Extraction of Pro- and Anti-Inflammatory Biomarkers from fish Cells Exposed to Polyunsaturated Fatty Acids and Quantification by Liquid Chromatography Tandem Mass Spectrometry

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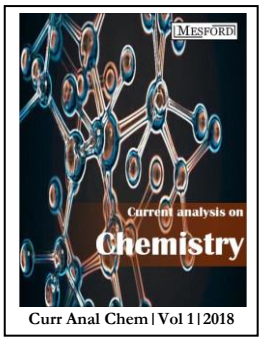

Abstract:

A solid-phase extraction method combined with liquid chromatography tandem mass spectrometry was developed to analyze simultaneously prostaglandins (PGE2, PGE3), prostacyclins (6-keto-PGF1α, Δ17-6-keto-PGF1α), resolvins (RvD1, RvD2) and leukotriene (LTB4) released into cL-15 medium by salmon liver cells. The optimal concentrations of different internal standards, for determining the analytical performance parameters, were selected by means of a uniform shell design. The limit of detection, quantification and recovery for the seven released pro- and anti-inflammatory biomarkers into cL-15 medium ranged from 0.3-1.0 ng/mL, 0.5-2.0 ng/mL and 83-127% respectively. The validated method was used to investigate the effect of polyunsaturated fatty acids (PUFA) on the production of prostaglandins, prostacyclins, resolvins and leukotriene by salmon liver cells. Statistically significant increases in the concentration of some eicosanoids were observed after adding arachidonic acid (PGE2, 6-keto-PGF1α and Δ17-6-keto-PGF1α) and eicosapentaenoic acid (PGE2, 6-keto-PGF1α, Δ17-6-keto-PGF1α and LTB4). Neither linoleic nor docosahexaenoic acid affected the production of both arachidonic acid or docosahexaenoic acid derived metabolites. Although RvD1 and RvD2 were not detected, there was some indication that the production of RvD3 and RvD4 was preferred over RvD1 and RvD2 after exposing the cells to different PUFA.

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Prostaglandins; Prostacyclins; Resolvins; Leukotriene; Solid Phase Extraction; Liquid Chromatography Mass Spectrometry; Cell Cultures; Atlantic salmon (Salmo salar).

1. INTRODUCTION

It is well known that arachidonic acid (AA, 20:4ω-6) is the substrate for two classes of enzymes, cyclooxygenases (COX) and lipooxygenases (LOX). The former enzyme is responsible for the production of 2-series prostaglandins, 2-series prostacyclins and 2-series thromboxanes and the latter enzyme is responsible for the biosynthesis of 4-series leukotrienes and hydroxyeicosatetraenoic acids (HETEs). Eicosapentaenoic acid (EPA, 20:5ω-3) exhibits a similar metabolism to AA, but it is metabolized by COX to 3-series prostaglandins, 3-series prostacyclins and 3-series thromboxanes and by LOX to 5-series leukotrienes and hydroxyeicosapentaenoic acids. Docosahexaenoic acid (DHA, 22:6ω-3) and EPA can be converted to D-series and E-series of resolvins through the action of LOX respectively [1].

AA-derived eicosanoids have pro-inflammatory effects and they are positively linked to arthritis [2], periodontal diseases [3] and also some diseases which are not considered to be of inflammatory etiology such as Alzheimer's disease [4], cardiovascular disease [5] and cancer [6, 7]. In contrast, metabolites derived from EPA and DHA, have anti-inflammatory properties. Resolvins are a new family of lipid mediators that possess both potent anti-inflammatory and immune-regulatory properties [8]. The production of classic eicosanoids and resolvin D1 (RvD1) by tissue and cells of salmon and trout respectively has been demonstrated [9, 10]. In addition, RvD2 and RvD5 were observed in the former and the latter fish.

Norway is a leading nation in fish research that consistently promotes fish welfare and implementation of 3Rs approaches (replacement, reduction, refinement) for the development of a sustainable aquaculture industry. In this regard, cell culture techniques are suitable substitute methods for animal

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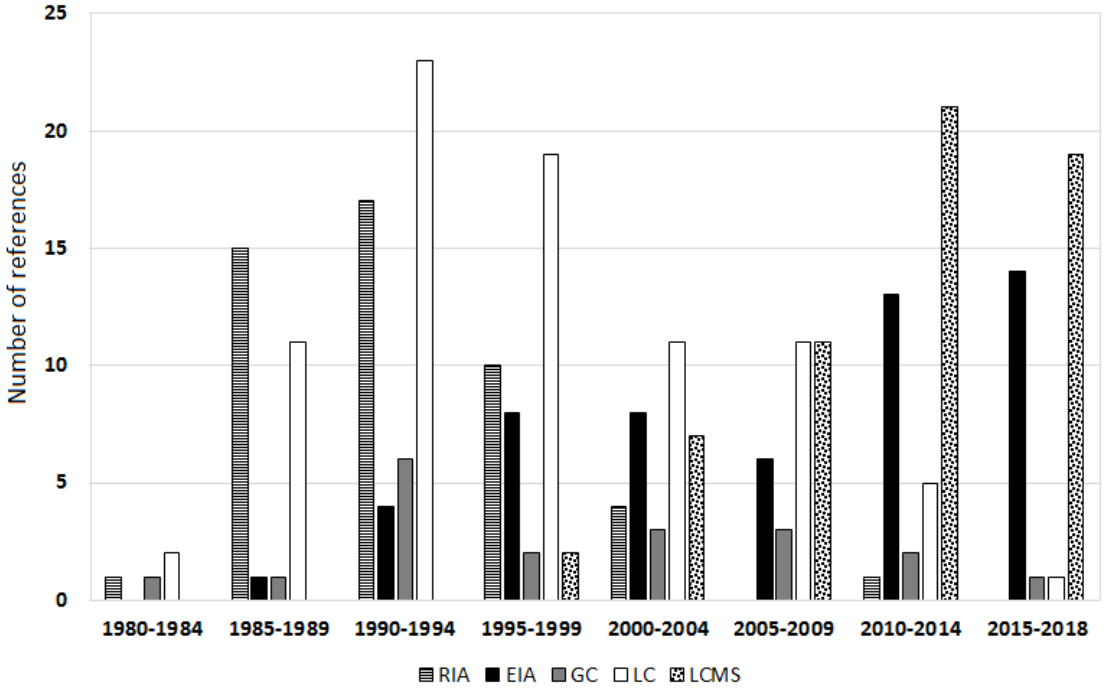


