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Optimizing the uptake of deuterated docosapentaenoic acid by salmonid liver cells using a uniform shell design and liquid chromatography triple-quadrupole mass spectrometry☆

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A B S T R A C T

Stable isotope-labelled fatty acids are valuable tracers for studying lipid metabolism in cell models. However, published methods rarely provide guidance on selecting the optimal concentration and incubation time to ensure accurate and reproducible measurements of cellular uptake and metabolism that reflect physiological conditions. This study systematically evaluated the uptake of deuterated docosapentaenoic acid (DPA-d_5) in Atlantic salmon (*Salmo salar* L.) liver cells using a uniform shell design methodology. Cultures were exposed to DPA-d_5 at concentrations ranging from 0 to 100 μM for 24, 48, or 72 h and the uptake was quantified in cell lysates by liquid chromatography–mass spectrometry. Results indicate that the concentration of DPA-d_5 has the strongest positive influence on signal intensity. Incubation time slightly reduces the signal, but when combined with higher concentration, it increases the uptake of DPA-d_5. Modelling of the data allowed selecting 60 μM DPA-d_5 and 48 h as the optimal culturing condition that provides a balance between achieving a strong signal and minimizing the risk of oversaturation or metabolic artifacts. This work provides a methodological foundation for future fatty acid tracer experiments in aquaculture research.

1. Introduction

Stable isotope-labelled fatty acids (FAs) are powerful tools for studying lipid metabolism, providing a safe and analytically precise alternative to radiolabelled tracers. By retaining the same chemical properties as their non-labelled counterparts, deuterated FAs enable unambiguous detection and quantification in metabolic studies due to their mass differences. [1,2]. This enables researchers to track lipid uptake, incorporation, and catabolism in complex biological systems without the risks associated with radiolabelled tracers. Such tracers are tightly regulated under frameworks like the Euratom Basic Safety Standards (2013/59/Euratom) [3], and their use requires formal licensing, trained personnel, continuous monitoring, and specialized waste disposal procedures [4,5], which substantially increase operational costs and limit accessibility, particularly in academic or shared research facilities.

Stable-isotope tracers, such as deuterated compounds, completely bypass these regulatory and safety constraints. They reduce administrative overhead, eliminate radiological waste, and permit studies in laboratories that lack authorization to handle radioactive substances. The safety, practicality, and scalability of deuterated tracers make them especially valuable for large-scale or routine applications, as well as for research sectors like aquaculture, where throughput and safety are critical considerations [6,7].

A variety of analytical techniques, including chromatography coupled to mass spectrometry [8–10], Raman vibrational spectroscopy [11], and nuclear magnetic resonance [12], have underscored the value of deuterium-labelled FAs as essential tools for investigating lipid metabolism, transport, and homeostatic regulation under both physiological and pathological conditions.

Advances in chromatography and high-resolution MS have further enhanced the simultaneous measurement of labelled and unlabelled species in complex biological matrices with minimal sample preparation, consistent run-to-run performance, and elimination of extra sample cleanup that are both labor-intensive and prone to error [13,14]. These instrumental and methodological improvements facilitate the tracking of isotopically distinct metabolite pools across metabolic networks, supporting kinetic analyses, flux studies, and mechanistic investigations [14–17].

Liquid chromatography-mass spectrometry (LC-MS), in particular, is an ideal technique for quantifying incorporation of deuterated/non-deuterated FAs. Its ability to discriminate the small mass differences

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introduced by deuterium labelling, with high sensitivity, wide dynamic range, and compatibility with multiplexed assays, has contributed to its adoption in cell culture studies. For example, LC-MS has been successfully used to monitor the uptake of deuterated docosahexaenoic acid (DHA) [18] and its enzymatic conversion by lipoxygenases in cell systems [19]. However, the potential long-term effects of tracer concentration on cellular systems remain poorly characterized. Some studies have indirectly underscored the importance of using a cautious concentration of polyunsaturated FAs (compounds that deuterated FAs are designed to mimic) to prevent oxidative stress and cytotoxicity [20], and few investigations have evaluated the impact of deuterated FAs on human cells [19], and synthetic liposomes vesicle [21]. For instance, a study on the supplementation of retinal epithelial cells with increasing concentrations of deuterated DHA (0–80 μM) for 24 h has demonstrated the influence of the tracer concentration on cell survival [19]. Although the results of this specific research indicated that a concentration of tracer between 40 and 80 μM were appropriate for further supplementation studies, due to the highest percentage of cell survival, it did not assess the potential effect of the incubation time. It is evident that a major gap in the literature is the lack of standardized approaches for determining both, optimal tracer concentrations and optimal incubation times in cell experiments. Most published protocols on deuterated tracers employ a single dose and exposure time with limited justification, making direct translation across models, and especially between mammalian and teleost (fish) systems, unreliable due to inherent differences in temperature and metabolic rate.

