Development of a solid phase extraction method for evaluating the production of classic and nonclassic eicosanoids in human and fish cells using liquid chromatography tandem mass spectrometry

By
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Thesis for the degree of European Master in Quality in Analytical Laboratories

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March, 2014

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Acknowledgments

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List of Publications

[1] Araujo, P.; Lucena, E.; Yang, Y.; Ceemala, B.; Mengesha, Z.; Holen, E. The impact of exogenous ω-6 and ω-3 polyunsaturated fatty acids on the induced production of pro- and anti-inflammatory prostaglandins and leukotrienes in Atlantic salmon head kidney cells using a full factorial design and LC-MS/MS. Journal of Chromatography B (Accepted).

[2] Lucena, E.; Yang, Y.; Aarsæther N.; Holen E.; Araujo P. Development of a solid phase extraction method for the determination of prostacyclins in human and fish cells by LC-MS/MS. American Journal of Modern Chromatography (to be submitted)

List of Abbreviations

Linoleic acid	LA
α-Linolenic acid	α-LNA
Arachidonic acid	AA
Eicosapentaenoic acid	EPA
Docosahexaenoic acid	DHA
Monounsaturated fatty acids	MUFAs
Polyunsaturated fatty acids	PUFAs
Enzyme immunoassay	EIA
Radioimmunoassay	RIA
Gas chromatography	GC
High performance liquid chromatography	HPLC
Extracted ion chromatograms	EICs
Diode array detector	DAD
Mass spectrometry	MS
Electrospray ionization	ESI
Atmospheric pressure chemical ionization	APCI
Flame ionization detector	FID
Human umbilical vein endothelial cells	HUVEC
Cyclooxygenases	COX
Lipooxygenases	LOX
Response factor	RF
Solid phase extraction	SPE
Liquid-liquid extraction	LLE
Limit of detection	LOD
Limit of quantification	LOQ
Analysis of variance	ANOVA
International Union of Pure and Applied Chemistry	IUPAC
Fetal bovine serum	FBS
Phosphate-buffered saline buffer	PBS
Least Significant Difference	LSD

Abstract

Eicosanoids are the major metabolites of fatty acids and they are correlated with many kinds of diseases such as Alzheimer's, cancer and cardiovascular diseases. Usually, most of the eicosanoids derived from ω-6 fatty acids have pro-inflammatory properties while those from ω-3 fatty acids are anti-inflammatory. The type and amount of the production of eicosanoids are affected by many factors including availability of fatty acids, activity of cyclo- and lipo- oxygenase and type of cells.

In this thesis, a solid-phase extraction method combined with HPLC-MS/MS is first developed to analyze simultaneously PGE_2, PGE_3, LTB_4, 6-keto-$PGF_{1\alpha}$, Δ17-6-keto-$PGF_{1\alpha}$, RvD_1 and RvD_2 in two types of cell culture medium, specifically EBM-2 medium and cL-15 medium. This method is systematically optimized and validated. The concentrations of internal standards are determined by a Doehlert design to keep the response factors constant in the analytical range. Method validation for the various eicosanoids in EBM-2 medium shows limits of quantification (LOQ) between 0.1-0.6 ng/ml, recovery between 57.1-127.2% and precision between 0.2- 20.7%. For cL-15 medium, LOQ is between 0.1-8 ng/ml, recovery 63.9-125.7% and precision 3.5-30.7% for the analyzed eicosanoids.

The validated method is then applied to investigate the effect of fatty acids on the production of eicosanoids in fish liver cells, fish head kidney cells and human umbilical vein endothelial cells (HUVEC). The various types of cells are incubated in medium with different combination of fatty acids according to a 2^4-factorial design and eicosanoids in the medium are analyzed after 24 hours. The results reveal that RvD_1 and RvD_2 cannot be detected in any type of cells. Addition of arachidonic acid (AA) increases the productions of all the other five eicosanoids except in fish head kidney cells where production of PGE_3 is reduced in a small degree. Individual linoleic acid (LA) reduces the productions of 6-keto-$PGF_{1\alpha}$ and Δ17-6-keto-$PGF_{1\alpha}$ and increases the production of PGE_3 in both types of fish cells. Eicosapentaenoic acid (EPA) remarkably increases the production of LTB_4 and docosahexaenoic acid (DHA) decreases the production of Δ17-6-keto-$PGF_{1\alpha}$ in three types of cells. The effects of interactions are complex and most of them are different in different types of cells.

1. Introduction

1.1 Fatty acids

1.1.1 Classification of the fatty acids

Fatty acids are carboxylic acids with an aliphatic chain. In nature, most of the fatty acids have an even number of carbon atoms, from 4 to 28. According to the length of the aliphatic chain, fatty acids can be categorized to short-chain fatty acids, medium-chain fatty acids, long-chain fatty acids and very long-chain fatty acids. A short-chain fatty acid has an aliphatic chain with less than 6 carbon atoms, a medium-chain has 6-12 carbons, a long-chain has 13-21 carbons, and a very long-chain fatty acid has more than 22 carbons. Besides, the aliphatic chain can be saturated or unsaturated, which divides the fatty acids into two classes: saturated and unsaturated fatty acids. Unsaturated fatty acids can be further classed to monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) according to the number of the double bounds. All of the fatty acids, especially PUFAs, are very important for the development and function of human and animal organisms.

1.1.2 Nomenclature of the unsaturated fatty acids

All of the fatty acids have their trivial names, systematic names defined by the International Union of Pure and Applied Chemistry (IUPAC) and symbol names. Since symbols are short and helpful for understanding the chemical structure of the fatty acids, they are widely applied names in scientific and popular literature.

A symbol name contains the number of carbon atoms, the number of double bounds and the position of the first double bound. To understand the position of the double bounds, the numbering of the carbon atoms should be first introduced. The carbons labelled as ω or n are those where the methyl group is numbered as carbon one. For example, linoleic acid (LA, 18:2n-6) has 18 carbons, two double bonds and the first double bound locates between the 6th and 7th carbons from the methyl end, so it is designated an n-6 (or ω-6) fatty acid, and the symbol name is 18:2n-6. More examples can be seen in **Figure 1.1**.

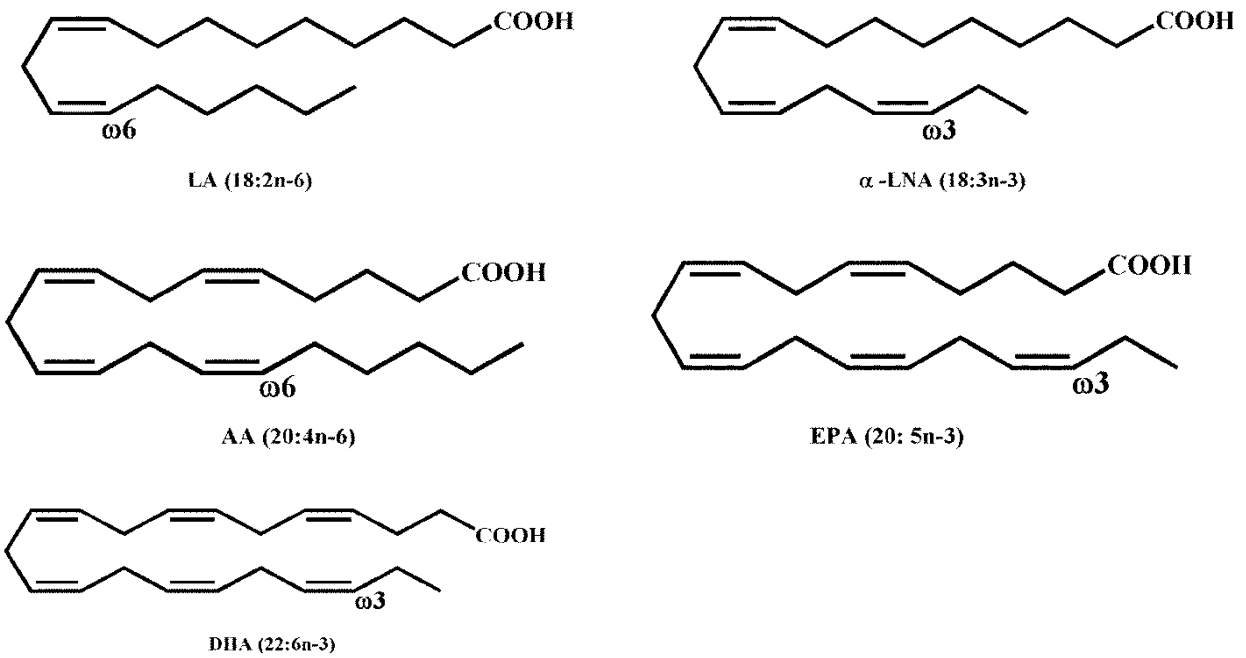


Figure 1.1 Chemical structures of five important fatty acids

1.1.3 Polyunsaturated fatty acids

PUFAs are the fatty acids that have more than one double bond in the aliphatic chain. Recently, dietary PUFAs are drawing more and more attention worldwide since they are correlated with many kinds of diseases. ω-3 and ω-6 fatty acids are two kinds of PUFAs. They cannot be synthesized by mammals, and so they must be obtained from the diet. Thus, the effect of different ω-3 and ω-6 fatty acids are becoming much more important.

Linoleic (LA, 18:2n-6) and α-linolenic (α-LNA, 18:3n-3) acids are the representative of ω-6 and ω-3 fatty acids respectively. They are the starting point to manufacture other ω-6 and ω-3 series fatty acids. LA can be metabolized to arachidonic acid (AA, 20:4n-6) by desaturase and elongase, while α-LNA is the precursor of the eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). In addition to the conversion from EPA to DHA, DHA can be reconverted to EPA also. The metabolism of different fatty acids is shown in **Figure 1.2**. It is worth noting that LA and α-LNA are involved in the similar metabolic system, so there is competition between them. Since α-LNA has a higher affinity for the enzymes involved in metabolism compared with LA, the recommendation ratio between LA and α-LNA is between 4/1 to 10/1[1].

1.2 Eicosanoids

1.2.1 Production of eicosanoids

One of the most important functions of PUFAs is related to their enzymatic conversion into eicosanoids. PUFAs are first released from membrane phospholipids by the action of various phospholipases. Then, they are metabolized to different eicosanoids. AA is the substrate for two classes of enzymes, cyclooxygenases (COX) which produce 2-series prostaglandins, 2-series prostacyclins and 2-series thromboxanes, and lipooxygenases (LOX) which catalyze the biosynthesis of hydroxyeicosatetraenoic acids (HETEs) and 4-series leokotrienes. EPA exhibits a similar metabolism to AA, but it is metabolized to 3-series prostaglandins, 3-series thromboxanes from COX and 5-series leokotrienes and hydroxyeicosapentaenoic acids from LOX. DHA are mainly converted to D-series resolvins by LOX. The metabolism of PUFAs is shown in **Figure 1.2**.

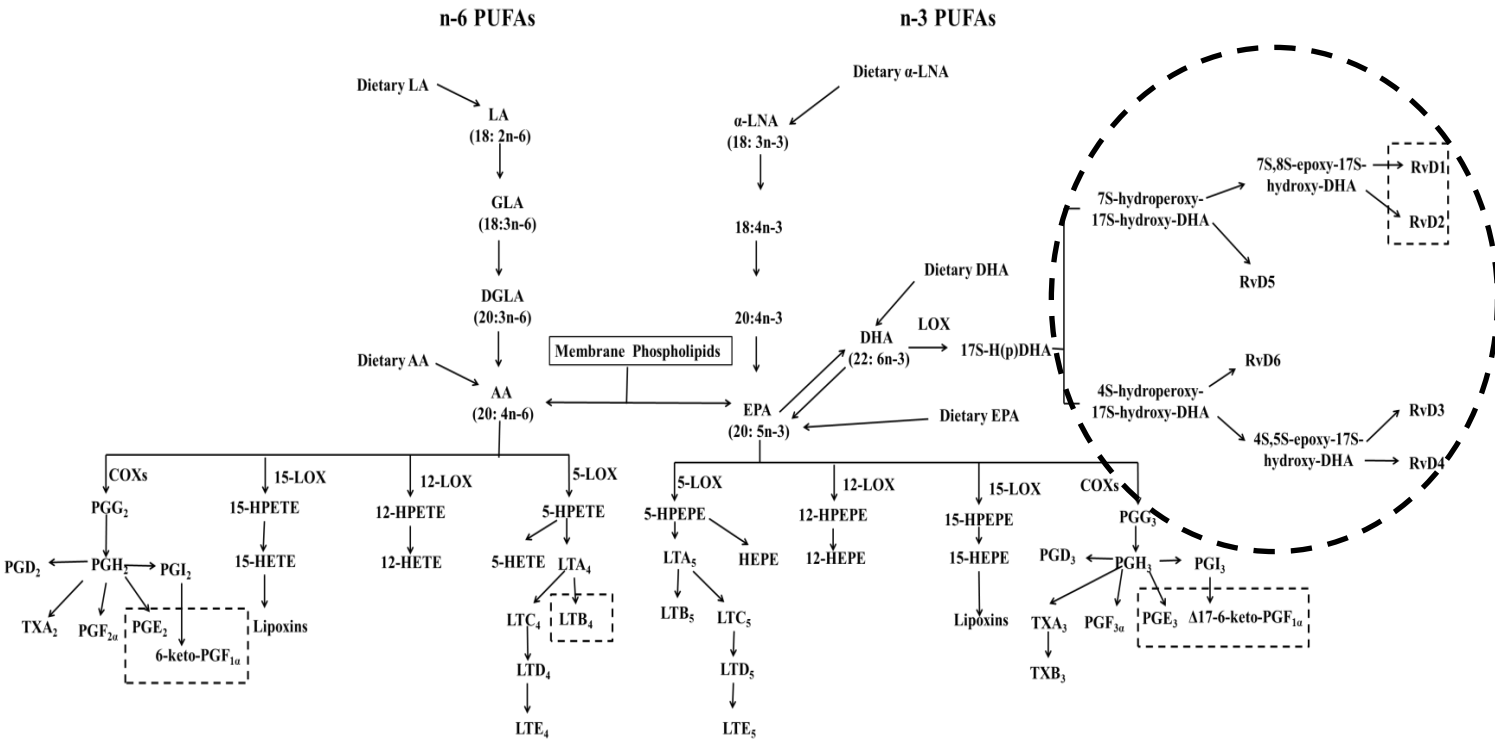
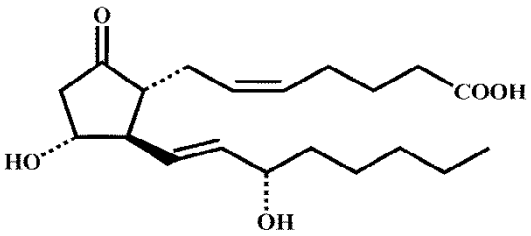


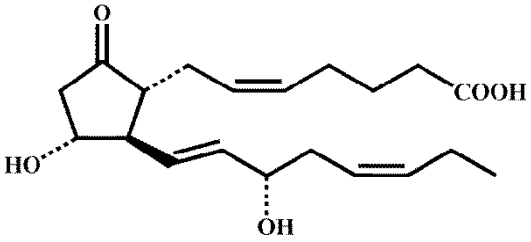
Figure1.2 The metabolism of different PUFAs into eicosanoids[2]

1.2.2 Chemical structure of eicosanoids

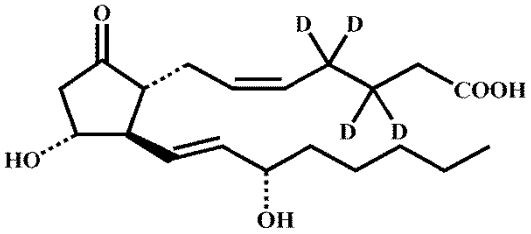
The chemical structures of the seven eicosanoids analyzed in this thesis and their corresponding internal standards are shown in **Figure 1.3**.