Fig. (1). Overview of the application of different instrumental techniques for analyzing eicosanoids in cell cultures during the last 37 years. The year 2018 comprised January and February. The list of references for the time segments is provided as supporting information.

experiments that allow studying the synthesis of valuable biological substances and the biochemistry of cells.

There is a growing body of evidence documenting the effective use of mammals and non-mammals cell lines for studying the production of pro- and anti-inflammatory biomarkers. A chronological overview of the application of different instrumental techniques for the analysis of eicosanoids in cell cultures is presented in Fig. (**1**). Radioimmunoassay (RIA) and enzyme immunoassay (EIA) are two types of immunoassay methods with comparable sensitivity and specificity. The rapid acceptance of RIA for the analysis of eicosanoids was mainly due to its high sensitivity, specificity and commercial availability. Nevertheless, the expense and hazards involved in the preparation and handling of radioactive materials led to a decline in the use of RIA and to an increase in the use of EIA in the middle nineties. The main drawbacks of EIA are the overestimation of the analytical concentrations due to cross-reactivity and the detection of a single analyte per commercial kit, which would amount to very expensive costs when different eicosanoids are assessed quantitatively. Gas chromatography (GC) and liquid chromatography (LC) have been effective alternatives to overcome the disadvantages imposed by EIA. It is clear from Fig. (**1**) that over the course of time the use of LC has always outperformed GC for determining eicosanoids in cell cultures due to the inherent limitation of the latter to volatile and thermally stable compounds which are prepared by time-consuming derivatization process and which ensure that samples are prepared to prevent non-volatile components from entering the chromatographic system [11, 12]. The main disadvantage of LC coupled to UV-visible or diode array (DAD) detectors is the coelution of eicosanoids from the same category or family (in some instances isomeric eicosanoids) exhibiting the same spectrum. The problems associated with the use of spectrophotometric detectors (e.g. UV or DAD) were circumvented with the use of mass detectors (MS). It is shown in Fig. (**1**) that LCMS and EIA have gained in popularity and surpassed the implementation of LC methods over recent years. An overview of the current year indicated that the trend is towards the implementation of LCMS instead of EIA as deducted from a remarkable number of references concerned with the quantification of eicosanoids in cell cultures by LCMS between 2015 and February 2018.

Liquid-liquid extraction (LLE) and solid phase extraction (SPE) are the main methods used for the extraction of eicosanoids from cell culture media. The chronological overview revealed that SPE is preferred over LLE for the analysis of eicosanoids in cell cultures by LCMS. (references associated with LLE or SPE are indicated in the supporting information by the letters L or S after the reference number respectively).

The majority of published SPE-LCMS methods are focused on a limited number of eicosanoids. In addition, some of these methods involve repeated multiple steps without the addition of of internal standard(s) to compensate for losses of the analytical eicosanoids during sample treatement [13]. In cases where the internal standard was used, the main drawbacks have been the lack of selectivity and the splitting of the analytes and internal standards observed elsewhere [14].

The present article aims at developing a SPE method to extract eicosanoids and resolvins from cell culture media and further quantification by liquid chromatography tandem mass spectrometry (LC-MS/MS). The developed method is applied in the analysis of released PGE2, PGE3, 6-keto-PGF1α, Δ17-6-keto-PGF1α, RvD1, RvD2 and LTB4 in culture media by

salmon liver cells exposed to AA, EPA, DHA or linoleic acid (LA, 18:2ω-6) that is the precursor of AA.

2. EXPERIMENTAL

2.1. Reagents

Prostaglandin E2 (PGE2, 99%), deuterated prostaglandin E2 (PGE2-d4, 99%), prostaglandin E3 (PGE3, 98%), 6-keto prostaglandin F1α (6-keto-PGF1α, 98%), deuterated 6-keto prostaglandin F1α (6-keto-PGF1α-d4, 99%), Δ17-6-keto-Prostaglandin F1α (Δ17-6-keto-PGF1α, 98%), resolvin D1 (RvD1, 95%), resolvin D2 (RvD2, 95%), deuterated resolvin D2 (RvD2-d5, 95%), leukotriene B4 (LTB4, 97%) and deuterated leukotriene B4 (LTB4-d4, 99%) were purchased from Cayman Chemical (Ann Arbor, MI, USA). Acetonitrile (99.8 %) and formic acid (98 %) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2-propanol (HPLC grade, 99.9 %) from Merck (Darmstadt, Germany). Chloroform (HPLC grade, 99.8%) was obtained from Merck (Darmstadt, Germany).

A Millipore Milli-Q system was used to produce ultra-pure water 18 MΩ (Millipore, Milford, USA). Cis-5,8,11,14,17-eicosapentaenoic acid (EPA, 99%), cis-5,8,11,14-eicosatetraenoic acid (ARA, 85%), cis-9,12-octadecadienoic acid (LA, 99%) and cis-4,7,10,13,16,19-docosahexaenoic acid (DHA, ≥98%) were purchased from Sigma–Aldrich (Oslo, Norway).

