] In addition, the method allows for an overall increase in accuracy for absolute DHA quantification, provided that internal standard (IS) can

correct for the matrix effect leading to signal suppression or enhancement. Recently, LC-ESI-MS/MS methods have been developed for the determination of DHA in canine and poultry species.[23,24] The objective of this article is to investigate whether this technique is fit for purpose for the determination of DHA in laying hen blood serum and to present the validation and verification undertaken in assessing its suitability. The disclosure of this work will be beneficial for researchers investigating laying hen enrichment with DHA whereby the blood serum can be collected at various stages throughout a study without having to euthanize test animals. It is worth considering that differences between blood and serum samples of various species have been reported, and therefore it cannot be assumed that a method used for other serum samples can be used effectively to obtain suitable results for DHA in poultry serum.[25] Porcine blood and plasma, for example, have been shown to have a higher protein content than that of poultry[26,27] and display different plasma amino acid profiles.[28] Protein variation and sequencing can affect solubility, while changes in impurities and salts can affect analyte separation and sample handling.[27,29] Together, these differences could have an influence on the efficiency and transferability of the LC-ESI-MS/MS methods in analyzing DHA in laying hen serum. Moreover, for non-clinical regulatory studies into the safety and efficacy of DHA-rich feed ingredients, it is necessary that only validated methods are employed for a given matrix, otherwise any resulting data would not be considered effective by officiating competent authorities.[30] The presentation of this method validation and verification therefore obviates the need for researchers interested in analyzing DHA in laying serum to invest considerable cost in validating LC-MS methodology for regulatory and toxicological studies.

Experimental

Instrumentation and chromatographic conditions

An Acquity UPLC® system (Waters Corporation, Hertfordshire, UK) coupled to a Sciex API 4000™ mass spectrometer (Sciex, Warrington, UK) was used for all validation experiments. Data were acquired and integrated using Analyst® 1.5.2 and 1.6.2 (Sciex) and calculated concentrations were determined using Watson LIMS™ software version 7.2 (Thermo, Loughborough, UK). For the verification study, experiments were performed on an Agilent 1100 HPLC system (Agilent Technologies, Mississauga, ON, Canada) coupled to an API 4000 mass spectrometer (AB Sciex, Concord, ON, Canada). Data were processed using Analyst 1.5 (AB Sciex) and concentrations were determined using MultiQuant software (AB Sciex).

A 50 mm × 2.1 mm, 1.7 µm BEH C8 column (Waters Corporation, Milford, MA, USA), which was maintained at 40°C, was used in the UPLC system, and the sample manager and sample organizer were maintained at a temperature of 4°C. The mobile phase flow rate was 0.6 mL/min and consisted of mobile phase A: 0.1% acetic acid (aq.) and mobile phase B: acetonitrile. The gradient profile was as follows: 0.0 to 1.0 min 72% B, 1.0 to 1.1 100% B, 1.1 to 1.3 100% B, 1.3 to 1.4 72% B, 1.4 to 1.7 72% B. For the verification study, a 50 mm × 2.1 mm, 3.6 µm XB-C8 column was used (Phenomenex, Torrance, CA, USA). Again, the sample manager and sample organizer were maintained at 4°C. A flow rate of 0.4 mL/min was used and, as with the validation procedure, the mobile phase consisted of A: 0.1% acetic acid (aq.) and mobile phase B: acetonitrile. The following gradient profile was used: 0.0 to 0.8 min 70% B, 0.8 to 0.81 100% B, 0.81 to 1.1 100% B, 1.1 to 1.2 70% B, 1.2 to 4.5 70% B.

The API 4000 mass spectrometer was operated in negative *TurboIonSpray* mode and used multiple reaction monitoring transitions *m/z* 327.3→283.0 and *m/z* 332.4→288.1 for DHA and docosahexaenoic acid-D_5 (DHA-D_5), respectively. Instrument conditions were as follows: temperature: 500°C, curtain gas: 30 psi, collision gas: 6, GS1: 60 psi, GS2: 40 psi, ionspray voltage: −4500 V. The remaining conditions were as follows: declustering potential: −85 V, collision energy: −16 eV, CXP: −13 V (DHA) and −15 V (DHA-D_5).