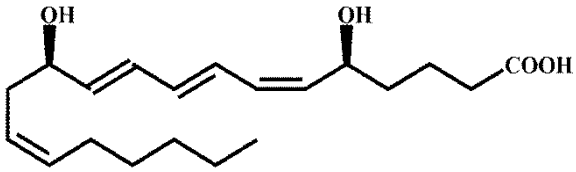
PGE_2 ($C_{20}H_{32}O_5$, M=352.5）



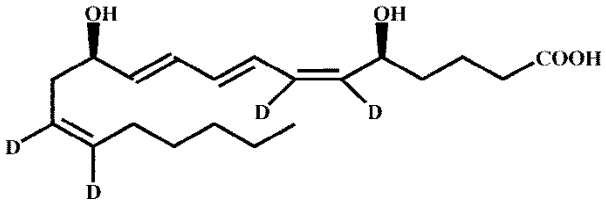
PGE_3 ($C_{20}H_{30}O_5$, M=350,5）



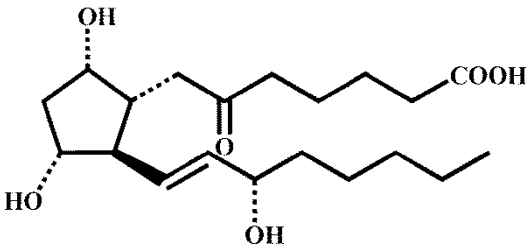
PGE_2-d_4 ($C_{20}H_{28}D_4O_5$, M=356.5)



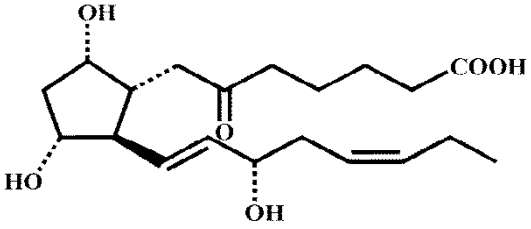
LTB_4 ($C_{20}H_{32}O_4$, M=336.5)



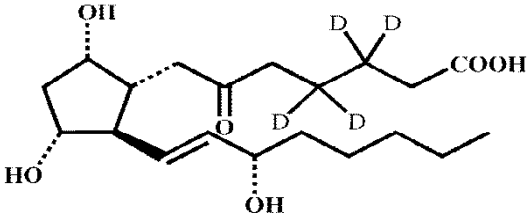
LTB_4-d_4 ($C_{20}H_{28}D_4O_4$, M=340.5)



6-keto-$PGF_{1\alpha}$ ($C_{20}H_{34}O_6$, M=370.5)

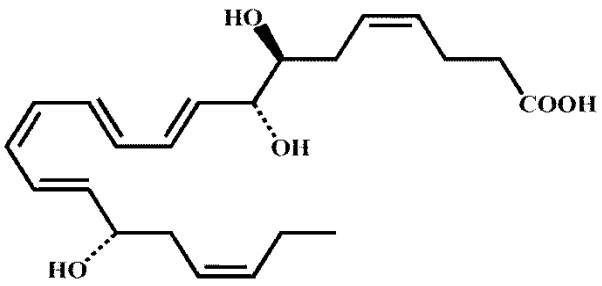


Δ17-6-keto-$PGF_{1\alpha}$ ($C_{20}H_{32}O_6$, M=368.5)

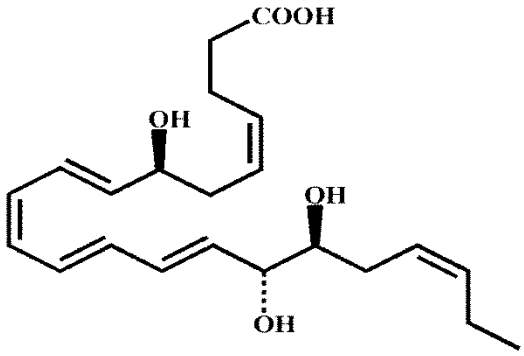


6-keto-$PGF_{1\alpha}$-d_4 ($C_{20}H_{30}D_4O_6$, M=374.5)

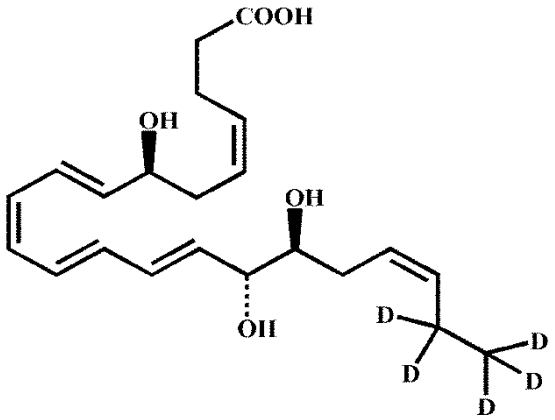
Figure 1.3-1 Chemical structures of different eicosanoids and associated internal standards



RvD_1 ($C_{22}H_{32}O_5$, M=376.5)



RvD_2 ($C_{22}H_{32}O_5$, M=376.5)



RvD_2-d_5 ($C_{22}H_{27}D_5O_5$, M=381.5)

Figure 1.3-2 Chemical structures of different eicosanoids and associated internal standards

1.2.3 Importance of eicosanoids

Generally, AA-derived eicosanoids have pro-inflammatory effects and they are positively linked to arthritis[3] and periodontal disease[4] and also some disease which are not considered to be of inflammatory etiology such as Alzheimer's disease[5], cardiovascular disease[2] and cancer[6,7]. In contrast, eicosanoids derived from ω-3 fatty acids have anti-inflammatory effects and they can inhibit the release of AA-derived eicosanoids. Resolvins is a new family of lipid mediators which possess both potent anti-inflammatory and immune-regulatory properties[8].

1.3 Cell culture

Culturing cells is a process where cells are isolated from plants or animals and cultivated in a

medium with appropriate nutrients and conditions. This technique became common in the 19th century[9] and nowadays, has enabled us to investigate the effect of different drugs or compounds on cells, to synthesize valuable biological substances and to study the biochemistry of cells. Hence, they are becoming suitable substitute methods for animal experiments. Compared to other biological samples (i.e. plasma, urine, tissue), the physical-chemical conditions (pH, temperature, O_2 and CO_2 tension) of the cell cultures are easily controlled which lead to the high consistency and reproducibility of the results. Thus, cell cultures are becoming the preferred techniques among researchers.

1.4 Analytical method

1.4.1 Instrumental techniques

Different techniques have been used for eicosanoids analysis. **Figure 1.4** and **Table 1.1** show a chronological overview of the application of different instrumental techniques for the analysis of eicosanoids in cell cultures.

Enzyme immunoassay (EIA) and radioimmunoassay (RIA) are two types of immunoassay methods. EIA carries out the quantitative analysis based on the color change caused by linking enzyme to antibody or antigen, while RIA based on the radioactivity resulting from the competition between radiolabeled and unlabeled antigens in an antigen-antibody reaction. Nowadays, EIA and RIA are easy to perform due to the availability of commercial kits. However, their main drawbacks are the involvement of dangerous radioactive substances in RIA, the overestimation of the analytical concentrations due to cross-reactivity and the detection of a single analyte per commercial kit.

Gas chromatography (GC) is a powerful separation technique. Various volatile compounds can be separated in a GC column, identified by different detectors such as flame ionization detector (FID) or mass spectrometry (MS) and quantified by using appropriate standard compounds. This technique is highly sensitive. However, its major disadvantage is that it is limited to volatile compounds which are generally synthesized by time-consuming derivatization process.

High-performance liquid chromatography (HPLC) largely extends the range of analytes. It is not only useful for volatile but also for non-volatile compounds. The traditional detectors for HPLC are UV-visible detector and diode array detector (DAD). The identification is made according the

spectrum and quantification based on the relationship between the absorption of light by the molecules of interest (e.g. absorbance) and the properties of these molecules (e.g. concentration). The main disadvantage of HPLC coupled to UV or DAD detectors is that the exact identification of compounds from the same category or family exhibiting same spectrum becomes expensive due to the need of several standards to achieve a reliable and positive identification.

Table 1.1 Overview of the application of different instrumental techniques for the analysis of eicosanoids in cell cultures

Year	RIA	EIA	GC	HPLC	HPLC-MS	Ref.
1980			1			10
1981						
1982				1		11
1983	1					12
1984				1		13
1985	1					14
1986	3		1	2		15-20
1987	3			5		21-26
1988	5			4		27-35
1989	3	1				36-39
1990	2	1				40-42
1991	1		1	3		43-46
1992	6	1	2	6		47-56
1993	2	1	2	7		57-67
1994	6	1	1	7		68-78
1995	2			2		79-82
1996	1	1	1	4		83-88
1997	4	4		5		89-98
1998	1	2		4	2	99-105
1999	2	1	1	4		106-111
2000	2	1	1	5		112-119
2001		1		1	2	120-123
2002	1	1	2	3	3	124-132
2003	1	3		1	1	133-138
2004		2		1	1	139-142
2005		4	2	3	3	143-154
2006				1	1	155,156
2007		1		4	1	157-162
2008		1	1	2	5	163-171
2009				1	1	172,173
2010		4			3	174-180
2011		2	1	1	6	181-190
2012		3	1	3	4	191-200
2013	1	4		1	7	201-212
2014					1	213

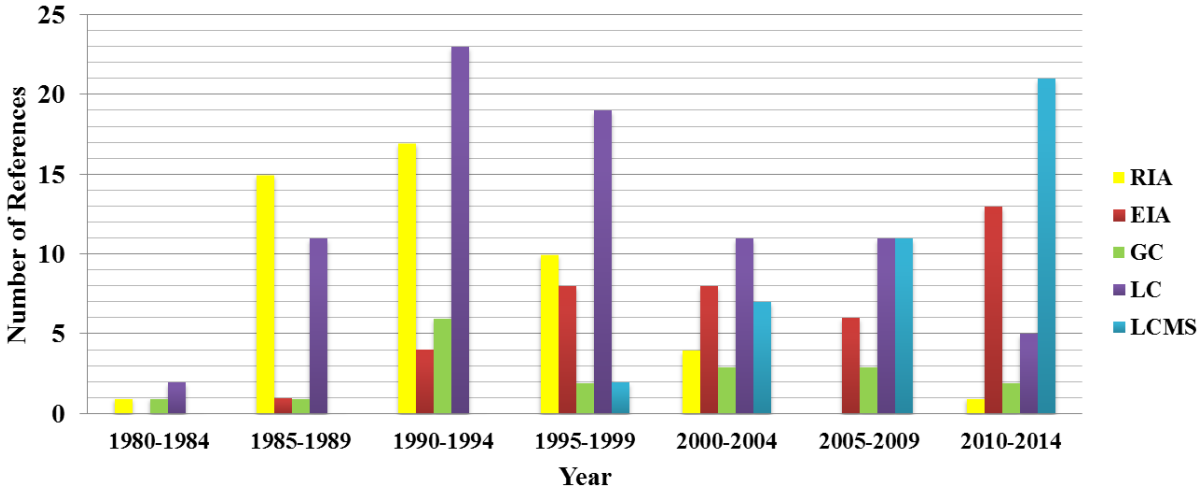


Figure 1.4 Overview of the application of different instrumental techniques for the analysis of eicosanoids in cell cultures

The introduction of mass spectrometers coupled to HPLC has emerged as a potent alternative to overcome the limitations imposed by the use of spectrophotometric detectors (e.g. UV or DAD). The key to combine a MS to HPLC is the ability to remove the solvent and transfer analytes into vacuum as ionic species. Since the first HPLC/MS interface was reported[214], many investigators attempted to improve the interface and then HPLC-MS got more and more extensive application.

Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are the two most widely used ion source in HPLC-MS. In ESI, the eluent solution passes through the electrospray capillary where a high voltage is applied to the tip of the capillary and as a consequence, the sample emerging from the tip is dispersed into an aerosol of highly charged droplets. The charged droplets are diminished in size by solvent evaporation. Eventually charged samples ions, free from solvent, are released from the droplets, some of which pass through a sampling cone into vacuum region and from there into the mass analyzer. It is worth mentioning that, in this step, it is easy to produce multiply charged ions. Thus, sample solutions for ESI are usually buffered or have added acids to enhance and control of the formation ions. In APCI, analyte solutions are first desolvated in a heated quartz tube, and then ions are produced by interacting with a corona discharge creating ions. Usually, the ESI is well-suited to the analysis of the polar molecules while APCI is a better choice for non-polar compounds.