Leibovitz`s L-15 medium from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS, cat# 14-801F) was from BioWhittaker (Petit Rechain, Belgium). The glutaMaxTM 100× (Gibco-BRL, cat# 35056) was from Gibco-BRL (Cergy-Pontoise, France).

2.2. Isolation of the Salmon Liver Cells

Liver cells were isolated from five Atlantic salmon (Salmo salar) with average body weight of 400-500 g (2 male and 3 female, not sexually mature) obtained from HIB, University of Bergen. The experimental protocol was approved by the Norwegian Board of Experiments with Living Animal.

Complete L-15 medium was prepared by mixing Leibovitz`s L-15 medium with 1% glutamax, 1% antibiotika and 10% FBS (cL-15). 1 M CaCl2 and perfusion buffer containing 1.4 M NaCl, 0.067 M KCl and 0.09 M HEPES sodium salt at pH 7.4 were prepared and used as stock solutions. Perfusion buffer containing EDTA was prepared by adding 1.11 g EDTA disodium salt to 20 mL of perfusion buffer and diluted to 200 mL using ultra-pure water; pH was finally adjusted to 7.4. Perfusion buffer containing collagenase was prepared by first diluting 10 mL of perfusion buffer to 100 mL and adjusting pH=7.4. Then, 100 µL 1 M CaCl2 and 100 mg collagenase were added. Both buffers, EDTA and collagenase, should be freshly prepared.

The fish were aestheticized by metacaine (MS222, 0.5 g/10 L) and the livers were perfused with a perfusion buffer containing EDTA at a flow of 4 mL/min until free of blood. Thereafter, the livers were digested with collagenase dissolved in above described stock perfusion buffer. The isolated cells were harvested in 10 mL 10% phosphate-buffered saline buffer (PBS buffer: 0.002 M KH2PO4, 0.02 M Na2HPO4, 0.03 M KCl and 0.14 M NaCl, pH 7.4), filtrated through a 100 µm mesh cell strainer, washed twice in the PBS buffer and re-suspended in cL-15 medium before the viability of the isolated cells was assessed. All centrifugations were performed at 50×g for 5 min. The viability of the liver cells was above 90% (range: 90.8-94.4%). Sterile equipment and buffers were used to isolate the cells.

2.3. Cell Cultures

Culture plates were first conditioned by adding 1% laminin (500 µL laminin in 50 mL PBS) 1920 µL/well and kept overnight. Once the cells were obtained, the laminin solution was removed from the plates and 1.67×106 liver cells were added to each well. Four cL-15 medium solutions containing LA, ARA, EPA or DHA were prepared by attaching the fatty acids to FBS and diluting with cL-15 medium to a concentration level of 46 µM of fatty acid. A blank solution was made by adding FBS and ethanol (the solvent used to dissolved the fatty acids) and diluting with cL-15 medium. The individual effect of the omega-6 (ω-6) LA, ARA and omega-3 (ω-3) EPA, DHA and the blank on the production of classical eicosanoids (PGE2, PGE3, 6-keto-PGF1α, Δ17-6-keto-PGF1α, LTB4) and resolvins (RvD1, RvD2) was tested by culturing salmon liver cells in 1.5 mL of cL-15 medium, adding 0.5 mL of fatty acid (or blank) and incubating for 24 h at 9 °C in an incubator device (Sanyo Electric CO., Ltd., Osaka, Japan). The experiments were performed in triplicate. The medium was collected carefully without disturbing the cells attached to the bottom of the plate and stored at -80 °C until SPE-LC-MS/MS analysis.

2.4. Optimal Concentrations of Internal Standards

The procedure to select optimal concentrations of internal standards has been published elsewhere [15]. Eleven stock solutions were prepared by dissolving pure standards of PGE2, PGE3, 6-keto-PGF1α, Δ17-6-keto-PGF1α, RvD1, RvD2, LTB4, PGE2-d4, 6-keto-PGF1α-d4, RvD2-d5 and LTB4-d4 in cL-15 medium. The stock solutions were combined and diluted with cL-15 at the seven levels of concentrations suggested by a uniform shell design (Table **1**). The experiments were performed in triplicate (7×3) and the resulting 21 mixtures submitted to SPE. The final reconstituted products from the SPE procedure were analyzed by LC-MS/MS and the response factor (RF) for every experimental point estimated by the expression:

$$\mathrm{RF} = \frac{[A]}{[IS]} \times \frac{I_{IS}}{I_A} \qquad \text{Eq. (1)}$$

where [A] and [IS] represent the concentrations and I_A and I_{IS} represent the recorded signals for the analyte and internal standard respectively. The Eq. (1) allows estimating 21 RF values (7×3) for every analytical eicosanoid and resolving. The behavior of RF wasexpressed as a function of [A] and [IS] by using a full second-order polynomial model of the form:

Table 1. Concentrations of pro- and anti-inflammatory biomarkers and corresponding internal standards used for modelling the response factor (RF) in the range of 0-200 ng/ml. Every experiment was measured in triplicate.