Chemicals and reagents

The validation study was performed at LGC (Fordham, Cambridgeshire, UK). Docosahexaenoic acid was purchased from Matreya, LLC (PA, USA) and the IS, DHA-D_5, was purchased from Cayman Chemical (Ann Arbor, MI, USA). High performance liquid chromatography grade hexane and acetonitrile, analytical reagent grade (~37%) hydrochloric acid, and laboratory reagent grade acetic acid (glacial) were all purchased from Fisher Scientific (Loughborough, Leicestershire, UK). Phosphate buffered saline (PBS) tablets (Dulbecco A) were purchased from Oxoid Ltd (Basingstoke, Hampshire, UK). Tween® 80 was purchased from Acros Organics (Geel, Belgium). Bovine serum albumin (BSA) was purchased from Sigma-Aldrich (Poole, Dorset, UK). Control laying hen whole blood containing lithium heparin anticoagulant and control laying hen serum were purchased from B&K Universal Ltd (Aldbrough, Hull, UK). The verification study was performed at Silliker JR Laboratories (Burnaby, BC, Canada). Docosahexaenoic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA) and DHA-D_5 was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). High performance liquid chromatography grade acetonitrile and hexane, analytical reagent grade (~37%) hydrochloric acid, and laboratory reagent grade acetic acid (glacial) were purchased from Fisher Scientific (ON, Canada). Phosphate buffered saline tablets, Tween 80 (polysorbate 80), and BSA were purchased from Sigma-Aldrich (St. Louis, MO, USA). Control laying hen serum was purchased from Life Technologies, Inc. (Burlington, ON, Canada).

Standards and quality control samples

Stock solutions of DHA at 10 mg/mL were prepared in acetonitrile and the IS DHA-D_5 was supplied as a 500 µg/mL

solution in ethanol. Calibration, quality control (QC), and IS working solutions were prepared by diluting stocks in acetonitrile and were stored in amber glass vials at −20°C. A surrogate matrix solution was prepared which was based on work by Bowen et al.[31] This involved preparing a 50 mg/mL fatty acid free BSA solution in PBS which contained 0.1% Tween 80. Calibration standards were prepared at the following concentrations; 1, 2, 5, 15, 50, 175, 450, and 500 µg/mL, by adding 5 µL of each calibration solution to 95 µL of surrogate matrix. Docosahexaenoic acid QC samples were prepared at 1 (LLOQ), ~3 (QCL), ~29 (QCM), and ~420 (QCH) µg/mL, depending on the endogenous DHA content of the serum and were stored at −20°C. Quality control LLOQ was prepared by adding 10 µL of spiking solution to 190 µL of surrogate matrix. QCL and QCM were prepared by diluting control laying hen serum with surrogate matrix, typically ~1:33 and 1:3.6 v/v, respectively. QCH was prepared by adding 8 µL of spiking solution to 192 µL of control laying hen serum. The mean endogenous DHA level of the control laying hen serum was determined by analyzing 12 replicates and was used to calculate QC concentrations.

Test sample preparation

The sample preparation procedure was based on a previously described method by Valianpour et al[32] with some modifications; 25 µL of sample was added to a 2-mL screwcap polypropylene tube, 20 µL of IS working solution (10 µg/mL) was added, and the tubes were vortex mixed. Acetonitrile:hydrochloric acid ~37% (150 µL; 80:20 v/v) was added and the tubes were sealed with screw caps containing an (ethylene-propylene diene monomer) EPDM O-ring, to ensure a tight seal. Tubes were then vortex mixed and incubated at 90°C for 3 h. After cooling to 20°C, 200 µL of water and 1 mL of hexane were added. The tubes were rotary mixed and centrifuged before 10 µL of the hexane layer was transferred to a 96-deep well plate containing glass inserts and evaporated under nitrogen at 40°C and reconstituted in 500 µL of acetonitrile:0.1% acetic acid (aq.) (70:30 v/v).

Validation and verification

The method validation followed the criteria outlined in the European Medicines Agency (EMA) recommendations,[33] with further reference to guidance from the U.S. Food and Drug Administration (FDA).[34]

Linearity

To examine the linearity, calibration curves were prepared by plotting the DHA:IS peak area ratio of the calibration standards against the concentration of DHA. Linear regression was performed using a $1/x^2$ weighting and the intercept, slope, and correlation coefficient ($R2$) were determined. The acceptance criteria for the LLOQ calibration standards were a relative error (%RE) of ±20% with a minimum signal-to-noise ratio of 5:1. For all other concentrations, the acceptance criteria were ±15% RE.

Precision and accuracy and lower limit of quantitation

The precision (%CV – coefficient of variation) and accuracy were determined by the analysis of QC samples LLOQ, QCL, QCM, and QCH on 3 separate occasions with 6 replicates per level. Inter- and intra-assay analyses were performed for precision. The acceptance criteria were %RE ± 20% and %CV ⩽ 20%.

Selectivity

The selectivity of the assay was assessed in laying hen serum from 6 individual determinations. Due to the endogenous nature of DHA, selectivity was only assessed for the IS. The peak area of any co-eluting interference was compared with the average IS response from the QCM samples. Any interference ⩽5% of the average IS peak area was considered acceptable.