A common drawback of these ion sources is that both of them involve a soft ionization process

where few fragments can be produced. In this case, it is easy to determine the molecular weight, but it cannot provide enough information about the structure of the compound. To solve this problem, a tandem mass spectrometry can be carried out. In this technique, ions produced in the ion source can be separated on the basis of their m/z ratios and the selected ion can be further fragmented by adding extra collisional energy. The resulting fragmentation can be useful for the identification analysis. In this thesis, a ESI-HPLC-tandem mass spectrometry method is applied.

1.4.2 Extraction methods

The main methods used for the extraction of eicosanoids from cell culture media and further analysis by HPLC-MS is liquid-liquid extraction (LLE) and solid phase extraction (SPE). A chronological overview of the application of these two particular methods for the analysis of eicosanoids in cell cultures by HPLC-MS is shown in **Figure 1.5** and **Table 1.2**.

Table 1.2 Application of different extraction methods prior to HPLC-MS for the analysis of eicosanoids

Year	LLE		SPE	
	Number of Ref.	Ref.	Number of Ref.	Ref.
1998	1	104	1	105
1999				
2000				
2001	1	123	1	122
2002	1	131	2	130,132
2003			1	138
2004			1	142
2005	1	154	2	152,153
2006			1	156
2007			1	162
2008	4	167-170	1	171
2009	1	173		
2010	1	179	2	178,180
2011	1	187	5	185,186,188-190
2012	2	197,198	2	199,200
2013	2	208,209	5	203,207,210-212
2014	1	213		

Figure 1.5 shows that SPE is preferred over LLE regardless of the simplicity and short extraction time of the latter. The main drawback of LLE is its low recovery and poor detection limit. In the present thesis, LLE was used initially to determine 6-keto-$PGF_{1\alpha}$ and Δ17-6-keto-$PGF_{1\alpha}$ by HPLC-MS. The latter compound (Δ17-6-keto-$PGF_{1\alpha}$) was not detected chromatographically as shown in **Figure 1.6**.

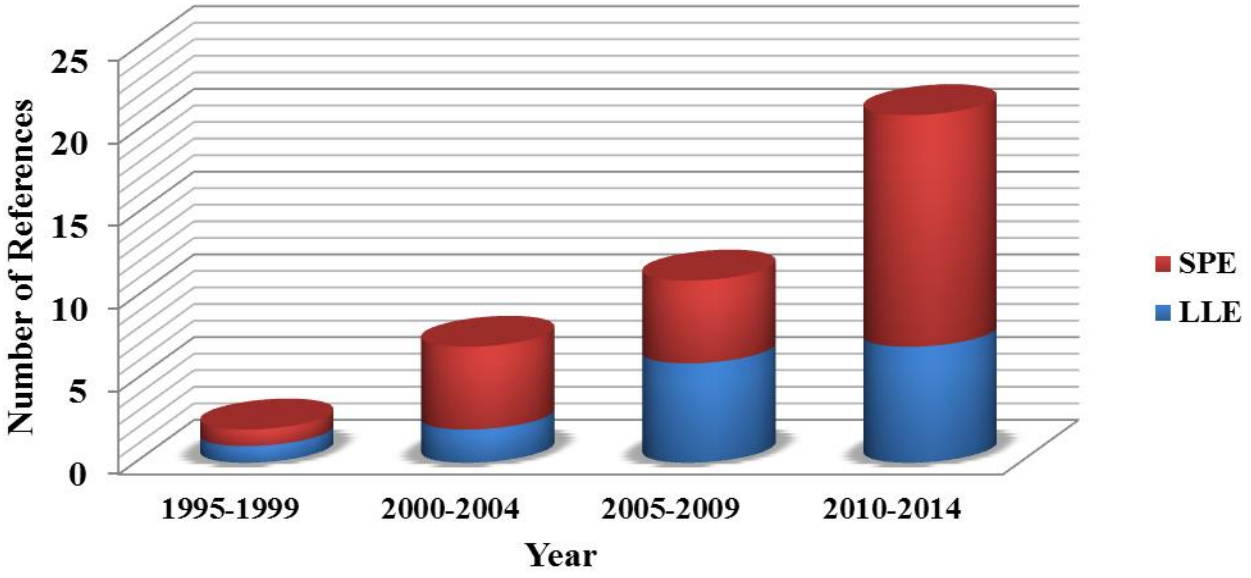


Figure 1.5 Application of different extraction methods in HPLC-MS analysis of eicosanoids.

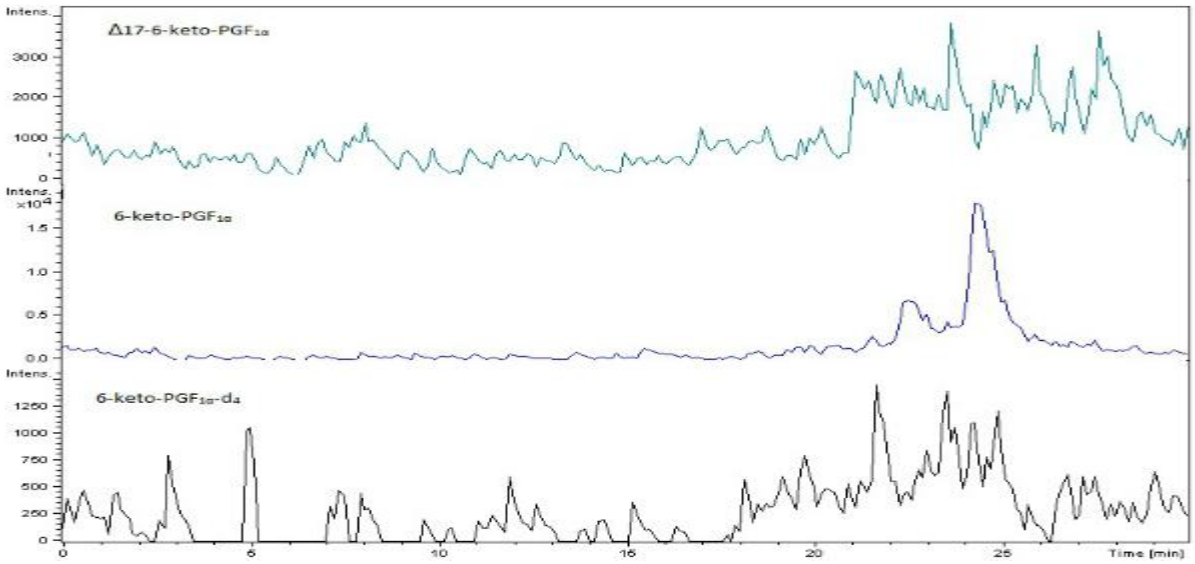


Figure 1.6 EICs of 6-keto-$PGF_{1\alpha}$ (369→351+315+289+323+307+205+220+149), Δ17-6-keto-$PGF_{1\alpha}$ (367→349+331+289+269+323+313+305+298+207+185+163), and 6-keto-$PGF_{1\alpha}$-d_4 (373→355+337+319+275+167) using liquid-liquid extraction combined with HPLC-MS/MS analysis

SPE can be seen as one type of liquid chromatography. Compounds are separated in SPE cartridges according to their chemical and physical properties by using different combination of solvents. A procedure of SPE usually contains conditioning of the cartridges, loading sample, washing and collecting the fractions of interest (**Figure 1.7**). Nowadays, it is commonly used for isolating compounds or concentrating and purifying compounds in analytical laboratories.

The majority of reported SPE methods are basically focused on limited number of eicosanoids. In this thesis, a new SPE method was developed to simultaneously extract LTB_4, PGE_3, PGE_2, 6-keto-$PGF_{1\alpha}$, Δ17-6-keto-$PGF_{1\alpha}$, RvD_1 and RvD_2 from human and fish cell culture medium and to determine quantitatively their concentrations in real samples by HPLC-MS/MS.

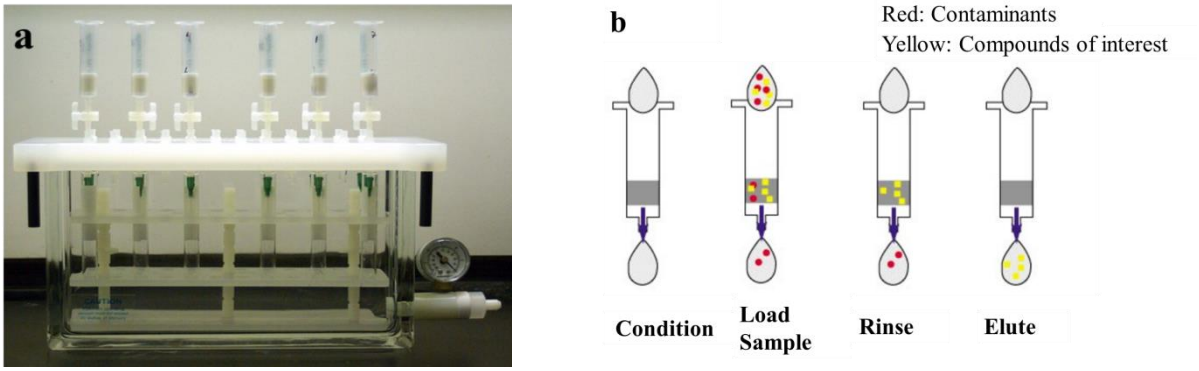


Figure 1.7 Device of SPE (**a**) and scheme of a SPE process (**b**)

1.5 Experimental design

1.5.1 Full factorial design

A 2-level full factorial design is the most popular design for the estimation of the significance of effects using one or more experimental responses. In this design, the factors are set at two levels (designated as +1 and -1) and all the possible combination of the factors are performed. An example for a 2-level three factors (2^3) design is shown in **Figure 1.8**, where positive (+1) means a high magnitude and negative (-1) represents a low magnitude of a particular factor. The number of experiments (N) of a 2-level full factorial design is calculated by **Eq. 1.1**.

$$N=2^k \quad (1.1)$$

where k is the number of studied factors.

After performing the experiments described in **Figure 1.8**, the effect of each variable can be calculated as **Eq. 1.2**.

$$R = \overline{R_{+1}} - \overline{R_{-1}} \quad (1.2)$$

Where $\overline{R_{+1}}$ is the average response when the variable is kept at high level and $\overline{R_{-1}}$ is the average response when the variable is kept at low level. A positive or negative relationship between a response and a variable is denoted by the final symbol of **R** (+ or - correlation).

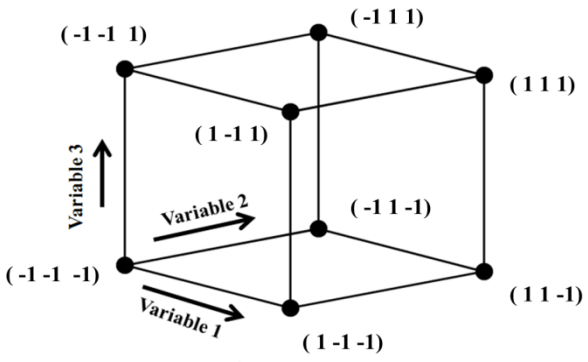


Figure 1.8 2^3- full factorial design

Once the magnitude of all the effects are obtained, a model describing the behaviour of R as a function of the variables can be built as shown in **Eq. 1.3**.

$$R = b_0 + b_1[x_1] + b_2[x_2] + b_{12}[x_1] \times [x_2] \quad (1.3)$$

The term b_0 is the intercept, b_1 and b_2 are the coefficents for variable x_1 and x_2 respectively, b_{12} the coefficient for the interaction between x_1 and x_2.

Although **Eq.1.3** can estimate the influence of each variable and the interaction between different variables, its main drawback is that curvature effects cannot be estimated.

1.5.2 Doehlert design

A full factorial design is a powerful tool for screening significant effects, but it cannot be used to determine an optimal value or region. To determine an optimum, it is necessary to implement more complex designs where the appropriateness of linear, interaction and quadratic terms from a mathematical model is evaluated by mathematical and statistical tools.

A Doehlert design is a response surface design that allows to determine an optimum region by performing a minimum number of experiments. The number of experiments (N) when 2 factors (k=2)

are evaluated can be calculated as **Eq. 1.4** and their spatial distribution is shown in **Figure 1.9**.

$$N = 2^k + k + 1 \tag{1.4}$$

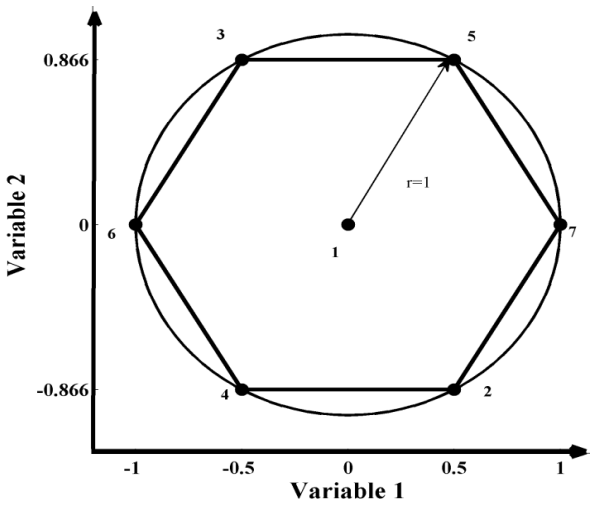


Figure 1.9 Distribution of experimental points for a 2-level Doehlert design

Figure 1.9 shows six experimental points are allocated in a regular hexagon with one point in the center. These points are also equally distributed in a circle of radius one. This uniformity makes the design generate information equally in all directions. Besides, a Doehlert design can be easily extended to study other experimental arrangements by using previous experiments[215]. For example, an initial experimental region (denoted in full line**)** can be extended to explore neighbouring domains as shown in **Figure 1.10**.