Number of Experiment	Concentration levels (ng/ml)										
	6-keto-$PGF_{1\alpha}$	Δ17-6-keto-$PGF_{1\alpha}$	LTB_4	PGE_2	PGE_3	RvD_2	RvD_1	6-keto-$PGF_{1\alpha}$-d_4	LTB_4-d_4	PGE_2-d_4	RvD_2-d_5
1	100	100	100	100	100	100	100	100.2	100.2	100.2	100.2
2	50	150	50	50	150	50	150	13.8	13.8	13.8	13.8
3	150	50	150	150	50	150	50	186.6	186.6	186.6	186.6
4	150	50	150	150	50	150	50	13.8	13.8	13.8	13.8
5	50	150	50	50	150	50	150	186.6	186.6	186.6	186.6
6	200	0.5	200	200	0.5	200	0.5	100.2	100.2	100.2	100.2
7	0.5	200	0.5	0.5	200	0.5	200	100.2	100.2	100.2	100.2

$$RF = b_o + b_A[A] + b_{IS}[IS] + b_A^2[A]^2 + b_{IS}^2[IS]^2 + b_{A\times IS}[A]\times[IS] \quad \text{Eq. (2)}$$

where b_o is the intercept, b_A and b_{IS} are the linear term coefficients, b_A^2 and b_{IS}^2 are second order curvature effect coefficients and $b_{A\times IS}$ is the first order interaction effect coefficient. The validity of the generated mathematical models (one model per analytical biomarker) was checked by monitoring the ratio lack-of-fit to pure error variances (aka F-test) at a 95% confidence level. Further simplification of the generated full second-order polynomial models was considered by removing the less significant coefficients in Eq. (2).

2.5. Extraction Procedure

The 21 mixtures (7×3) prepared according to a uniform shell design (Table **1**) were submitted to the following SPE protocol. An aliquot of medium (1 mL) containing the concentrations of analytes and internal standards indicated in Table 1 was combined with 175 µl of ethanol and 20 µl of acetic acid, vortex-mixed and applied on a SPE column (Agilent, ASPEC Bond Elute C18, 500 mg, 3 mL, USA) previously preconditioned with 2 mL of methanol and 2 mL of water. The cartridge was washed with 4 mL of distilled water and 4 mL of hexane. The analytes were eluted with 1 mL of hexane/ethyl acetate (1:2 v/v), collected into glass tubes and the solvent evaporated under a stream of nitrogen. The dried sample was dissolved in 70 µL of acetonitrile, vortex-mixed 30 s, centrifuged at 1620 ×g for 3 min and transferred to an auto sampler vial for LC-MS/MS analysis.

2.6. Method Validation

The proposed SPE-LC-MS/MS method was submitted to analytical validation after selecting the optimal concentrations of the internal standards by means of the mathematical models describing the behavior of RF as a function of [A] and [IS]. The validation parameters considered were: selectivity, limit of detection (LOD), limit of quantification (LOQ), linearity, analytical range and recovery.

The selectivity of the method was assessed by comparing the extracted ion chromatograms from spiked and unspiked cL-15 medium samples and establishing whether the proposed SPE method can determine the various biomarkers without interference from other components in the culture medium. The LOD and LOQ were determined by comparing the analytical signals at known low concentrations with those of a blank sample up to an analytical level where the analytical signals are equivalent to three and six times the standard deviation of the blank sample (3×σblank and 6×σblank) respectively. The analytical range was prepared by using medium spiked with six concentration levels of PGE2, PGE3, 6-keto PGF1α Δ17-6 keto PGF1α RvD1, RvD2 and LTB4 (0.0-200 ng/mL) and optimal levels of internal standards were selected by means of a uniform shell design and mathematical modelling (specifically 180 ng/mL PGE2-d4, 45 ng/mL 6-keto-PGF1α-d4, 40 ng/mL RvD2-d5 and 30 ng/mL LTB4-d4). These samples were submitted to the proposed SPE protocol and the relationship between IA/IIS versus [A] evaluated. The recovery of the method was determined by comparing the back-calculated against the nominal concentrations.

2.7. Liquid Chromatography Ion-Trap Mass Spectrometry

The LC-MS was an Agilent 1100 series LC/MSD trap, SL model equipped with an electrospray interface (ESI), a quaternary pump, degasser, autosampler, thermostatted column compartment and a variable-wavelength UV detector. A column C18 RP 250×4.6 mm, 5µm (Alltech, USA) kept at 40 °C and injection volume of 20 µL were used. For the analysis of PGE2, PGE3, 6-keto-PGF1α, Δ17-6-keto-PGF1α, LTB4, PGE2-d4, 6-keto-PGF1α-d4 and LTB4-d4 the solvent system, operated in isocratic mode at 0.50 mL/min, consisted of acetonitrile with 0.1% formic acid (v/v) and the analysis time was 15 min. For the analysis of RvD1, RvD2 and RvD2-d5 the solvent system, operated in gradient mode at 0.5 mL/min, consisted of solvent A: water:acetonitrile:formic-acid (63:37:0.02 v/v/v) and solvent B: 2-propanol:acetonitrile (50:50 v/v) and was delivered as follows: 100% A (0-5 min), 60% A (5-11 min), 10% A (11-13 min), 10% A (13-15 min), 100% A (15-30 min).

The ESI source was operated in negative ion mode with nitrogen as nebulizing and drying gas at 350 °C, 8 mL/min and 50 psi. The ion optics responsible for getting the ions in the ion-trap such as capillary exit, skimmer, lens and octapoles voltages were controlled by using the Smart View option with a resolution of 13000 m/z/s (FWHM/m/z = 0.6-0.7). Complete system control, data acquisition and processing were done using the ChemStation for LC/MSD trap software, version 5.3 from Agilent (Agilent Technologies, Inc., 2005). The areas of the extracted ion chromatograms for the different biomarkers were computed in ion counts per second (icps) and used as analytical signals. The recorded fragmentation patterns were m/z 351→ 333, 315, 271 for PGE2; m/z 349 → 331, 313, 269 for PGE3; m/z 355→337, 319, 275 for PGE2-d4; m/z 369→351, 315, 289, 323, 307, 205, 220, 149 for 6-keto-PGF1α; m/z 367→349, 331, 289, 269, 323, 313, 305, 298, 207, 185, 163 for Δ17-6-keto-PGF1α; m/z 373→355, 337, 319, 275, 167 for 6-keto-PGF1α -d4; m/z 375→141 for RvD1 and RvD2; m/z 380→362, 344, 326, 282, 141 for RvD2-d5; m/z 335→317, 273, 151, 129, 109 for LTB4; m/z 339→321, 277, 319, 293, 275, 197,179, 153, 125 for LTB4-d4.