Traditionally, optimization of two experimental factors (e.g., tracer concentration and incubation time) relies on a one-factor-at-a-time (OFaT) approach, in which one variable is adjusted while keeping the other constant. Although simple to apply, OFaT neglects potential interactions between factors that may substantially affect the outcomes. As a result, this sequential method is often time-consuming, resource-intensive, and less efficient than modern multivariate optimization techniques such as response surface methodology.

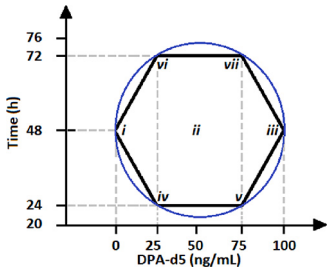
Design of experiments (DoE) approaches provide a systematic framework for identifying optimal experimental conditions. Among them, the Doehlert design (aka uniform shell design) is particularly efficient, offering near-uniform coverage of the experimental space with fewer runs than factorial designs, while enabling estimation of main effects, interactions, and curvature [22,23]. These designs have been widely applied in analytical chemistry and chromatographic method development [23] but have not yet been used to optimize deuterated FAs uptake in cell models. Despite the clear advantages of multivariate approaches, optimal conditions for stable-isotope tracer experiments are seldom established or reported, whether using OFaT or DoE strategies.

In the field of aquaculture research, FA tracers are increasingly employed to study nutrient utilization and lipid deposition [24,25]. Yet, systematic dose-time optimization remains largely unexplored, even though such optimization is crucial for balancing analytical precision with biological relevance.

Docosapentaenoic acid (DPA; 22:5n-3) is an omega-3 long-chain polyunsaturated FA of both physiological and nutritional importance. In Atlantic salmon, DPA is a key component of tissue lipidomes and plays a role in growth, health, and the nutritional value of fillets [26,27]. DPA is an intermediate FA between eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3). Although, no direct dietary DPA-supplementation studies in Atlantic salmon have been reported yet, DHA accumulation in salmon is directly linked to the conversion efficiency of DPA [28,29], consequently understanding the uptake dynamics of DPA and its metabolism at the cellular level is therefore relevant for both basic lipid biology and applied aquaculture strategies, including feed formulation and selective breeding for lipid traits.

The present study applies a Doehlert design to systematically evaluate the uptake of DPA-d_5 in Atlantic salmon liver cells. This approach allowed quantitative assessment of the effects of concentration, exposure time, and their interaction on the analytical signal. To our knowledge, this is the first systematic optimisation of deuterated FA uptake in salmonid liver cells, providing a methodological framework that can be adapted to other stable-isotope tracer experiments in aquaculture and related fields.

Table 1
Doehlert design used to study simultaneously the effects of DPA-d_5 concentration and culturing time on salmon liver cells in L-15 medium. The effect of the solvent used to dissolve DPA-d_5 was evaluated by adding pure ethanol at the same volume level as in the seven experimental preparations. Additionally, pure L-15 cultures without ethanol or fatty acid were prepared in a separate experiment. The biological replicates were submitted to different conditions.

Doehlert design	Condition	DPA-d_5 (μM)	Time (h)
	*i*	0	48
	ii	50	48
	iii	100	48
	iv	25	24
	v	75	24
	vi	25	72
	vii	75	72

2. Materials and methods

2.1. Reagents

(7Z,10Z,13Z,16Z,19Z)-cis-docosapentaenoic-21,21,22,22,22-d_5 acid was purchase from Cayman Chemical (Ann Arbor, MI, USA). Acetonitrile (99.8 %), methanol (99.8 %), and formic acid (98 %) were from Sigma Aldrich (St. Louis, MO, USA). Leibovitz's L-15 medium (21083027) and PBS tablets were purchased from Thermo Fisher (Waltham, Ma, USA). Chloroform (HPLC grade, 99.8 %) and 2-propanol (HPLC grade, 99.9 %) were obtained from Merck (Darmstadt, Germany). A Millipore Milli-Q system was used to produce ultra-pure water 18 MΩ (Millipore, Milford, CT, USA). Fetal bovine serum (FBS, F7524) and antibiotics (A5955) were purchased from Merck (Darmstadt, Germany). Erythrosin B stain (L13002) was purchased from BioNordika (Oslo, Norge)