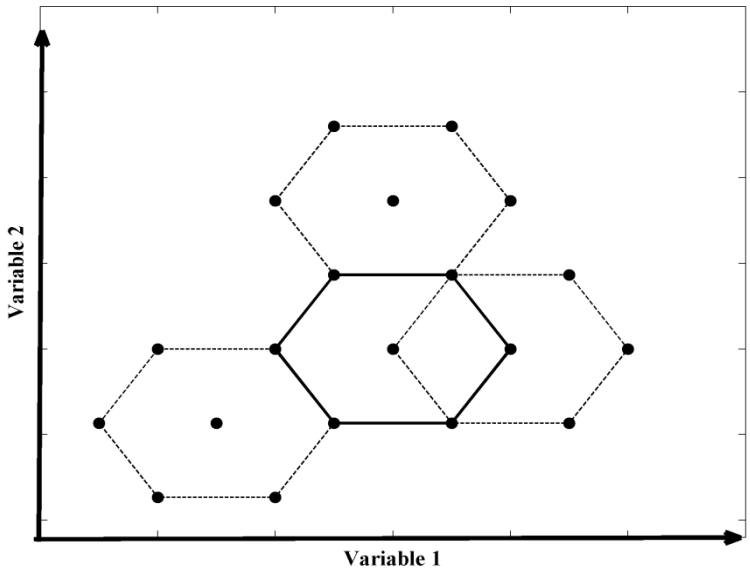


Figure 1.10 Extension of a Doehlert experimental matrix from the initial 2-level matrix

The final model of a Doehlert design for 2 variables is given by **Eq. 1.5**, which describes the main effects, the interaction and the second order curvature effects of the variables.

$$R = b_0 + b_1[x_1] + b_2[x_2] + b_{12}[x_1] \times [x_2] + b_{11}[x_1]^2 + b_{22}[x_2]^2 \quad (1.5)$$

Where b_0 is the intercept, b_1 and b_2 are the coefficents for variable x_1 and x_2 respectively, b_{12} the coefficient for the interaction of the two variables and b_{11} and b_{22} are the second order curvature coefficients.

1.6 Objectives of the thesis

1) To develop a new SPE-HPLC-MS/MS method to analyze prostaglandins, leukotrienes, prostacyclyns and resolvins in cultured fish and human cells in EBM-2 medium and cL-15 medium.

2) To determine the optimal internal standard concentrations by means of a two-factor Doehlert uniform shell design in two different cell culture medium (EBM-2 and cL-15).

3) To validate the new SPE-HPLC-MS/MS method.

4) To apply the method to salmon head kidney, salmon liver and HUVEC exposed to different combinations of ω-6 and ω-3 fatty acids.

2. Method development and method validation

2.1 Experimental

2.1.1Reagents

Prostaglandin E_2 (PGE_2, 99%), deuterated prostaglandin E_2 (PGE_2-d_4, 99%), prostaglandin E_3 (PGE_3, 98%), leukotriene B_4 (LTB_4, 97%), deuterated leukotriene B_4 (LTB_4-d_4, 99%), 6-keto prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$, 98%), deuterated 6-keto prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$-d_4, 99%), Δ17-6-keto-Prostaglandin $F_{1\alpha}$ (Δ17-6-keto-$PGF_{1\alpha}$, 98%), resolvin D_1 (RvD_1, 95%), resolvin D_2 (RvD_2, 95%) and deuterated resolvin D_2 (RvD_2-d_5, 95%) 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). A Millipore Milli-Q system was used to produce ultra-pure water 18 MΩ (Millipore, Milford, USA).

Complete cL-15 medium was prepared by mixing Leibowitch cL-15 medium with 1% glutamax, 1% antibiotika and 10% fetal bovine serum (FBS). Complete EBM-2 medium contained EBM^{TM}-2 basal medium supplemented with 0.1 % Heparin, 0.1 % R^3-IGF-1, 0.1 % ascorbic acid, 0.04 % hydrocortisone, 0.4 % h-FGF-B, 0.1 % h-EGF, 0.1 % GA-1000 and 2% FBS.

2.1.2 Preparation of standard solutions for determining the concentration of internal standards

The initial concentrations of PGE_2, LTB_4, 6-keto-$PGF_{1\alpha}$, Δ17-6-keto-$PGF_{1\alpha}$, RvD_1, RvD_2, LTB_4-d_4, 6-keto-$PGF_{1\alpha}$-d_4 and RvD_2-d_5 were 100 µg/ml, the concentration of PGE_3 was 1000 µg/ml and PGE_2-d_4 was 500 µg/ml. All of them were prepared by dissolving the pure standards in ethanol.

From the initial solutions : *i)* stock solution 'A' containing 2000 ng/ml PGE_2, LTB_4, 6-keto-$PGF_{1\alpha}$; *ii)* stock solution 'B' containing 2000 ng/ml PGE_3, Δ 17-6-keto-$PGF_{1\alpha}$, RvD_1, RvD_2 and *iii)* stock solution 'C' containing 1000 ng/ml PGE_2-d_4, LTB_4-d_4, 6-keto-$PGF_{1\alpha}$-d_4, RvD_2-d_5 were prepared in two different mediums (complete cL-15 and complete EBM-2) respectively. And then, these stock solutions were diluted to a series of five equally spaced solutions in the range of 0.5-200

ng/ml containing three different levels of internal standards, according to a Doehlert design (**Figure 2.1**). Every experimental point in the Doehlert design was prepared in triplicate.

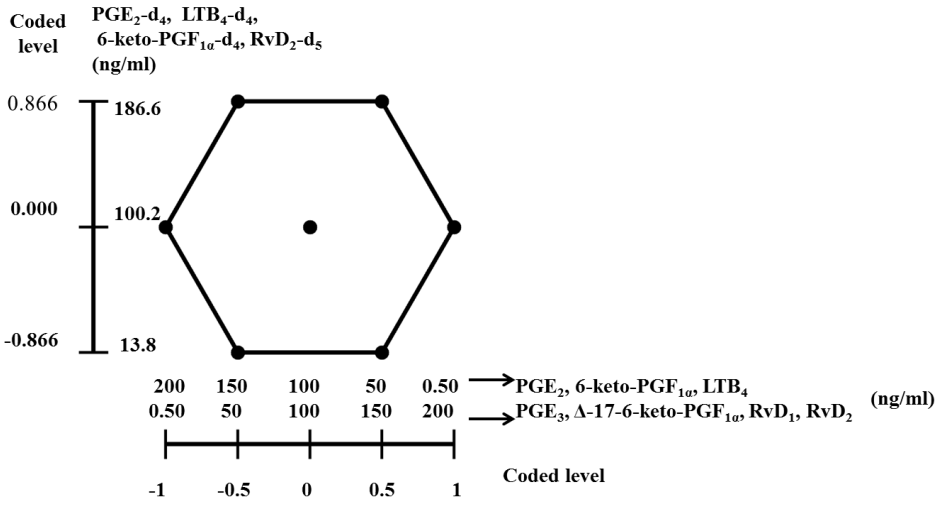


Figure 2.1 Doehlert design used for the determination the optimal concentration of internal standards in the analytical range 0.5-200 ng/ml.

2.1.3 Extraction procedure

The extraction method is a modification of a previously published protocol[162]. First, an aliquot of 100 µL of internal standard (for cL-15 medium: PGE_2-d_4 2000 ng/mL, LTB_4-d_4 250 ng/mL, 6-keto-$PGF_{1\alpha}$-d_4 500 ng/mL, RvD_2-d_5 450 ng/ml; for EBM-2 medium: PGE_2-d_4 2000 ng/mL, LTB_4-d_4 1000 ng/mL, 6-keto-$PGF_{1\alpha}$-d_4 300 ng/mL, RvD_2-d_5 300ng/ml) was added to 1 mL of sample. Then, 175 µL of ethanol and 20 µL of acetic acid were added. The mixture was vortex-mixed and applied to SPE columns (Agilent, ASPEC Bond Elute C18, 500 mg, 3 ml, USA) which had been 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 to remove peptides and salts as well as polar and nonpolar interfering substance. The analytes were eluted with 1 ml of hexane/ethyl acetate (1:2 v/v) collected into glass tubes and the solvent was evaporated under a stream of nitrogen gas. The dried sample was redissolved in 70 µL of acetonitrile, vortex-mixed 30 s, centrifuged at 3000 rpm (1620 g) for 3 min and transferred to an auto sampler vial for HPLC-MS/MS analysis.

2.1.4 HPLC-MS/MS analysis

The HPLC-MS used 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) was used and kept at 40 ℃ in the column compartment. Nitrogen was used as nebulizing and drying gas at 350 ℃. The ESI source was operated in negative ion mode and 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 5.3 from Agilent.

For the analysis of prostaglandins, leukotrienes and prostacylins, the solvent system was acetonitrile with 0.1% formic acid (v/v）and operated in isocratic mode at 0.75 ml/min. The injection volume was 20 μl and the analysis time 15 min.

Table 2.1 Fragmentation patters for various eicosanoids in cL15 and EBM-2 medium

Name of eicosanoids	Parent ion	m/z common to both media	m/z unique to cL-15	m/z unique to EBM-2
PGE_3	349	313, 269		331
PGE_2	351	333, 315, 271		
PGE_2-d_4	355	337, 319, 275		
LTB_4	335	317, 275, 151, 129, 109		289, 255, 195, 273, 177, 203
LTB_4-d_4	339	321, 277, 319, 293, 275, 197, 179, 153, 125		
6-keto-$PGF_{1\alpha}$	369	351, 315, 289, 307, 205, 220, 149	323	333
Δ17-6-keto-$PGF_{1\alpha}$	367	349, 331, 289, 269, 313, 305, 207, 185, 163	323, 298,	
6-keto-$PGF_{1\alpha}$ -d_4	373	355, 337, 319, 275, 167		
RvD_1	375	141		
RvD_2	375	141		
RvD_2-d_5	380	362, 344, 326, 282, 146, 141		

For the analysis of resolvins, the mobile phase 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). The mobile phase was delivered in gradient mode as follows: 100% of solvent A was held for 5 min and changed into 60% in the following 6 min and into 10% in the subsequent 2 min. Solvent A was kept in 10% for 2

min and then increased to 100% in 15 min. The total analysis time was 30 min, the flow rate 0.5 mL/min and the injection volume 25 μL.

Different fragmentation patterns were chosen for cL-15 and EBM-2 medium and they are shown in **Table 2.1**.

2.2 Mathematics and statistics

2.2.1 Response Factor (RF)

In chromatography analysis, a peak area is usually proportional to the concentration of its corresponding compound. However, the ratio between peak area and concentration for different compounds at the same level of concentration could vary due to different chemical behavior under the same instrumental conditions. Besides, the ratio for the same compound may also have slight variation because of the changing of instrumental conditions. Thus, in order to make a quantitation analysis, an internal standard is always introduced and the response factor (RF) for every analyte and internal standard is determined as **Eq. 2.1**.

$$\mathrm{RF} = \frac{[A]}{[IS]} \cdot \frac{S_{IS}}{S_A} \tag{2.1}$$

Where [A] and [IS] represent the concentration of analyte and internal standard respectively, while S_{IS} and S_A are the chromatographic peak area of the internal standard and analyte.

2.2.2 F-test

F-test is a statistical parametric test commonly used to compare the lack-of-fit to pure error variances of a predetermined mathematical model. This statistical test was applied to check the regression models generated from the Doehlert design and to determine the optimal concentration of internal standards. The process of an F-test can be seen in **Table 2.2**.

Firstly, experimental RF (RF_{exp}) was calculated according to **Eq. 2.1** and the averages of each triplicate were obtained. Then, a regression model like **Eq. 2.2** can be built and the calculated RF (RF_{cal}) were obtained from this model.

$$\mathrm{RF} = b_0 + b_1[A] + b_2[IS] + b_{12}[A] \times [IS] + b_{11}[A]^2 + b_{22}[IS]^2 \tag{2.2}$$

After that, the sum of residual error (V_{RE}), pure error (V_{PE}) and lack-of-fit (V_{LOF}) were calculated according to **Eq. 2.3—Eq. 2.5.** And the F-value can be calculated by the ratio between V_{LOF} and $V_{PE.}$

$$V_{RE} = \frac{\sum(RF_{exp} - RF_{cal})^2}{N-P} \tag{2.3}$$

$$V_{PE} = \frac{\sum(RF_{exp} - \overline{RF})^2}{N-K} \tag{2.4}$$

$$V_{LOF} = \frac{\sum(RF_{cal} - \overline{RF})^2}{K-P} \tag{2.5}$$

$$F_{cal} = \frac{V_{LOF}}{V_{PE}} \tag{2.6}$$

N, P, K are the number of total experiments, number of regression coefficients and number of experimental points respectively.

If F_{cal} is lower than the theoretical F-value (calculated by using Excel 2010), it means the model is fitted. In some instances, it is possible to remove the non-significant regression coefficients from **Eq. 2.2** to increase the degree of freedom and obtain a simplest model (aka reduced models). In this thesis, all the theoretical F-values were calculated at the 95 % confidence level of F-distribution.

Table 2.2 Schematic explanation of the F-test applied to determine the validity of the various regression models of the response factor (RF). The best models are subsequently used to determine optimal concentrations of internal standards

[A]	[IS]	RF_{exp}	RF_{cal}	$\overline{RF}$	Residual error (V_{RE})	Pure error (V_{PE})	Lack of fit (V_{LOF})
$[A]_{11}$	$[IS]_{11}$	RF_{exp11}	RF_{cal11}	$\overline{RF_1}$	$(RF_{exp11} - RF_{cal11})^2$	$(RF_{exp11} - \overline{RF_1})^2$	$(RF_{cal11} - \overline{RF_1})^2$
$[A]_{12}$	$[IS]_{12}$	RF_{exp12}	RF_{cal12}	$\overline{RF_1}$	$(RF_{exp12} - RF_{cal12})^2$	$(RF_{exp12} - \overline{RF_1})^2$	$(RF_{cal12} - \overline{RF_1})^2$
$[A]_{13}$	$[IS]_{13}$	RF_{exp13}	RF_{cal13}	$\overline{RF_1}$	$(RF_{exp13} - RF_{cal13})^2$	$(RF_{exp13} - \overline{RF_1})^2$	$(RF_{cal13} - \overline{RF_1})^2$
.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.
$[A]_{71}$	$[IS]_{71}$	RF_{exp71}	RF_{cal71}	$\overline{RF_7}$	$(RF_{exp71} - RF_{cal71})^2$	$(RF_{exp71} - \overline{RF_7})^2$	$(RF_{cal71} - \overline{RF_7})^2$
$[A]_{72}$	$[IS]_{72}$	RF_{exp72}	RF_{cal72}	$\overline{RF_7}$	$(RF_{exp72} - RF_{cal72})^2$	$(RF_{exp72} - \overline{RF_7})^2$	$(RF_{cal72} - \overline{RF_7})^2$
$[A]_{73}$	$[IS]_{73}$	RF_{exp73}	RF_{cal73}	$\overline{RF_7}$	$(RF_{exp73} - RF_{cal73})^2$	$(RF_{exp73} - \overline{RF_7})^2$	$(RF_{cal73} - \overline{RF_7})^2$
DF.		N	P	K	N-P	N-K	K-P

2.2.3 Statistical Software

All of the basic calculation and F-test were carried out in Excel (Microsoft Office Excel 2010).