2.8. Statistics

Excel (Microsoft Office Excel 2013) was used for determining the adequacy of the mathematical regressions. The lack-of-fit (SSlof) and pure experimental (SSE) error sum of squares were calculated and divided by their corresponding degrees of freedom to obtain the variance components σ^2_{lof} and σ^2_E respectively. The ratio $\sigma^2_{lof}/\sigma^2_E$ (Festimated) is computed and compared against the tabulated Fisher value (Ftabulated) at the 95% confidence level to judge the linearity of the proposed models.

3. RESULTS AND DISCUSSION

3.1. Selection of the Optimal Concentrations of Internal Standards

The RF for the different biomarkers was estimated by Eq. (1) and modelled as a function of the analyte and corresponding internal standard concentrations by using polynomial models (Eq. (2)). The computed regression models for PGE2, PGE3, 6-keto-PGF1α, Δ17-6-keto-PGF1α, RvD1, RvD2, LTB4 and their corresponding internal standards are presented in Fig. (**2**).

3.1.1. Internal Standard for Prostaglandins (PGE2 and PGE3)

The concentration plots for PGE2-d4 vs PGE2 (Fig. **2a**) and PGE2-d4 Vs. PGE3 (Fig. **2b**) showed three well differentiated regions (indicated in blue, red and green) along the PGE2-d4 axis where the RF remains constant over the entire PGE2 range. By considering simultaneously the magnitude of RF in both plots (Figs. **2a-b**) for PGE2 and PGE3, it is possible to conclude that a concentration of PGE2-d4 in the range of 175-185 ng/mL is optimal for obtaining a constant RF with the lowest relative standard deviation (~11%). Consequently, 180 ng/mL of PGE2-d4 is chosen for further analysis of PGE2 and PGE3 in cL-15 medium.

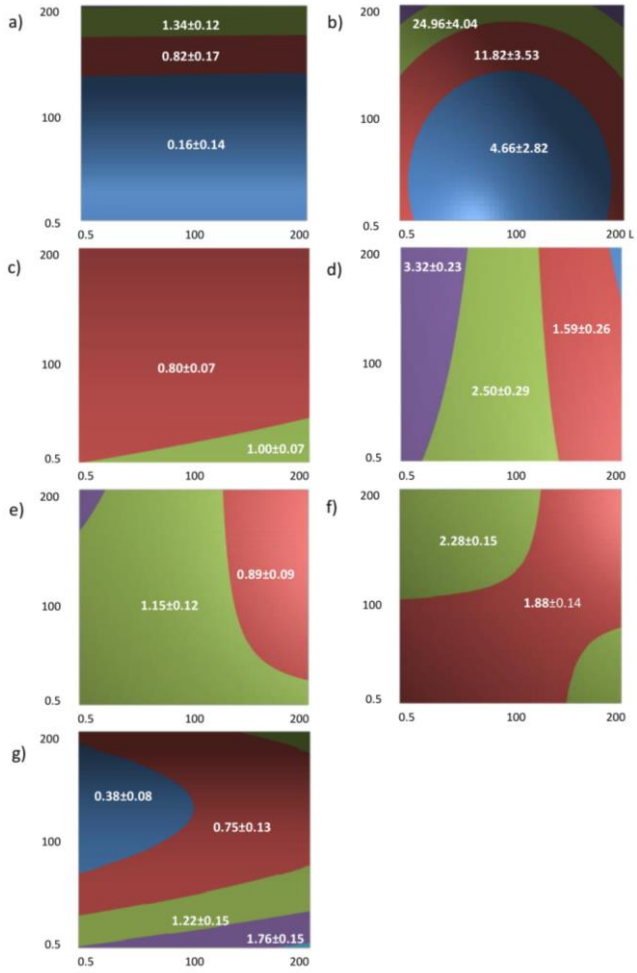


Fig. (2). Response factor (RF) as a function of the analyte concentration (horizontal axis) and internal standard concentration (vertical axis) for the pro- and anti-inflammatory biomarkers released in cL-15 medium. The axis are expressed in ng/mL and the values on the contours represent the mean ± standard deviation of the RF (n=3). a) PGE2-d4 vs PGE2, b) PGE2-d4 vs PGE3, c) 6-keto-PGF1α-d4 vs 6-keto-PGF1α, d) 6-keto-PGF1α-d4 vs Δ17-6-keto-PGF1α, e) RvD2-d5 vs RvD1, f) RvD2-d5 vs RvD2 and g) LTB4-d4 vs LTB4.

3.1.2. Internal Standard for Prostacyclins (6-keto-PGF1α and Δ17-6-keto-PGF1α)

The contour plot in Fig. (**2c**) showed an optimal concentration region between 40-200 ng/mL of 6-keto-PGF1α-d4 over the entire 6-keto-PGF1α range. The contour plot in Fig. (**2d**) revealed three different RF values in different concentration regions of Δ17-6-keto-PGF1α that were independent of the concentration of internal standard (6-keto-PGF1α-d4). The Δ17-6-keto-PGF1α concentration regions were 0.5-22 ng/mL (3.32±0.23), 22-148 ng/mL (2.50±0.29) and 148-200 ng/mL (1.59±0.26). Based on both contour plots (Figs. **2c-d**)), a level of 45 ng/mL of 6-keto-PGF1α-d4 was selected for the analysis of 6-keto-PGF1α and Δ17-6-keto-PGF1α produced by salmon liver cells in cL-15 medium.

Table 2. Statistical validation for the RF-models in Fig. 2 and validation parameters in cL-15 medium.