2.2. Salmon liver cells: Isolation and culture

The cells were isolated from six Atlantic salmon with a mean body weight of 650 g which had been fed a commercial diet. Three biological replicates were used and the cells from every individual fish were submitted to the different experimental conditions suggested by the Doehlert design (Table 1). A stock buffer containing1.4 M NaCl, 0.067 M KCl and 0.09 M Hepes sodium salt were made and pH adjusted to 7.4. A perfusion buffer was prepared by adding 1.11 g EDTA disodium salt to 20 mL of the stock buffer and diluted to 200 mL using ultra-pure water, then pH was adjusted to 7.4. A collagenase buffer was prepared by diluting 10 mL of perfusion buffer to 100 mL. Phosphate-buffered saline buffer (PBS) was prepared by dissolving two PBS tablets in 1 L ultra-pure water and then pH was adjusted to 7.4. All buffers were autoclaved. Afterwards, 100 μL 1 M $CaCl_2$ (pH 7.4) and 100 mg collagenase were added to the collagenase buffer. A complete Leibovitz's L-15 medium (cL-15) was mixed with 1 % antibiotic and 10 % FBS. The fish was anesthetized by metacaine (MS222, 1 g/10 L), opened with a sterile scalpel and the exposed liver was slightly lifted to get access to vena porta. The hepatic portal vein was perfused via cannulation (PE50 cannula, BD Venflon Pro, Oslo, Norway) with the perfusion buffer at a flow of 4 mL/min until free of blood. The free-of-blood liver was digested with collagenase buffer for 5 min, and the isolated cells were

Table 2
Summary of the uptake of DPA-d$_5$ by salmon liver cells, its metabolic conversion to DHA-d$_5$, and the endogenous levels of DPA and DHA at different experimental points of a Doehlert design, determined by LC-MS/MS. Each treatment was performed in triplicate.

Condition	Volume of DPA-d5 (~6 mM) in ethanol added to 2 mL media	DPA-d$_5$ (μM)	Time (h)	Signal area (counts×seconds)			
				Deuterated PUFA		Endogenous PUFA	
				DPA-d$_5$	DHA-d$_5$	DPA	DHA
i	0	0	48	0 ± 0	0 ± 0	2015 ± 1638	1592 ± 1117
ii	17	50	48	1736 ± 491	143 ± 40	2614 ± 591	1090 ± 401
iii	34	100	48	5521 ± 1723	279 ± 127	3411 ± 868	1282 ± 575
iv	8	25	24	254 ± 70	28 ± 11	1004 ± 178	671 ± 291
v	25	75	24	1863 ± 449	96 ± 61	1599 ± 369	773 ± 422
vi	8	25	72	421 ± 59	65 ± 29	2174.43 ± 155	1133 ± 516
vii	25	75	72	2067 ± 419	205.08 ± 79	2110 ± 318	1183 ± 488

harvested in 10 mL 10 % PBS at 5 °C, filtrated through a 100 μm mesh cell strainer, washed twice in the PBS buffer at 5 °C and resuspended in cL-15 medium before the viability of the isolated cells was assessed. All centrifugation steps were done using 50 ×*g* for 5 min. The cells were counted using a Kova glasstic slides and erythrosin B stain. The viability of the liver cells was above 90 %. Sterile equipment and buffers were used to isolate the cells.

Cell culture plates (Costar, Cambridge, MA) were conditioned by adding 1 % laminin (500 μL laminin in 50 mL PBS) 1920 μL/well and kept overnight. The initial laminin solution was removed from the plates and ~ 7.5×10^6 liver cells were cultured into each well containing 2 mL cL-15 medium. DPA-d$_5$ was dissolved in ethanol (stock solution of 5961 μM) and added to the wells to give concentrations suggested by the Doehlert design (namely: 0, 50, 75, 100 μM). A media culture (0 μM DPA-d$_5$) was used as control and also a media culture with pure ethanol added (the solvent used to dissolve the FAs) and diluted with cL-15 medium. The cell culture plates were incubated in a normal atmosphere incubator (Sanyo Electric Company Ltd. Osaka, Japan) at 9 °C at the times indicated in Table 1 (namely: 24, 48, 72 h) under dark conditions. The suspensions of cells were prepared in triplicate. The medium from the liver cells was removed carefully without disturbing the cells attached to the bottom of the plate and stored at −80 °C. The cells were detached from the plate by non-gentle wash with PBS and centrifuged at 50 ×*g* for 5 min. The supernatant was discarded, and the pellet was stored at −80 °C until extraction followed by liquid chromatography tandem mass spectrometry (LC-MS/MS) quantitative analysis.