2.3 Results and discussion

2.3.1Determination of the concentrations of internal standards

The seven standards in Doehlert design, prepared in triplicates, were injected randomly into the chromatography system. Extracted ion chromatograms (EICs) were used for the quantitative analysis.

2.3.1.1 Optimal concentrations of internal standards in EBM-2 medium

The EICs and corresponding mass spectra of different analytes and internal standards in EBM-2 medium are shown in **Figure 2.2-2.5**.

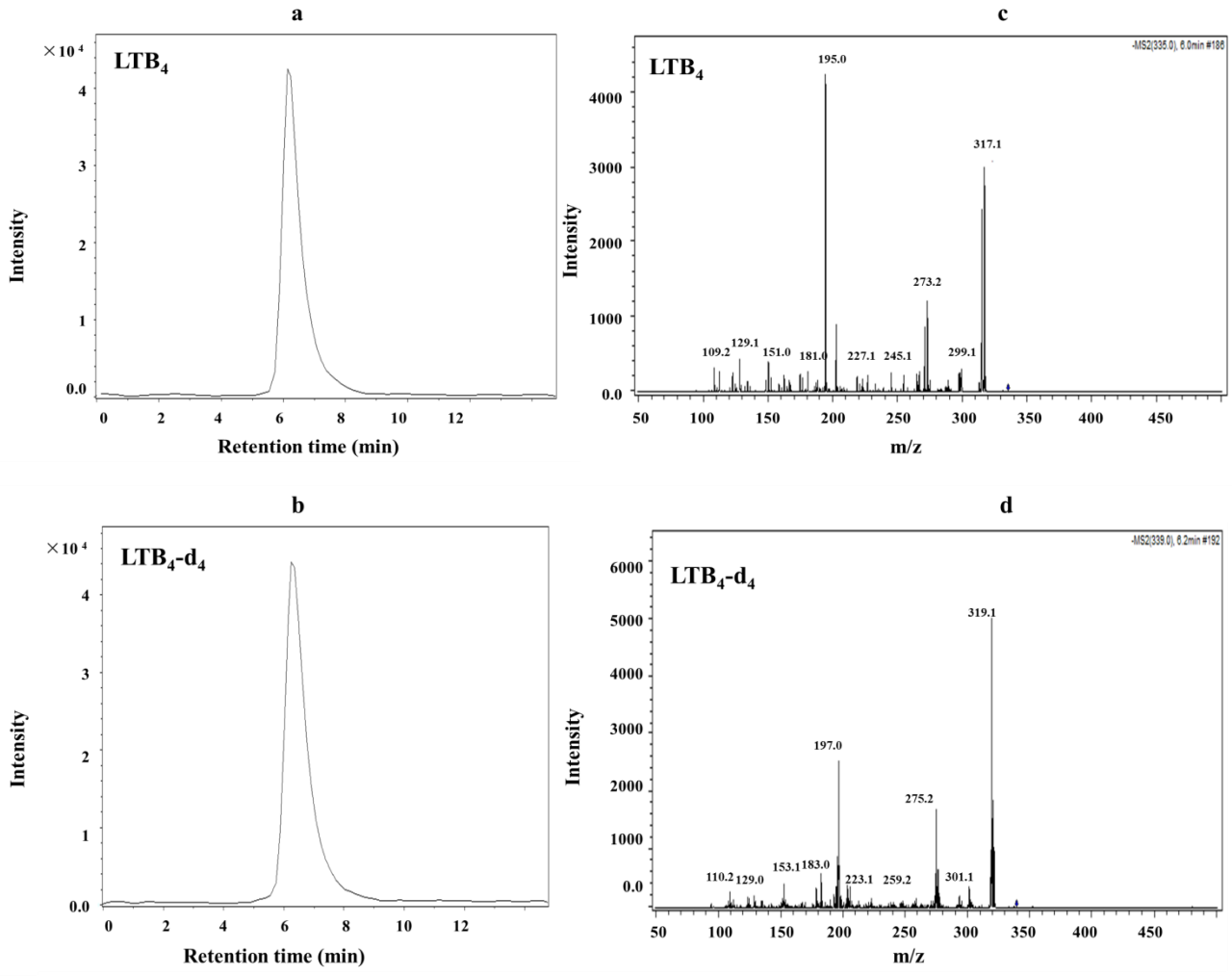


Figure 2.2 EICs (**a-b**) and mass spectra(**c-d**) of leukotrienes in EBM-2 medium

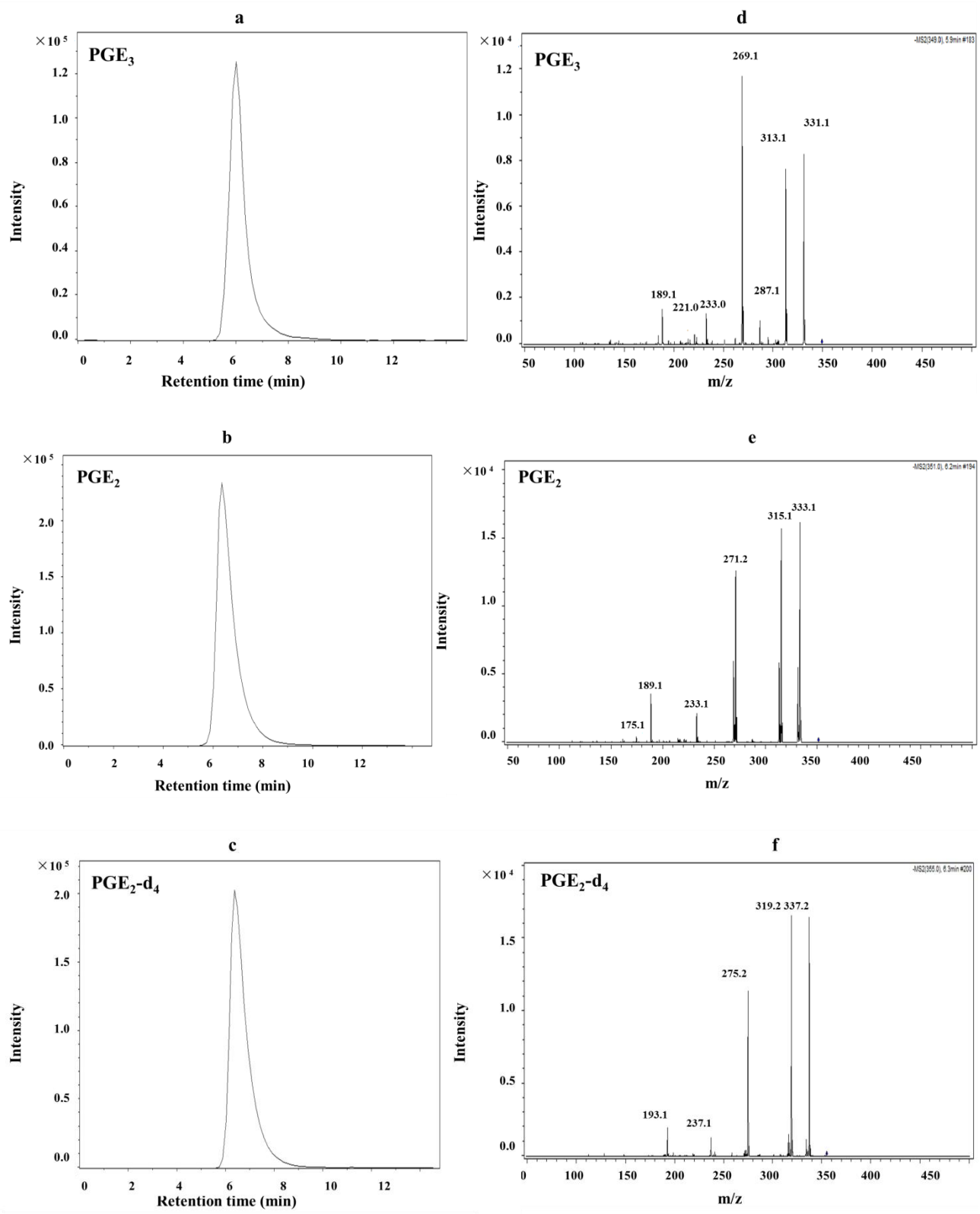


Figure 2.3 EICs (**a-c**) and mass spectra (**d-f**) of prostaglandins in EBM-2 medium

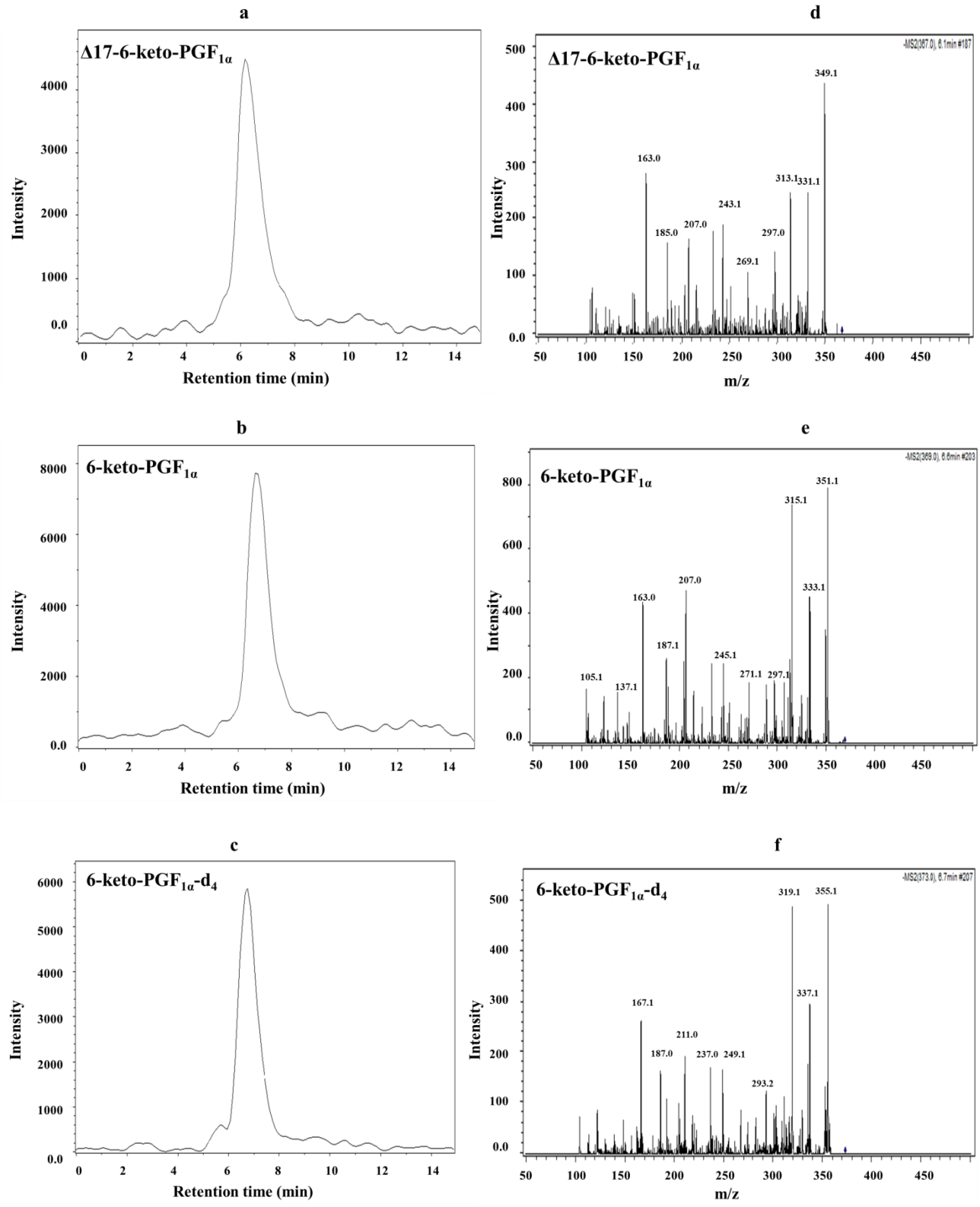


Figure 2.4 EICs (**a-c**) and mass spectra (**d-f**) of prostacylins in EBM-2 medium