	Parameter	PGE2 Fig. 2A	PGE3 Fig. 2B	6-keto-PGF1α Fig. 2C	Δ17-6-keto-PGF1α Fig. 2D	RvD1 Fig. 2E	RvD2 Fig. 2F	LTB_4 Fig. 2G
RF Modelling	SS_r	0.389 (17)	198.46 (16)	0.600 (14)	10.636 (14)	0.696 (14)	1.376 (14)	2.715 (17)
	SS_E	0.324 (14)	197.09 (14)	0.587 (12)	10.019 (12)	0.634 (12)	1.056 (12)	2.157 (14)
	SS_{lof}	0.066 (3)	1.375(2)	0.013 (2)	0.617 (2)	0.062 (2)	0.320 (2)	0.558 (3)
	$F_{estimated}$	0.944	0.0488	0.137	0.369	0.590	1.819	1.206
	$F_{tabulated}$	3.344	3.739	3.885	3.885	3.885	3.885	3.344
Validation	LOD (ng/ml)	0.3	0.3	0.8	1.0	0.3	0.3	0.3
	LOQ (ng/ml)	0.5	0.5	1.5	2.0	0.5	0.5	0.5
	Recovery (%)	92-100	92-106	88-111	83-127	89-127	80-121	80-121
	Range ng/ml)	0.5-200	0.5-200	1.5-200	2.0-100	0.5-200	0.5-200	0.5-150

Ftabulated at the 95% confidence level. Degrees of freedom in parentheses

3.1.3. Internal Standard for Resolvins (RvD1 and RvD2)

Reduced models with equivalent number of coefficients were obtained for the resolvins (Table **2**). For RvD1, the RF was constant (1.15±0.12) at concentration levels below 20 ng/mL of RvD2-d5 (Fig. **2e**). However, at this particular level the chromatographic peak for RvD2 exhibited a noisy baseline. For RvD2, the RF was constant (1.88±0.14) in the range of 40-110 ng/mL of RvD2-d5 (Fig. **2f**). It was decided that 40 ng/mL of RvD2-d5 (representing RF values of 1.06±0.06 and 1.84±0.15 for RvD1 and RvD2 respectively) was an optimal concentration level for the analysis of released resolvins in cL-15 medium by salmon liver cells.

3.1.4. Internal Standard for Leukotriene (LTB4)

The concentration plot LTB4-d4 vs LTB4 (Fig. **2g**) revealed that the major variation of RF was along LTB4–d4 axis. Three concentrations of LTB4-d4 (30, 70 and 190 ng/mL) rendered RF values (1.28±0.17, 0.78±0.17 and 0.79±0.17 respectively) that remained constant in the range of 0.5-200 ng/mL of LTB4 (Fig. (**2g**). The lowest internal standard concentration (30 ng/mL) was selected for the analysis of LTB4 in cL-15 medium.