2.3. Extraction protocol

The extraction protocol has been described elsewhere [30]. Briefly, two aliquots of 500 μL each of acetonitrile and chloroform were added successively to an Eppendorf tube containing the cell pellet. The mixture was vortexed for 30 s (Bandelin RK 100 Ultra Mixer, Berlin, Germany)

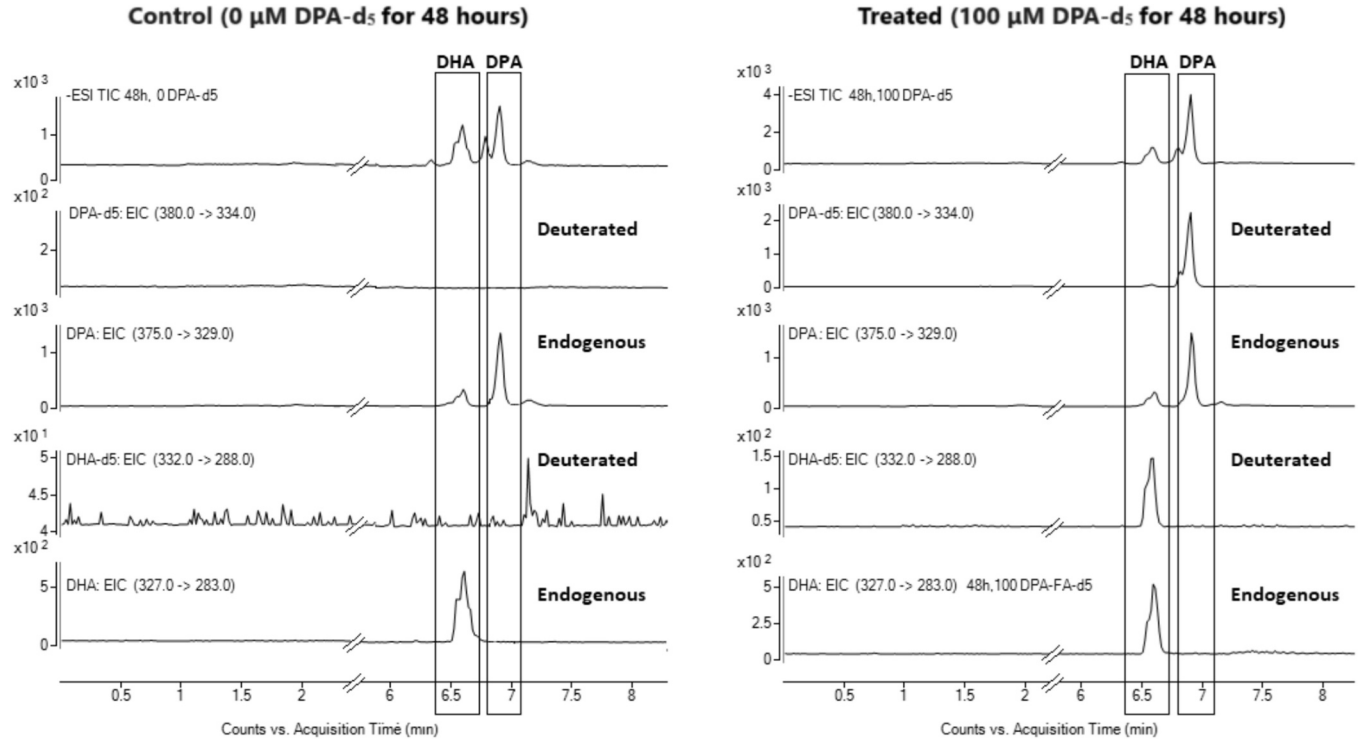


Fig. 1. Representative total ion chromatograms (TICs) and extracted ion chromatograms (EICs) of salmon liver cell lysates after incubation with 0 or 100 μM DPA-d$_5$ for 48 h. Retention times of DHA and DPA are indicated by vertical boxes. The control sample (left) shows only endogenous DPA and DHA, whereas the sample incubated with 100 μM DPA-d$_5$ (right) demonstrates uptake of DPA-d$_5$ and its metabolic conversion to DHA-d$_5$. Endogenous DPA and DHA levels remained comparable between treatments. These results confirm that the biological system actively incorporates exogenous DPA and enzymatically converts it to DHA, consistent with established ω-3 fatty acid metabolic pathways.

and centrifuged at 1620 ×*g* for 3 min (Eppendorf AG centrifuge, Hamburg, Germany). The supernatant was collected into a 15 mL high-performance centrifuge tube (VWR AS, Brynsalleen, Oslo). This extraction protocol was repeated, and the supernatants were combined and vacuum-dried at room temperature (Labconco Vacuum Dryer System, Kansas, MO, USA). The residue was then diluted to 50 µL with acetonitrile, transferred to an autosampler vial, and analyzed using LC-MS/MS.