Recovery

Surrogate matrix recovery samples were spiked with DHA at QCL and QCH levels, and extracted and spiked with IS, post extraction. Reference samples were prepared by spiking DHA and IS into surrogate matrix post extraction. Internal standard recovery from surrogate matrix was performed in an analogous way at the IS working solution concentration. Recovery of free DHA from laying hen serum was assessed at QCH level by extracting QCH samples and adding DHA-D_5 post extraction. This was compared with a reference sample prepared by post-spiking extracted laying hen serum with DHA and DHA-D_5. Recovery of IS from laying hen serum was assessed in a similar way, at the IS working level. Efficiency of the hydrolysis procedure at recovering total DHA was not tested.

Parallelism Test

A parallelism test was performed based on the approach described by Houghton et al.[35] Parallelism samples were prepared in laying hen serum from 6 individuals by adding 5 µL of QCH spiking solution to 195 µL of serum, giving ~300 µg/mL DHA. The undiluted samples were analyzed alongside aliquots diluted 1:9 with surrogate matrix (n = 6 replicates in each case). Acceptance criteria were %CV ⩽ 20% between replicates and, for the diluted samples, %RE ± 20% of the undiluted sample.

Stability

Stability of DHA in laying hen serum was assessed at endogenous and QCH level. Long-term stability was assessed for

Table 1. Parallelism in laying hen serum and laying hen serum diluted in surrogate matrix.

LAYING HEN SERUM BATCH	MEAN PARALLELISM SAMPLE CONCENTRATION (µG/ML)		
	UNDILUTED	DILUTED 1:9 (BACK-CALCULATED)	%RE[a]
1	282	309	9.6
2	300	328	9.3
3	296	325	9.8
4	301	320	6.3
5	315	344	9.2
6	279	308	10.4

Abbreviation: RE, relative error.
[a]%RE = ((Back-calculated concentration − Undiluted concentration) / Undiluted concentration) × 100.

196 days at −80°C and 210 days at −20°C. To cover sample processing conditions, 25 h room temperature stability was assessed as well as 4 freeze-thaw cycles at both −20°C and −80°C. Stability in laying hen whole blood (lithium heparin anticoagulant) was assessed at endogenous level after 4 h at room temperature. Endogenous stability samples were compared with a $T = 0$ measurement; QCH stability samples were compared with the theoretical concentration. Stability acceptance criterion was ±20% RE. Stability of DHA stock and LLOQ calibration solutions (the highest and lowest concentration DHA solutions, respectively) was assessed after storage at −20°C. Acceptance criteria were ⩽5% change for the stock solution and ⩽10% change for the LLOQ calibration solution.

Results and Discussion

Parallelism

The purpose of the parallelism experiment is to demonstrate that there is no significant bias observed when using a surrogate matrix calibration line to quantify DHA in serum. Bias can occur as a result of uncorrected matrix effects or differential recovery between 2 matrices. This therefore makes parallelism a pivotal test for a surrogate matrix. The results of parallelism are given in Table 1. Back-calculated concentrations of parallelism samples diluted in surrogate matrix agreed with theoretical values. As well as the 6 individual results, each precision and accuracy batch is also a test of parallelism, as it contains QCs prepared in surrogate matrix, that is, (LLOQ), serum (QCH), and a mixture of the 2 (QCL and QCM).

Linearity

The method was shown to be linear over the desired range of 1 to 500 µg/mL. The slopes and intercepts were determined, and the coefficients of determination ($R2$) were found to be between 0.9968 and 0.9978. This was analogous to the linear range determined for DHA in canine and pig serum using the same analytical technique.

Precision and accuracy and selectivity

The sensitivity at LLOQ was acceptable, and no interference peaks were observed in the IS. Intra- and inter-assay precision and accuracy, summarized in Table 2, met acceptance criteria at all levels, which confirmed that the lower limit of quantitation was 1 µg/mL.

Recovery

Recovery in surrogate matrix was 75.5%, 86.6%, and 73.5% at QCL, QCH, and IS working level, respectively. Recovery from laying hen serum was 80.0% and 76.5% at QCH and IS working level, respectively.

Stability

Stability of DHA at endogenous and QCH in laying hen serum is summarized in Table 3. Stability was confirmed after 4 freeze-thaw cycles at −20°C and −80°C, 25 h at room temperature, and up to 30 days at −20°C. After 210 days at −20°C, the endogenous level remained stable, whereas QCH had decreased to −74.1 %RE. The QCH result demonstrated a greater degree instability compared with DHA in canine serum but was comparable with that in pig serum. at −80°C, stability, however, was proven after storage for 196 days at both endogenous and QCH levels. The majority of DHA present in a QCH stability sample was added as free DHA, whereas endogenous DHA would also be present in bound forms such as phospholipids. We suspect that the instability at QCH at −20°C may be specific to free DHA and therefore may not reflect the integrity of genuine samples, particularly if the proportion of endogenous free DHA is low. Arguably, the most relevant stability data are from the endogenous laying hen serum; however, without further investigation, long-term storage at −80°C is recommended as the most suitable option. Whole blood stability, assessed at 63.9 µg/mL, resulted in −5.2 %RE after 4 h at room temperature.