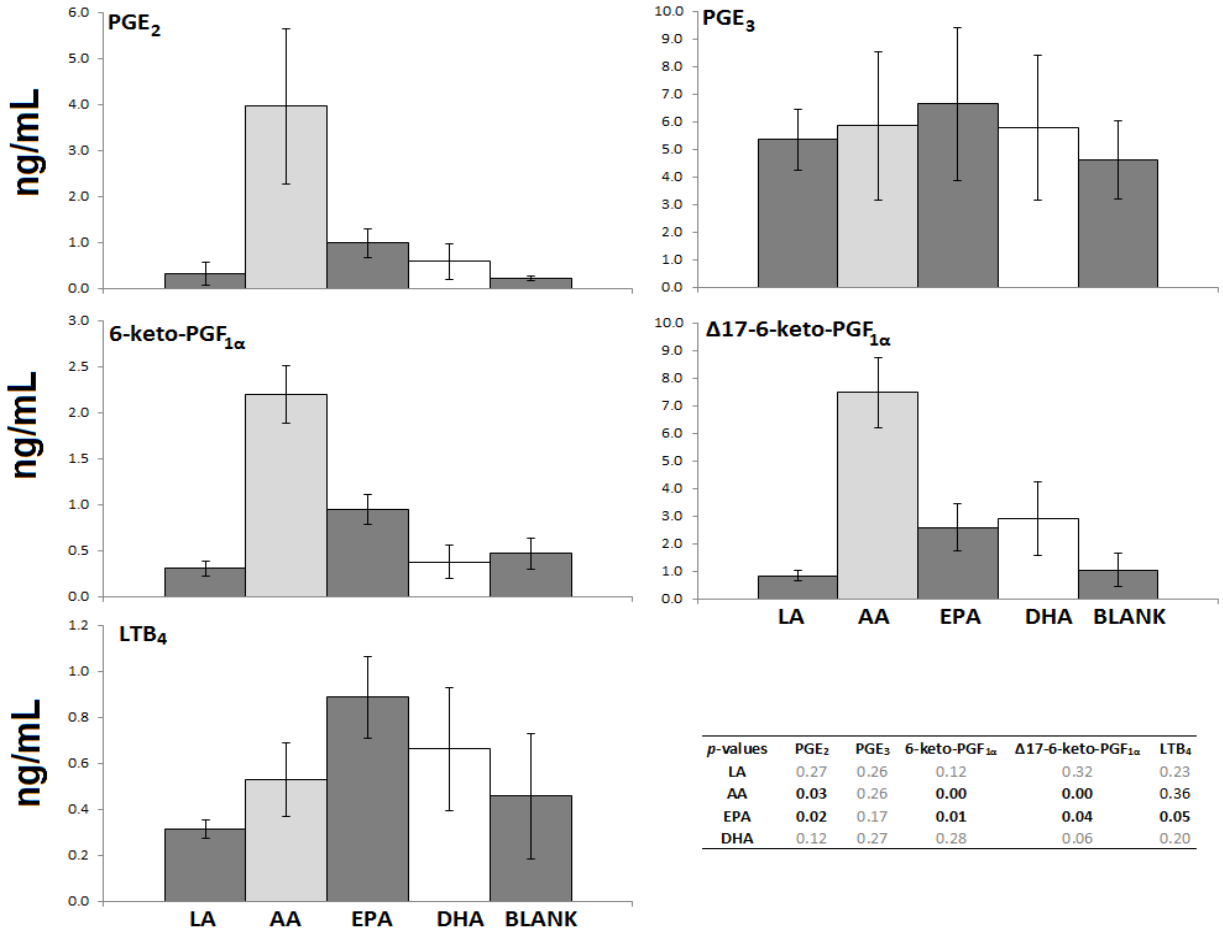
3.2. Analytical Performance

The proposed SPE-LC-MS/MS was submitted to analytical validation by using the previously selected optimal concentrations of internal standards (180 ng/mL PGE2-d4, 45 ng/mL 6-keto-PGF1α-d4, 40 ng/mL RvD2-d5 and 30 ng/mL LTB4-d4).

The analysis of the extracted ion chromatograms from spiked and unspiked cL-15 medium samples revealed that the various fragmentation patterns were clearly distinguished from each other and that the SPE protocol is highly selective towards the non-deuterated and deuterated classic and non-classic pro- and anti-inflammatory biomarkers.

The linearity of the method was assessed by preparing calibration mixtures of PGE2, PGE3, 6-keto-PGF1α, Δ17-6-keto-PGF1α, RvD1, RvD2, LTB4 in cL-15 medium in the range of 0.0-200 ng/mL and containing constant amounts of PGE2-d4 (180 ng/mL) 6-keto-PGF1α-d4 (45 ng/mL) RvD2-d5 (40 ng/mL) and LTB4-d4 (30 ng/mL). The proposed regression models for the various biomarkers (Table **2**) exhibited consistently lower experimental Fisher ratios than the tabulated value at the 95% confidence level with 4 and 12 degrees of freedom (< F4/12=3.259), hence the mathematical models described in Table **2** can be regarded as linear in the range of concentration of 0.5-200 ng/mL for PGE2, PGE3, RvD1 and RvD2, 1.5-200 ng/mL for 6-keto-PGF1α, 2.0-100 ng/mL for Δ17-6-keto-PGF1α and 0.5-150 ng/mL for LTB4. The narrower concentration ranges for Δ17-6-keto-PGF1α (2.0-100 ng/mL) and LTB4 (0.5-150 ng/mL) are the result of variation in their RF towards high concentrations which in turn prevent the modelling of these system as linear function of the signal in the whole range of analytical concentrations (0-200 ng/mL).

The LOD, estimated by diluting successively a 1.0 ng/mL cL-15 medium solution of the analytical prostaglandins, resolvins and leukotriene and determining the confidence with which it is possible to detect a concentration larger than that in a blank of cL-15 medium with a statistical power of 84%, were 0.3 ng/mL for PGE2, PGE3, RvD1, RvD2 and LTB4, 0.8 ng/mL for 6-keto-PGF1α and 1.0 ng/mL for Δ17-6-keto-PGF1α. The LOQ values were encompassed in the dynamic range as the minimum concentration of spiked cL-15 medium yielding an analytical signal equivalent to 6×σblank that can be quantified with acceptable level of precision and accuracy. The recovery values, estimated as the ratio between back-calculated and the nominal concentrations, varied between 92-100% for PGE2, 92-106% for PGE3, 88-111% for 6-keto-PGF1α, 83-127% for Δ17-6-keto-PGF1α, 89-127% for RvD1, 80-121% for RvD2 and LTB4, with average values close to 100% in all cases (Table **2**).



p-values	PGE_2	PGE_3	6-keto-$PGF_{1\alpha}$	Δ17-6-keto-$PGF_{1\alpha}$	LTB_4
LA	0.27	0.26	0.12	0.32	0.23
AA	**0.03**	0.26	**0.00**	**0.00**	0.36
EPA	**0.02**	0.17	**0.01**	**0.04**	**0.05**
DHA	0.12	0.27	0.28	0.06	0.20

Fig. (3). Production of eicosanoids by salmon liver cells exposed to linoleic (LA), arachidonic (AA), eicosapentaenoic (EPA) and docosahexaenoic (DHA) fatty acids and their significance ($p < 0.050$).

3.3. Stimulated Production of Prostaglandins, Resolvins and Leukotriene in Salmon Liver Cell Cultures by PUFA

The validated SPE-LC-MS/MS was used for the simultaneous determination of prostaglandins, prostacyclins, leukotriene and resolvins generated by salmon liver cells in cL-15 medium exposed to four different ω-6 (LA and AA) and ω-3 (EPA and DHA) PUFA.

The graphical representations of the mean concentrations of the generated eicosanoids with their corresponding 95% least significant intervals (Fig. **3**) revealed a statistically significant increase in the production of PGE2, 6-keto-PGF1α, Δ17-6-keto-PGF1α and LTB4 in salmon liver cell cultures containing EPA. Arachidonic acid (AA) increased the production of these eicosanoids but LTB4. The production of eicosanoids was not affected by exposing the salmon liver cells to LA or DHA.