2.4. Liquid chromatography mass spectrometry

An Agilent ultra-high performance liquid chromatography (UHPLC), coupled to a 6495 QQQ triple quadrupole (Agilent Technologies, Waldbronn, Germany) with an electrospray ionization (ESI) interface and iFunnel ionization.The UHPLC system was equipped with a Zorbax Eclipse Plus C18, rapid resolution HD column (2.1 × 50 mm, 1.8 µm, 95 Å). The injection volume was 5 µL and the mobile phase was delivered at 0.4 mL/min in gradient mode and consisted of ultra-pure water with 0.1 % formic acid (solution A) and an equal-volume mixture of acetonitrile and methanol with 0.1 % formic acid (solution B). The solvent gradient was as follows: solution A was reduced from 60 to 5 % from 0.00 to 4.00 min, kept at 5 % between 4.00 and 5.50 min, increased to 60 % between 5.50 and 5.51 min and kept at 60 % between 5.51- and 10.00 min.

The adducts $[DPA\text{-}d_5 + HCOOH\text{-}H]^-$, $[DPA + HCOOH\text{-}H]^-$). $[DHA\text{-}d_5\text{-}H]^-$, and $[DHA\text{-}H]^-$ were automatically selected as the most abundant precursors by the software MassHunter Workstation Optimizer (version 10.0 SR1, Agilent Technologies, Waldbronn, Germany), respectively. After collision activation, the loss of HCOOH (46 *m/z*) from $[DPA\text{-}d_5 + HCOOH\text{-}H]^-$ and $[DPA + HCOOH\text{-}H]^-$, and COO^- (44 m/z) from $[DHA\text{-}d_5\text{-}H]^-$ and $[DHA\text{-}H]^-$ were recorded by monitoring the transitions: 380 → 334 m/z; 375 → 329 m/z; 332 → 288 m/z; and 327 → 283 m/z, respectively. Multiple reactions monitoring (MRM) in negative mode was used and the analytical signals recorded in counts×seconds. The ESI parameters were gas temperature (120 °C), gas flow rate (19 L/min), nebulizer pressure (20 psi), sheath gas temperature (300 °C), sheath gas flow (10 L/min), capillary voltage (3500 V) and nozzle voltage (2000 V). The integration of the chromatograms was performed using the MassHunter Qualitative Navigator software (version 10.0, Agilent Technologies, Waldbronn, Germany).

2.5. Statistics

Statgraphics Centurion XVI (Version 16.1.11, StatPoint Technologies, Inc., Warrenton, VA, USA) was used to perform the variance check, analysis of variance, and multiple range tests of the experimental data from the different treatments. Microsoft® Excel® for Microsoft 365 MSO (Version 2505 Build 16.0.18827.20060) was used for determining the coefficient of determination, the experimental and theoretical Fisher values and establishing the validity of the regression model.

3. Results and discussion

The results of the uptake of DPA-d₅ and its metabolic conversion to DHA-d₅ by salmonid liver cells using a Doehlert design are presented in Table 2. Representative chromatograms for 0 and 100 µM DPA-d_5 and 48 h incubation (Fig. 1) show that in the absence of DPA-d_5 (control condition), only endogenous DPA (*m/z* 375 → 329 m/z) and endogenous DHA (m/z 327 → 283 *m/z*) are detected, with clear peaks eluting at approximately 6.9 and 6.6 min, respectively (Fig. 1, left panel). As expected, no signals were observed for the deuterated species (DPA-d_5 and DHA-d_5) in the controls, confirming the specificity of the method and absence of contamination. In contrast, incubation with 100 µM DPA-d_5 (Fig. 1, right panel) resulted in strong detection of deuterated DPA (*m/z* 380 → 334 m/z), eluting at the same retention time as endogenous DPA. Notably, a distinct peak corresponding to deuterated DHA (DHA-d_5; m/z 332 → 288 m/z) was observed at 6.6 min, indicating endogenous/metabolic conversion of exogenously supplied DPA-d_5 into DHA-d_5. Endogenous DPA and DHA levels remained consistent with control samples, suggesting that deuterated precursor incorporation does not suppress endogenous FA production over the 48 h period.

Table 3
Summary of the effect of pure ethanol (without DPA-d₅) on the endogenous levels of DPA and DHA at various experimental points of a Doehlert design, determined by LC-MS/MS. Each treatment was performed in triplicate.