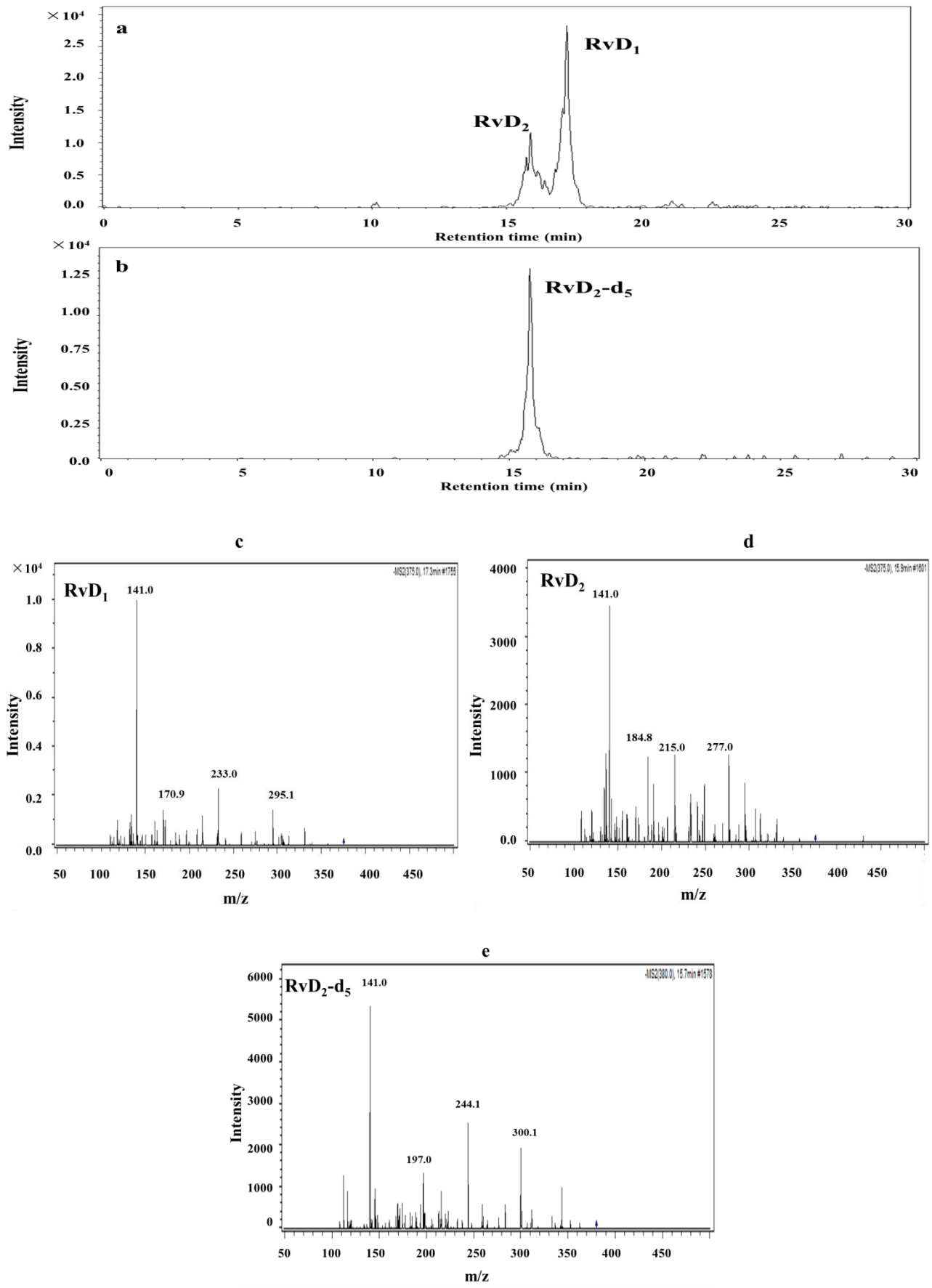


Figure 2.5 EICs (**a-b**) and mass spectra (**c-e**) of resolvins in EBM-2 medium

The regression models for different RFs as a function of the concentration of analytes and internal standards were built in Excel 2010 and F-test was used to check the fitness of the models. After reduction, the final models are listed in **Eq. 2.7—Eq. 2.13** and the corresponding response surface are shown in **Figure 2.6-2.9. Table 2.3** summarizes the results of F-test of the final models.

Eq. 2.2 is valid for describing RF as a function of the concentration of PGE_2 and PGE_2-d_4, and a six-parameter model is built (**Eq. 2.7**). It is clear from **Figure 2.6a** that between 100-140 ng/ml of PGE_2-d_4, the RF remains constant in the analytical range 50-200 ng/ml of PGE_2. However, a larger variation occurs below the above mentioned analytical concentration range.

$$\mathrm{RF} = -3.76 \times 10^{-1} + 1.21 \times 10^{-2}[PGE_2] + 8.21 \times 10^{-3}[PGE_2 - d_4] + 1.72 \times 10^{-5}[PGE_2] \times [PGE_2 - d_4] - 6.19 \times 10^{-5}[PGE_2]^2 - 2.98 \times 10^{-5}[PGE_2 - d_4]^2 \quad (2.7)$$

The calculated F-values for the various constructed RF regression models as a function of PGE_3 and PGE_2-d_4 are higher than the theoretical F-values (95% confidence), thus, the modeling of PGE_3 was carried out by using the inverse of RF (1/RF). The fitted model (**Eq. 2.8**) is shown in **Figure 2.6b.** The RF remains constant in the whole studied PGE_3 range when the concentration of PGE_2-d_4 is in the region of 0.5-50 ng/ml and 180-200 ng/ml. The relative standard deviation of RF in region of 0.5-50 ng/ml (35%) is higher than that in 180-200 ng/ml (11%).

$$\frac{1}{\mathrm{RF}} = 4.14 - 1.12 \times 10^{-2}[PGE_3] - 3.64 \times 10^{-2}[PGE_2 - d_4] + 5.83 \times 10^{-5}[PGE_3] \times [PGE_2 - d_4] + 8.96 \times 10^{-5}[PGE_2 - d_4]^2 \quad (2.8)$$

The concentration of 182 ng/ml of PGE_2-d_4 is selected as the optimal concentration to analyze quantitatively PGE_2 and PGE_3 in EBM-2 medium, based on the experimental evidence summarized in **Figure 2.6**.

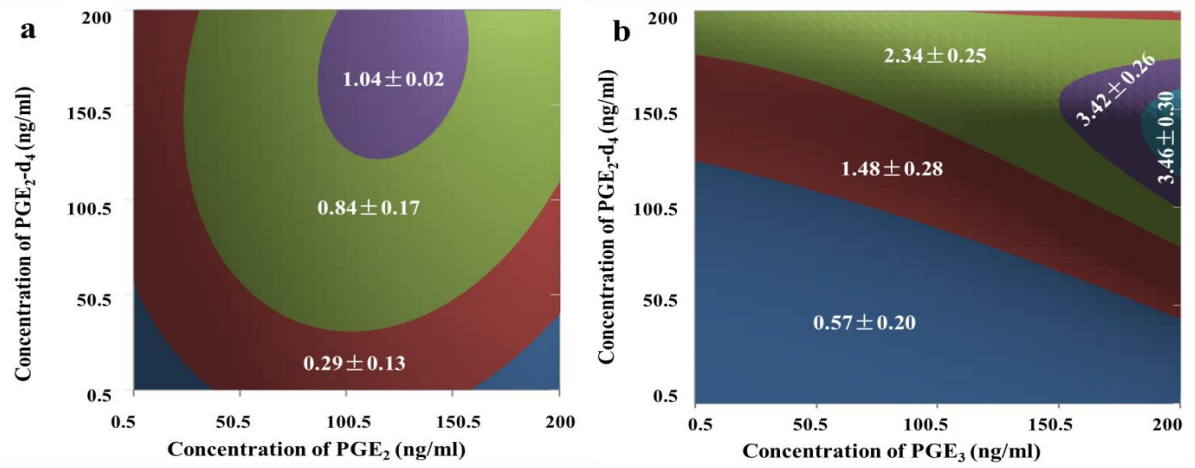


Figure 2.6 Behavior of RFs as a function of **(a)** PGE_2 and PGE_2-d_4; **(b)** PGE_3 and PGE_2-d_4 in EBM-2 medium, values in the figures represent the mean ±standard deviation of RFs.

A four-parameter polynomial function was built between RF and concentrations of LTB_4 and LTB_4-d_4 (**Eq. 2.9**) and it exhibited a non-significant lack-of fit to pure error variance ratio of 2.343 (**Table 2.3**). The graph (**Figure 2.7**) displays three major regions, in which the major variation of RF is along LTB_4–d_4 axis. RF keeps constant when LTB_4-d_4 is between 75-125 ng/ml. Thus, 91 ng/ml is chosen as the optimal concentration of LTB_4-d_4 for the analysis of LTB_4 in EBM-2 medium.

$$\mathrm{RF} = -4.02 \times 10^{-1} + 8.27 \times 10^{-3}[LTB_4] + 1.90 \times 10^{-2}[LTB_4 - d_4] - 9.70 \times 10^{-5}[LTB_4] \times [LTB_4 - d_4] \quad (2.9)$$

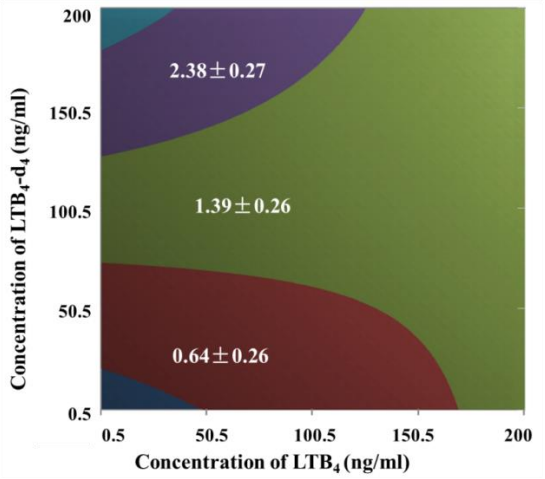


Figure 2.7 Behavior of RF as a function of LTB_4 and LTB_4-d_4 in EBM-2 medium, values in the figures represent the mean ± standard deviation of RFs

The modeling of 6-keto-$PGF_{1\alpha}$ was also carried out by using 1/RF. The model (**Eq. 2.10**) was only a function of the concentration of 6-keto-$PGF_{1\alpha}$ and independent of the concentration of internal standard (**Eq. 2.10**). There are three different RF values in three different 6-keto-$PGF_{1\alpha}$ concentration regions, namely 0.5-45 ng/ml (0.65±0.01), 45-148 ng/ml (0.69±0.02) and 148-200 ng/ml (0.74± 0.01) regardless of the concentration of the internal standard.

$$\frac{1}{RF} = 1.56 - 1.19 \times 10^{-3}[6 - keto - PGF_{1\alpha}] \quad (2.10)$$

For Δ17-6-keto-$PGF_{1\alpha}$, RF was a linear function of the concentration of Δ17-6-keto-$PGF_{1\alpha}$ and 6-keto-$PGF_{1\alpha}$-d_4 (**Eq. 2.11**). There are two main concentration regions (**Figure 2.8)** where RF remains constant between 0.5-50 ng/ml (green region in **Figure 2.8b**) and 100-150 ng/ml (purple region in **Figure 2.8b**) of 6-keto-$PGF_{1\alpha}$-d_4 in the analytical range of 0.5-200 ng/ml of Δ17-6-keto-$PGF_{1\alpha}$. In general, it is advisable to use low concentrations of internal standard due to the high cost of the eicosanoid standards used in the present investigation (for example, 1 mg of Δ17-6-keto-$PGF_{1\alpha}$ costs 1200 Euros). Based on this particular consideration, a concentration of 27 ng/ml of internal standard was selected as the optimal level for the determination of 6-keto-$PGF_{1\alpha}$ and Δ17-6-keto-$PGF_{1\alpha}$ in EBM-2 medium.

$$RF = 1.33 - 1.36 \times 10^{-3}[\Delta 17 - 6 - keto - PGF_{1\alpha}] + 4.09 \times 10^{-3}[6 - keto - PGF_{1\alpha} - d_4] \quad (2.11)$$

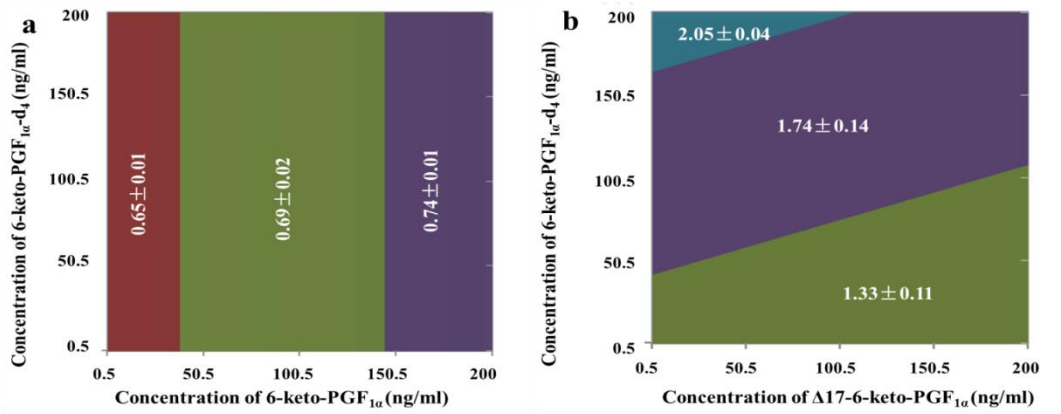


Figure 2.8 Behavior of RF as a function of **(a)** 6-keto-$PGF_{1\alpha}$ and 6-keto-$PGF_{1\alpha}$-d_4; **(b)** Δ17-6-keto-$PGF_{1\alpha}$ and 6-keto-$PGF_{1\alpha}$-d_4 in EBM-2 medium, values in the figures represent the mean ± standard deviation of RFs

For RvD_1, the RF was only affected by the concentration of RvD_2-d_5 (**Eq. 2.12**) and was independent of the concentration of RvD_1 in the range 0.5-200 ng/ml as demonstrated in **Figure 2.9a and Eq. 2.12**.

$$RF = 6.59 \times 10^{-1} - 8.45 \times 10^{-4}[RvD_2 - d_5] \quad (2.12)$$

For RvD_2, a three-parameter function was built (**Eq. 2.13**). The graph displayed in **Figure 2.9b** shows that RF is constant between 50-200 ng/ml of RvD_2 when RvD_2-d_5 is varied between 0.5-50 ng/ml. This particular region is regarded as optimal for the analysis of real samples. Based on the previous results (**Figure 2.9 and Eq. 2.12 -2.13**), 27 ng/ml of RvD_2-d_5 is considered as the optimal level for the determination of RvD_1 and RvD_2 in EBM-2 medium.

$$RF = 5.01 \times 10^{-1} + 1.70 \times 10^{-3}[RvD_2] + 1.09 \times 10^{-3}[RvD_2 - d_5] \quad (2.13)$$