Verification

The verification study showed that the method was linear over the same calibration range. The precision and accuracy for DHA were determined by the measurement of samples at the concentrations; LLOQ, low, medium, and high were found to be within acceptance criteria (Table 4). The lower limit of quantitation was accepted as the lowest standard on the

Table 2. Precision and accuracy of docosahexaenoic acid in QC samples.

QC LEVEL (µG/ML)	%RE[a] (%CV)			
	LLOQ 1.00	LOW 3.21	MEDIUM 29.4	HIGH 423
Intra-assay 1 (n = 6)	4.0 (9.4)	0.3 (3.9)	−3.1 (2.7)	0.5 (2.6)
Intra-assay 2 (n = 6)	−14.5 (8.6)	−14.3 (8.9)	−5.1 (2.6)	−5.7 (1.9)
Intra-assay 3 (n = 6)	−1.2 (4.5)	−11.2 (4.6)	−6.9 (2.1)	−5.5 (2.3)
Inter-assay (n = 18)	−4.1 (11.0)	−8.4 (9.0)	−5.0 (2.9)	−3.6 (3.8)

Abbreviations: QC, quality control; RE, relative error.
[a]%RE = ((Mean calculated concentration − Theoretical concentration) / Theoretical concentration) × 100.

Table 3. Stability of docosahexaenoic acid in laying hen serum.

STORAGE AT ROOM TEMPERATURE	%RE[a] (%CV)	
	ENDOGENOUS	QC HIGH
25 h	5.4 (3.0)	−6.5 (2.3)
STORAGE AT −20°C	ENDOGENOUS	QC HIGH
Freeze-thaw 4 cycles	−6.5 (2.1)	−14.9 (4.6)
7 days	−9.3 (2.1)	−6.9 (3.7)
14 days	−7.4 (3.2)	−4.4 (1.9)
30 days	−5.7 (1.0)	−2.8 (4.8)
210 days	−8.9 (1.8)	−74.1 (6.7)
STORAGE AT −80°C	ENDOGENOUS	QC HIGH
Freeze-thaw 4 cycles	−4.0 (2.3)	−14.5 (2.3)
7 days	−6.9 (4.0)	−8.6 (2.0)
14 days	−8.3 (2.8)	−4.6 (1.8)
30 days	−7.2 (2.4)	−3.3 (2.6)
196 days	6.4 (1.9)	−2.0 (2.5)

Abbreviations: QC, quality control; RE, relative error.
[a]%RE = ((Mean calculated concentration − Theoretical concentration) / Theoretical concentration) × 100.

Table 4. Precision and accuracy of docosahexaenoic acid in QC samples of verification study.

	%RE[a] (%CV)			
QC LEVEL (µG/ML)	LLOQ 0.994	LOW 3.01	MEDIUM 41.8	HIGH 427
Intra-assay 1 (n = 6)	−0.6 (8.7)	6.2 (5.4)	3.4 (6.0)	10 (4.7)
Intra-assay 2 (n = 3)	−8.5 (1.8)	4.4 (1.7)	0.9 (3.0)	−1.4 (1.3)
Intra-assay 3 (n = 3)	−13.0 (9.4)	−8.7 (4.1)	−11.0 (11)	0.16 (2.0)
Inter-assay (n = 12)	−5.7 (9.4)	−2.0 (7.6)	−0.83 (8.7)	4.9 (6.5)

Abbreviations: QC, quality control; RE, relative error.
[a]%RE = ((Mean calculated concentration − Theoretical concentration) / Theoretical concentration) × 100.

calibration line, that is, 1 µg/mL. In terms of the selectivity of the IS, no significant interfering peaks were observed at any of the retention times of interest in the blank serum samples. The verification was performed in a different laboratory, operator, and analytical system, and thus, it can be concluded that the method is suitably robust and fit for purpose.

Conclusion

An LC-ESI-MS/MS method for the analysis of DHA in laying hen serum was successfully validated over a calibration range of 1 to 500 µg/mL DHA. The method was considered suitable for measuring concentrations of DHA in laying hen serum samples in a second laboratory. Its use and application will be of relevance in a regulatory, toxicological, and clinical context given its ability to demonstrate the safety and efficaciousness of enrichment in laying hens with DHA-rich feed additives and ingredients.

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Author Contributions

The text of the manuscript was prepared with input from all authors.

Supplemental material

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