Condition	Pure ethanol added to 2 mL media (µL)	DPA-d_5 (µM)	Time (h)	Signal area (counts×seconds)			
				Deuterated PUFA		Endogenous PUFA	
				DPA-d_5	DHA-d_5	DPA	DHA
i	0	0	48	0 ± 0	0 ± 0	2015 ± 1638	1592 ± 1117
ii	17	0	48	0 ± 0	0 ± 0	3105 ± 596	1212 ± 358
iii	34	0	48	0 ± 0	0 ± 0	2464 ± 873	1078 ± 524
iv	8	0	24	0 ± 0	0 ± 0	1132 ± 397	1082 ± 804
v	25	0	24	0 ± 0	0 ± 0	1198 ± 441	624 ± 324
vi	8	0	72	0 ± 0	0 ± 0	1701 ± 1171	1061 ± 831
vii	25	0	72	0 ± 0	0 ± 0	1091 ± 484	778.88 ± 504

Control cells were either treated with ethanol alone, the solvent used to dissolve DPA-d₅, or only with pure media in triplicate to account for any potential effects attributable to the vehicle rather than the stable isotope-labelled FA itself (Table 3). The results for ethanol-treated control cells and pure media control cells exhibited comparable variability across conditions, and Levene's test confirmed homogeneity of variances for both deuterated and endogenous analytes. This observation supports the robustness of the dataset and is consistent with previous reports of reproducible LC–MS/MS quantification of FAs in cellular systems [31,32].

Comparison of endogenous DPA and DHA in Tables 2 and 3 revealed no significant differences between treated and controls at the 0.05 significance level. The overall pools of non-deuterated FAs remained stable regardless of DPA-d_5 addition. This stability indicates that tracer supplementation did not perturb basal lipid metabolism, corroborating earlier findings that isotope-labelled FAs can be used to monitor fluxes without significantly altering endogenous pools [32,33]. In addition, these endogenous levels results highlight the presence of a baseline omega-3 polyunsaturated FA pool in the salmon liver cells prior to supplementation.

A notable feature of the dataset is the degree of variability between biological replicates within the same condition (Supplementary Table 1). For example, at 50 ng/mL DPA-d₅ (condition *ii*), recorded DPA-d₅ areas ranged from 765.89 to 2357.46 counts×seconds despite identical exposure conditions. Such variability could arise from differences in cell confluence, metabolic state, or minor handling differences during dosing and washing. LC-MS/MS analytical reproducibility is generally high for these analytes [34], so biological variation is likely the primary contributor. This highlights the importance of sufficient replication and statistical treatment in quantitative lipidomic studies.

DHA-d₅ was undetectable in control cells. However, a partial but measurable bioconversion appeared in nearly all treated samples (25, 50, 75, and 100 µM DPA-d₅) suggesting that the elongation/desaturation pathway occur at all levels of investigated concentrations. The time effect on DHA-d₅ accumulation was particularly evident at 75 ng/mL. After 24 h (condition *v*), DHA-d₅ areas reached up to 216.48