RvD1, RvD2, RvD3 and RvD4 are isobaric molecules (MW= 376) and their formation proceed through two different lipid mediators, more specifically 7S,8S-epoxy-17S-hydroxy-DHA for RvD1, RvD2 (quantitatively analyzed in this work) and 4S,5S-epoxy-17S-hydroxy-DHA for RvD3, RvD4 [5]. The mass spectrometry base peaks in negative mode for RvD1 and RvD2 (m/z 375→141) and for RvD3 and RvD4 (m/z 375→147) constitute a practical basis for their discrimination [16]. These base peaks were extracted from the chromatograms of analyzed medium and the results did not show the presence of the fragment m/z 141 (Fig. **4a**). However, a significant peak at m/z 147 with an intensity similar to the internal standard (RvD2-d5 at m/z 380→141) was observed (Fig. **4b**). Unfortunately, the standards RvD3 and RvD4 were not characterized in the present targeted analysis. However, these results might indicate that the major metabolites of DHA in salmon liver cells are RvD3 and RvD4, and not RvD1 and RvD2 (as initially assumed in the present work).

Lack of RvD1 and RvD2 production by salmon cells was unexpected, considering that a previous work on salmon tissue demonstrated their production in salmon tissue [10]. These apparently contradictory results might indicate that RvD1 and RvD2 are metabolized locally which emphasized their paracrine signaling mechanism.

The observed increase in PGE2, 6-keto-PGF1α indicates that during the incubation period, AA is incorporated into the cell membrane resulting in the release of AA into the cells and the subsequent synthesis of AA-derived eicosanoids. The increased levels of PGE2 and 6-keto-PGF1α compared to the unaffected levels of LTB4 produced after exposing the cells to AA, are indicative that the added ω-6 PUFA is basically enrolled in

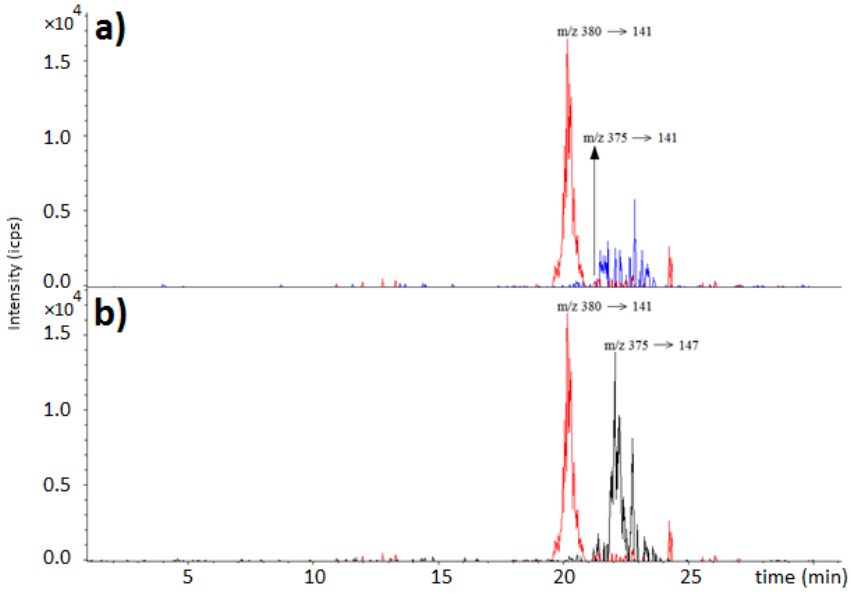


Fig. (4). Extracted ion chromatograms indicating the production of resolvins by salmon liver cells in cL-15 399 medium. a) RvD1 and RvD2 (m/z 375→141) and b) RvD3 and RvD4 (m/z 375→147) with their corresponding RvD2-d5 internal standard (m/z 380→141). Pure commercial standards of RvD3 or RvD4 were not available in the present research to confirm their involvement in the transition m/z 375→141.

COX pathway instead of LOX pathway. The increased production of the EPA metabolite, Δ17-6-keto-PGF1α, after exposure to AA was unexpected. Some researchers has argued that AA can stimulate in some degree the production of Δ17-6-keto-PGF1α in human endothelial cells [17]. In addition, increased levels of EPA-metabolites have also been observed in skin cells from dogs fed ω-6/ω-3 diet ratios of 5/1 and 10/1 [18].

The enhanced production of LTB4 by the presence of EPA confirms that EPA is incorporated into the by-layer phospholipid cell membrane after releasing AA in a dose- and time-dependent manner [19,20]. At the first 24 h, EPA is incorporated into the cell membrane at the expense of AA that is released and converted into AA-derived eicosanoids.

The significant increasing in the production of Δ17-6-keto-PGF1α compared to PGE3 in salmon liver cell cultures exposed to EPA could be explained on the basis that PGH3 synthase (the common substrate for the production of both Δ17-6-keto-PGF1α and PGE3) has more affinity towards the production of the prostacyclin.

CONCLUSIONS

The validated SPE protocol in conjunction with LC-MS/MS is a reliable strategy for the quantitative monitoring of pro- and anti-inflammatory biomarkers released in culture media by fish cells exposed to different PUFA. The SPE protocol demonstrated to be an effective tool for understanding the impact of PUFA on the production of prostaglandins, resolvins and leukotriene and for gaining insights into the potential mechanism behind the production of lipid mediators derived from AA, EPA and DHA. The stimulated production of prostaglandins, resolvins and leukotriene in cell cultures after exposure to AA and EPA indicated that these particular PUFA can be incorporated into salmon liver cells after releasing EPA and AA from the membrane, which in turn promote the production of Δ17-6-keto-PGF1α and LTB4 respectively. The results seem to indicate that the production of RvD3 and RvD4 is preferred over RvD1 and RvD2 in salmon liver cells. However, this conclusion is derived from qualitative analysis of the experimental chromatograms. Hence, it is advisable to generate quantitative models for RvD3 and RvD4 to substantiate this observation.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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SUPPLEMENTARY MATERIAL

The following references correspond with the eight time segments portrayed in Fig. **1**. The bold and italics L or S letters after a reference number indicate liquid-liquid extraction or solid phase extraction respectively.

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