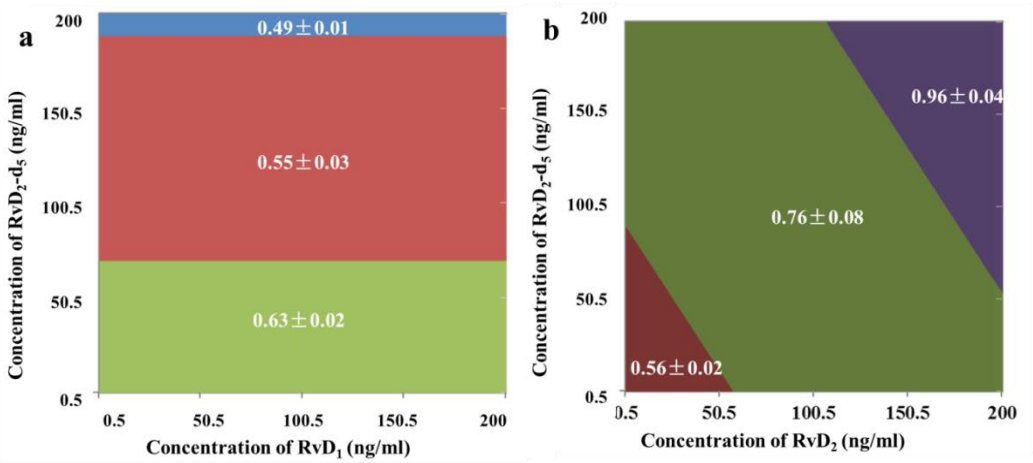


Figure 2.9 Behavior of RF as a function of **(a)** RvD_1 and RvD_2-d_5; **(b)** RvD_2 and RvD_2-d_5 in EBM-2 medium, values in the figures represent the mean ± standard deviation of RFs

Table 2.3 Statistical validation results of the RF models for selecting optimal levels of internal standards associated to the analysis of different eicosanoids generated by human cells in EBM-2 medium

	PGE_3	PGE_2	LTB_4	6-keto-$PGF_{1\alpha}$	Δ-17-6-keto-$PGF_{1\alpha}$	RvD_1	RvD_2
Residual variance	1.566 (14)	0.233(13)	28.190(17)	0.321(16)	2.880(15)	0.110(11)	0.316(10)
Pure error variance	0.986(12)	0.213(12)	18.767(14)	0.204(12)	2.097(12)	0.051(7)	0.192(7)
Lack-of-fit variance	0.580(2)	0.020(1)	9.423(3)	0.117(4)	0.784(3)	0.059(4)	0.125(3)
$F_{estimated}$	3.528	1.109	2.343	1.720	1.495	2.017	1.518
$F_{theoretical}$	3.885	4.747	3.344	3.259	3.490	4.120	4.347

2.3.1.2 Optimal concentrations of internal standards in cL-15 medium

The procedure to select the optimal levels of internal standards in cL-15 medium was similar to the procedure described in the previous section. The EICs and corresponding mass spectra of different eicosanoids are shown in **Figure 2.10-2.13**. Some of the characteristic peaks for LTB_4 (specifically m/z 192, 255, 289 in **Figure 2.10c**) were observed in cL-15 blank medium (**Figure 2.14**) and consequently they were excluded for quantification purposes.

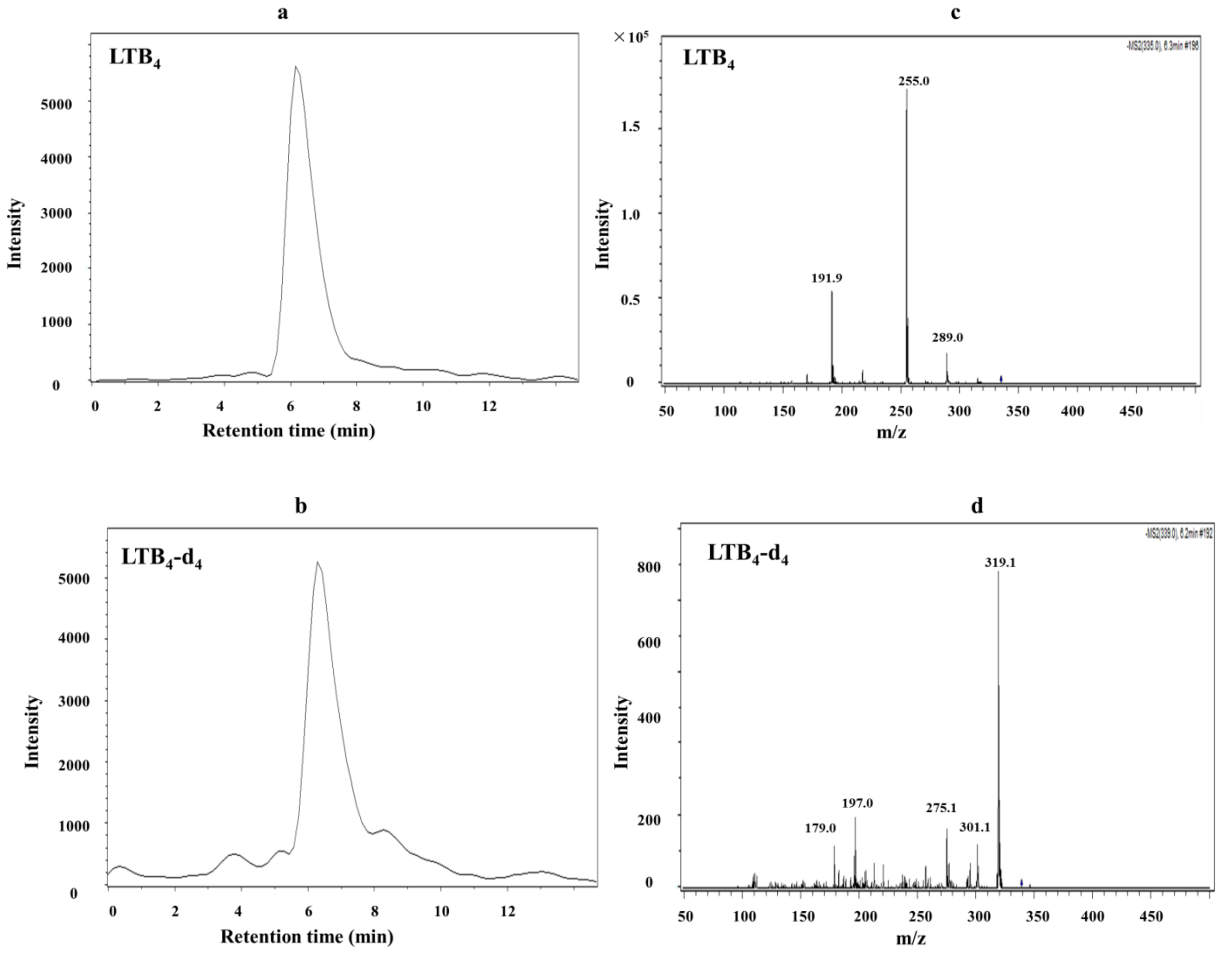


Figure 2.10 EICs (**a-b**) and mass spectra (**c-d**) of leukotrienes in cL-15 medium

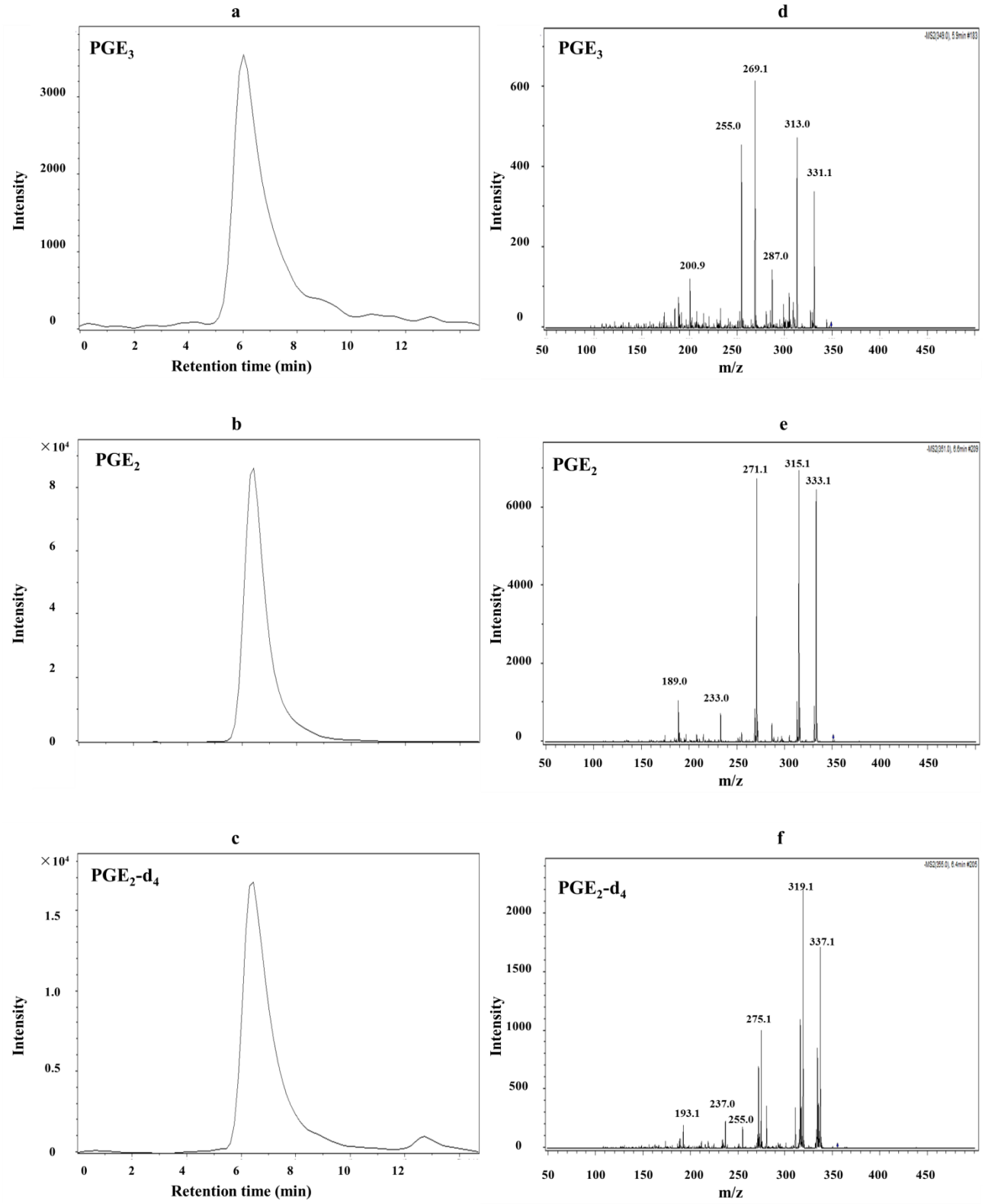


Figure 2.11 EICs (**a-c**) and mass spectra (**d-f**) of prostaglandins in cL-15 medium

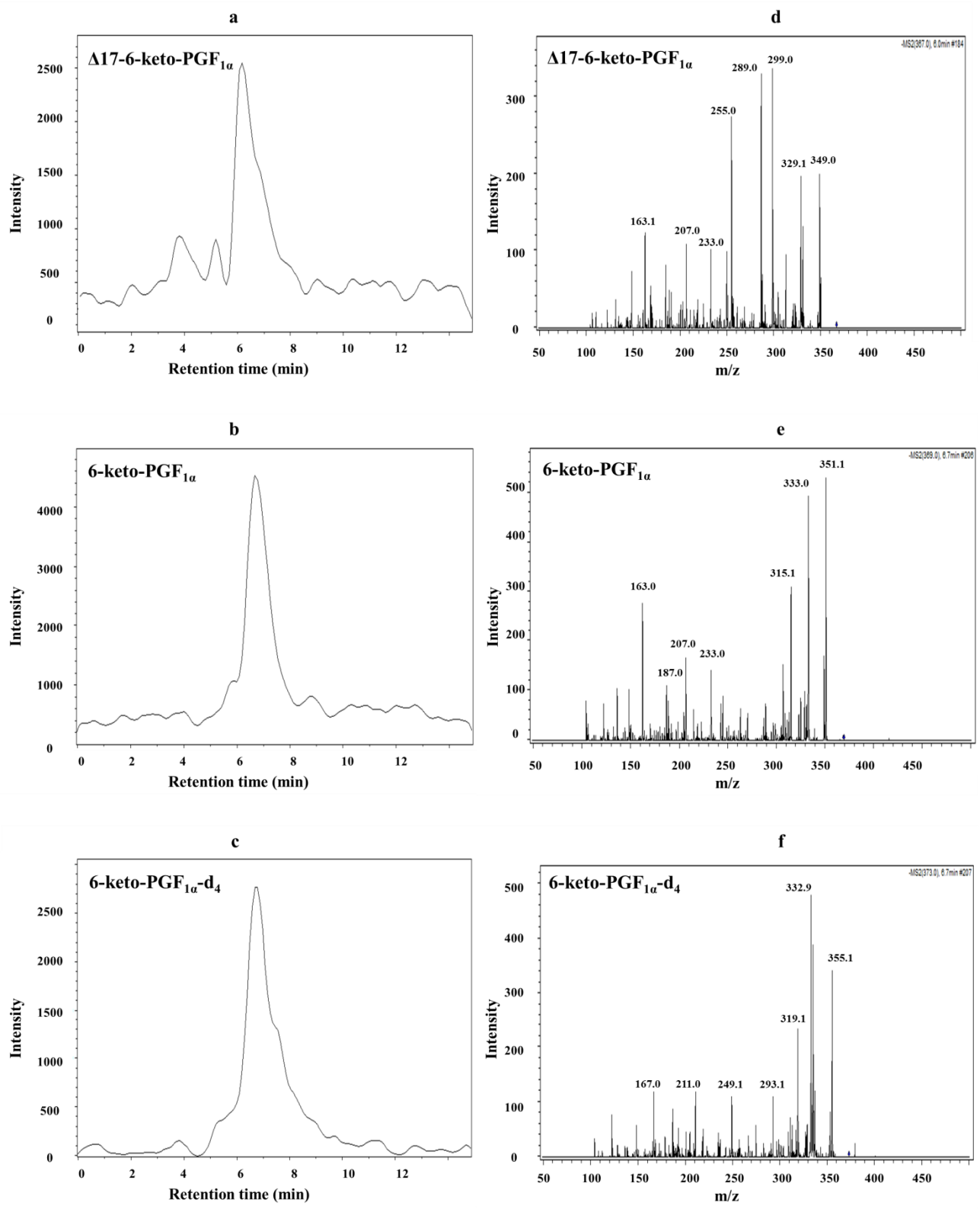


Figure 2.12 EICs (**a-c**) and mass spectra (**d-f**) of prostacylins in cL-15 medium