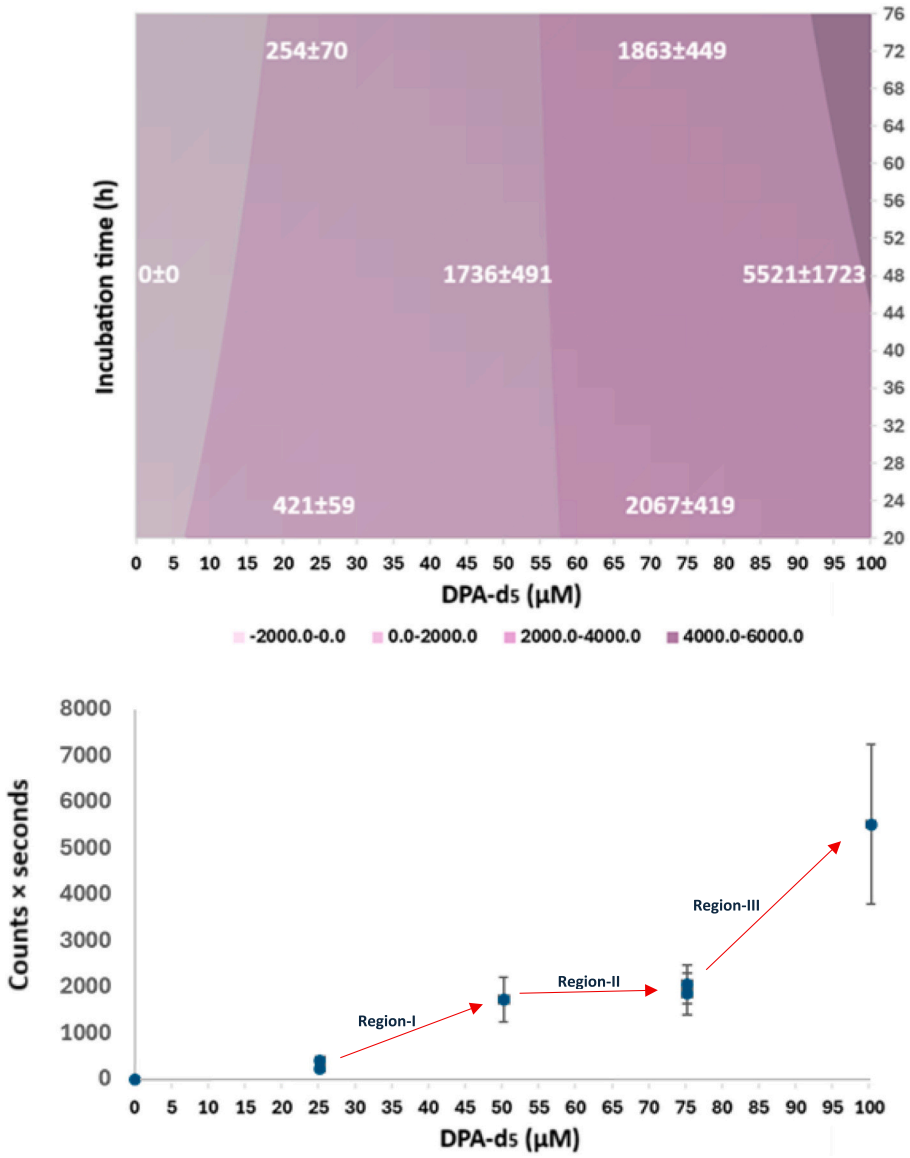


Fig. 2. Uptake of exogenous DPA-d_5 by salmon hepatocytes. Top: response surface showing signal intensity as a function of DPA-d_5 concentration, incubation time, and their interaction. Signal intensity is represented by a continuous colour gradient, where higher intensities correspond to warmer colours. Bottom: experimental signals versus DPA-d_5 concentration indicating the three concentration regions characterized by an initial increase (25–50 μM), plateau (50–75 μM) and second increase (75–100 μM). Mean experimental values ± standard errors are overlaid on both panels.

counts×seconds, whereas after 72 h (condition *vii*), values were substantially higher in some replicates (e.g., 362.75 counts×seconds). This suggests that the enzymatic omega-3 PUFA elongation/desaturation in salmon hepatocytes operates continuously over extended periods, with ongoing accumulation of DHA-d₅.

The results in Table 2 revealed a strong positive relationship between the supplied DPA-d₅ concentration and the recorded intracellular DPA-d₅ peak areas. Under 48-h incubations, conditions with 100 ng/mL DPA-d₅ supplementation (condition *iii*) yielded the highest recorded DPA-d₅ areas. This contrasts sharply with the absence of DPA-d₅ signal in condition *i* (0 ng/mL), indicating the lack of contamination by endogenous DPA-d₅ or background interference in the analytical workflow. These results are further validated by the ethanol blanks in Table 3, where the absence of signals for both deuterated compounds, DPA-d₅ and DHA-d₅, at all experimental conditions is a clear indication that the cell cultures do not contribute with any unwanted DPA-d₅ or DHA-d₅. Also, the lack of signal indicates that the analytical workflow (sample preparation, LC-MS/MS method, solvents, consumables) is not introducing spurious DPA-d₅ or DHA-d₅. Consequently, the results in Tables 2 and 3 demonstrates that any detected DPA-d₅ or DHA-d_5 truly come from an uptake or a conversion process, respectively, after supplementation, and not from contamination or baseline artifacts.

In general, Table 2 highlights the strongest responses at high concentration and time (e.g., 100 μM, 72 h). However, the selection of an optimal experimental condition cannot be based on visual inspection of raw signal intensities alone, because it does not capture the underlying interactions or potential curvature in the response surface. A more rigorous strategy is to use the structure of the experiments described by the Doehlert design to fit a mathematical model describing the response as a function of both concentration and incubation time. The geometry of the Doehlert design distributes points uniformly across the time-concentration space, ensuring that predictions have nearly constant precision in all directions [23]. This property makes it especially well-suited for analytical optimization problems, where both factor interactions and nonlinear responses are expected. Importantly, such designs are widely used in chromatography and mass spectrometry method

development, where subtle parameter interplay can markedly affect sensitivity and selectivity [35,36].

The uptake of DPA-d$_5$ by salmon hepatocytes was described by expressing the LC-MS/MS chromatographic signals (S) as a function of the factors time (T), DPA − d_5 concentration (C) and their interaction (T × C). The resulting empirical model was:

$$S = 33.68 \times C - 12.82 \times T + 0.27 \times T \times C \quad (1)$$

The validity of Eq. 1 was confirmed, as the experimental Fisher value ($F_{exp} = 2.161$), representing the ratio of lack-of-fit to pure-error variances with 4 and 14 degrees of freedom, was lower than the theoretical Fisher value ($F_{theo} = 3.112$) at the 95 % confidence level, as recommended by the Analytical Methods Committee [37]. The positive coefficient (+33.68) for the factor concentration in Eq. 1 highlights extracellular DPA-d$_5$ as the major determinant of uptake, consistent with diffusion-driven or transporter-mediated entry into hepatocytes [38]. The negative time coefficient in Eq. 1 (−12.82) suggests that over time the cells can take up and lose DPA-d$_5$. Consequently, prolonged exposure may be accompanied by counterbalancing processes such as metabolism or efflux, which tend to reduce net intracellular signal over time when considered independently. The positive interaction coefficient (+0.27) demonstrates that concentration and time act synergistically, therefore high exposure times and concentrations yield a greater uptake. The graphical representation of Eq. 1 indicates three main concentration regions (Fig. 2). The first region (25–50 μM) is characterized by an abrupt rise of 4.1-fold at 24 h and 6.8-fold at 72 h, relative to the 50 μM at 48 h. The second region (50–75 μM) is characterized by a near-plateau, 1.2-fold at 24 h and 1.1-fold at 72 h relative to the 50 μM condition at 48 h. And the third region (75–100 μM)) is characterized by a second jump of 2.7-fold at 24 h and 3.0-fold at 72 h, relative to 100 μM at 48 h. Fig. 2 indicates that the stable and reproducible time-concentration range of 48–72 h and 50–75 μM DPA-d$_5$ is a less sensitive window to experimental fluctuations, enhancing reproducibility.

The concentration region 50–75 μM at 72 h aligns with a previous study on human and murine cells treated with 0–75 μM DPA for 72 h, where increases of around 1.1 and 1.2-fold were observed in cell lysates between 50 and 75 μM DPA [39,40]. Based on Fig. 2, the condition 48 h and 60 μM (inside the stable region 50–75 μM and 48–72 h**)** represents an optimal compromise that provides robust signal intensity, minimizes variability and artifacts associated with extreme concentrations or extended incubations. Therefore, condition 48 h and 60 μM constitutes a rational choice for subsequent uptake studies and comparative experiments. It should be emphasized that the optimized concentration-time condition is specific to the culture settings used in this study. Future work may extend the design-of-experiments approach to additional incubation parameters (e.g., temperature, pH, gas composition) when adapting the protocol to other species, developmental stages, or defined stress/perturbation models. In addition, the present study focuses on the conversion of DPA-d$_5$ to DHA-d$_5$ in Atlantic salmon hepatocytes cultured in a serum-containing medium with a fixed background of unlabelled fatty acids. Further studies should: i) consider the retroconversion of DPA-d$_5$ to EPA-d$_5$; ii) incorporate broader lipidomic profiling; iii) and potentially employ more defined or serum-reduced media to better disentangle the influence of competing fatty acids on DPA-d$_5$ uptake. Moreover, while cell viability was confirmed under the optimized condition, other physiological responses (e.g., oxidative stress, mitochondrial activity, lipid peroxidation, or inflammatory signalling) were not investigated, and future studies should include these endpoints to provide a more comprehensive understanding of the cellular impact of DPA-d$_5$ uptake and metabolism.

4. Conclusion

The LC-MS/MS method applied here provided clear resolution of both deuterated and endogenous FAs, with minimal interference. The lack of DPA-d$_5$ and DHA-d$_5$ signals in control samples confirms negligible carryover and good analytical specificity. From a nutritional perspective, these findings have implications for aquaculture feed formulation. If dietary DPA is efficiently taken up and partially converted to DHA in salmon hepatocytes, it may represent a viable strategy to enhance DHA content in fish tissues without relying solely on direct DHA supplementation, which can be more expensive and less stable in feed formulations.

CRediT authorship contribution statement

Pedro Araujo: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Zhenxiao Zhuang:** Writing – review & editing, Methodology, Investigation, Data curation. **Maren Hoff Austgulen:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis. **Bjørg Kristine Hundal:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2025.124863.

Data availability

Data will be made available on request.

References

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