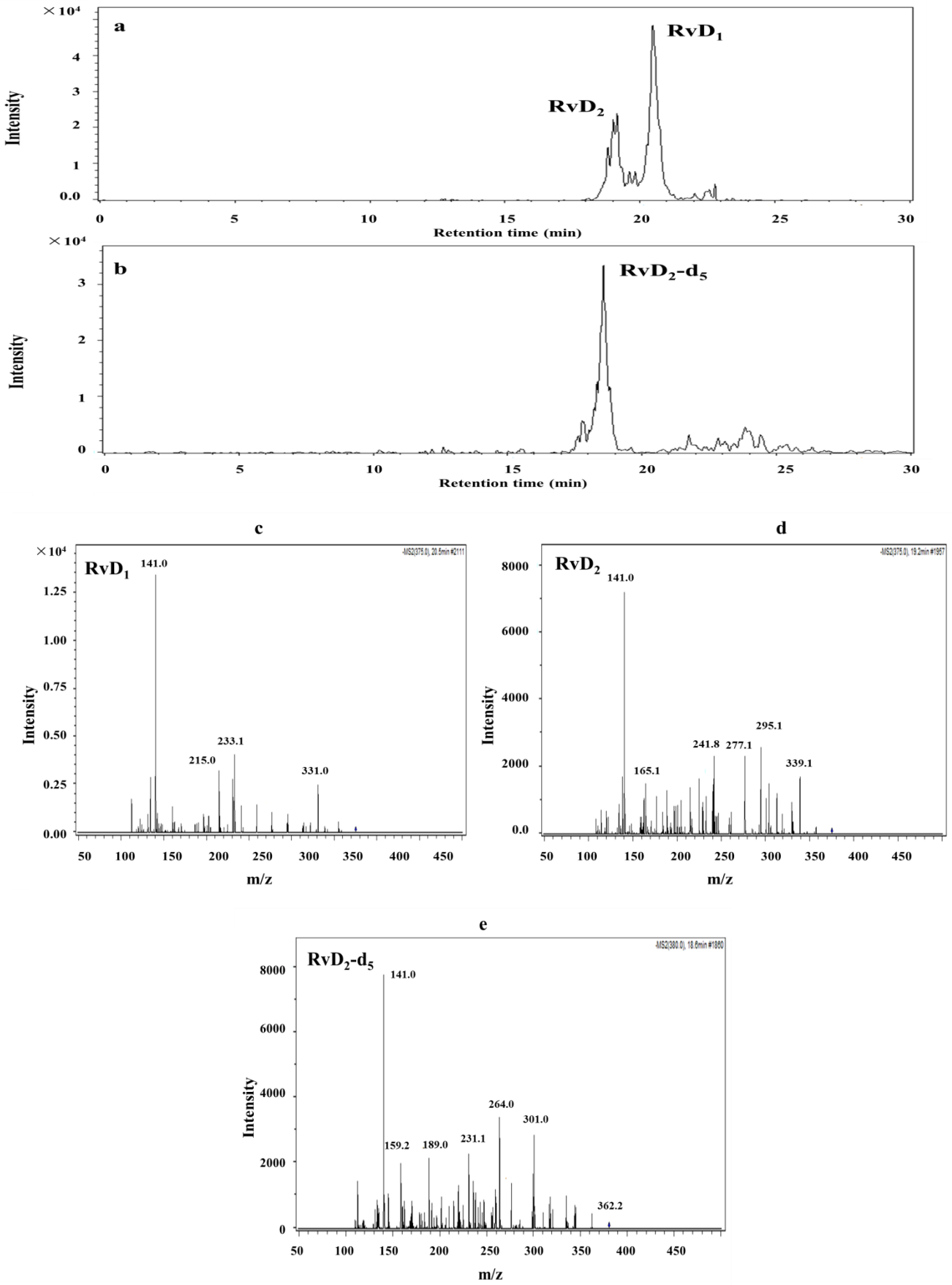


Figure 2.13 EICs (**a-b**) and mass spectra (**c-e**) of resolvins in cL-15 medium.

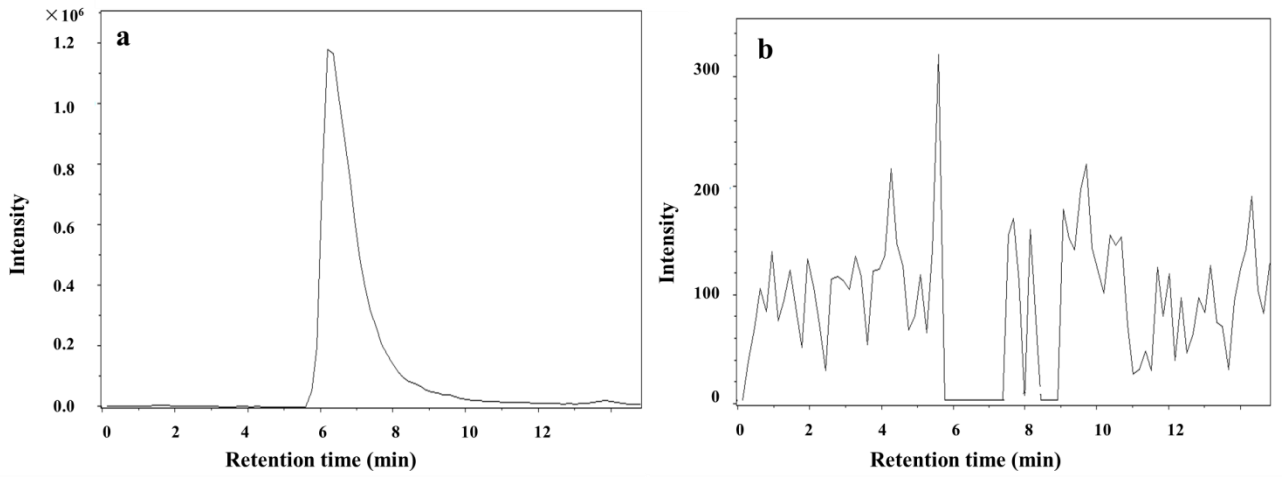


Figure 2.14 EICs of LTB_4 in blank medium when the fragmentation is: **(a)** 335→317+275+289+273+255+192+203+177+151+129+109; **(b)** 335→317+275+151+129+109 in cL-15 medium

The validated models describing the behavior of RF as a function of different internal standards and analytes are described in **Eqs 2.14 - 2.20**.

The optimal concentration of PGE_2- d_4 was selected by simultaneously considering the behavior of RF for PGE_2 and PGE_3 (**Figure 2.15**). The concentration plot of PGE_2-d_4 versus PGE_2 shows three well differentiated regions (indicated in blue, brown and red in **Figure 2.15a**) along the PGE_2-d_4 axis. In these particular regions, RF remains constant over the entire PGE_2 range. It has been mentioned that low concentration levels are preferred due to the high cost of the pure eicosanoid standards. However, based on **Figure 2.15a**, it is advisable to use high concentrations of PGE_2-d_4 (between 175-200 ng/ml) due to the high coefficient of variation (CV= 100×0.13/0.15=87%) observed in the concentration range of 0.5-130 ng/ml of PGE_2-d_4. Similar observations to those described above were derived from **Figure 2.15b** for PGE_3. The high coefficient of variation (approx. 56%) in the concentration range 0.5-130 ng/ml of PGE_2-d_4 precluded the use of this particular range. Consequently, 182 ng/ml of PGE_2-d_4 is chosen for further analysis of PGE_2 and PGE_3 in cL-15 medium.

$$\mathrm{RF} = 2.48 \times 10^{-1} - 1.77 \times 10^{-4}[PGE_2] - 7.47 \times 10^{-3}[PGE_2 - d_4] + 6.94 \times 10^{-5}[PGE_2 - d_4]^2 \quad (2.14)$$

$$\mathrm{RF} = 1.27 \times 10^{1} - 2.22 \times 10^{-1}[PGE_3] - 6.03 \times 10^{-2}[PGE_2 - d_4] + 1.13 \times 10^{-3}[PGE_3]^2 + 8.59 \times 10^{-4}[PGE_2 - d_4]^2 \quad (2.15)$$

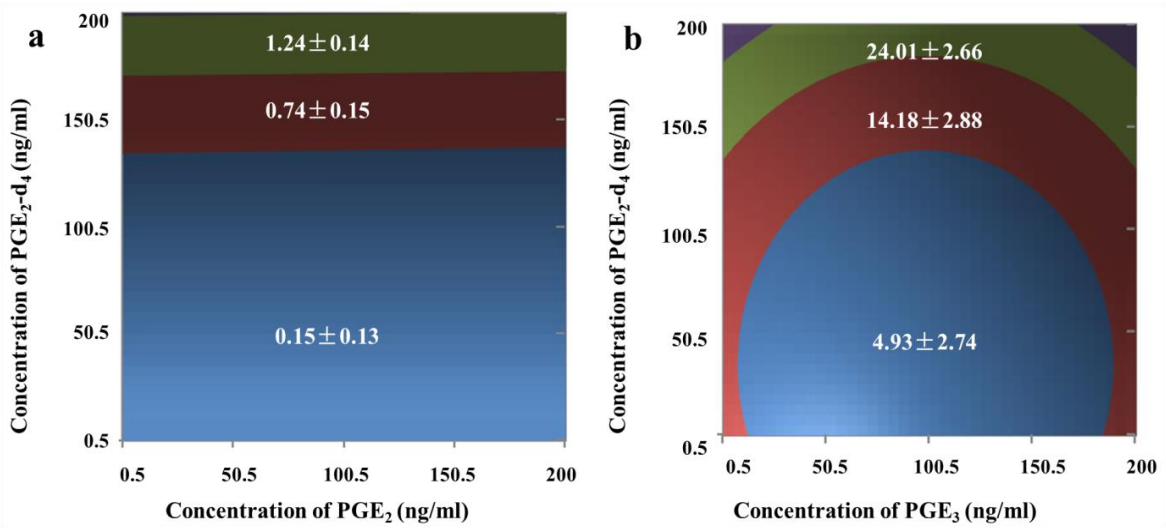


Figure 2.15 Behavior of RF as a function of **(a)** PGE_2 and PGE_2-d_4; **(b)** PGE_3 and PGE_2-d_4 in cL-15 medium, values in the figures represent the mean ± standard deviation of RFs

Three concentrations of LTB_4-d_4 (27, 70 and 190 ng/ml) were observed where RF values remain almost constant in the whole analytical range (**Figure 2.16**). Based on eicosanoid price considerations, a concentration of 27 ng/ml of LTB_4-d_4 was selected for the analysis of LTB_4 in cL-15 medium.

$$RF = 1.53 + 2.82 \times 10^{-3}[LTB_4] - 2.03 \times 10^{-2}[LTB_4 - d_4] + 7.84 \times 10^{-5}[LTB_4 - d_4]^2 \qquad (2.16)$$

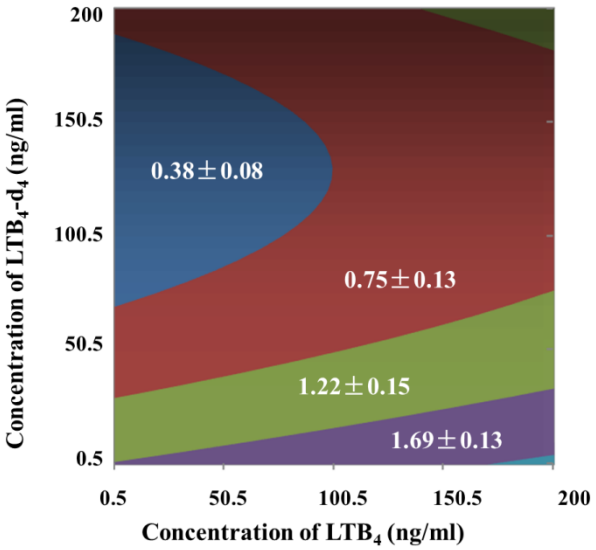


Figure 2.16 Behavior of RF as a function of LTB_4 and LTB_4-d_4 in cL-15 medium, values in the figures represent the mean ± standard deviation of RFs

The behavior of RF for 6-keto-$PGF_{1\alpha}$ (**Figure 2.17a**) was similar to that described in **Figure 2.16**. An optimal concentration region between 35-50 ng/ml of 6-keto-$PGF_{1\alpha}$-d_4 over the entire

6-keto-$PGF_{1\alpha}$ range was observed (**Figure 2.17a**). Considering that the behavior of RF for Δ17-6-keto-$PGF_{1\alpha}$ was independent of 6-keto-$PGF_{1\alpha}$-d_4 (**Figure 2.17b**), it was decided that 45 ng/ml of internal standard represents the optimal amount of 6-keto-$PGF_{1\alpha}$-d_4 for the analysis of 6-keto-$PGF_{1\alpha}$ and Δ17-6-keto-$PGF_{1\alpha}$ produced by fish cells in cL-15 medium.

$$RF = 9.98 \times 10^{-1} + 8.04 \times 10^{-4}[6-keto-PGF_{1\alpha}] - 4.41 \times 10^{-3}[6-keto-PGF_{1\alpha}-d_4] + 1.49 \times 10^{-5}[6-keto-PGF_{1\alpha}-d_4]^2 \quad (2.17)$$

$$RF = 3.16 - 8.07 \times 10^{-3}[\Delta 17-6-keto-PGF_{1\alpha}] + 4.00 \times 10^{-3}[6-keto-PGF_{1\alpha}-d_4] - 3.81 \times 10^{-5}[\Delta 17-6-keto-PGF_{1\alpha}][6-keto-PGF_{1\alpha}-d_4] \quad (2.18)$$

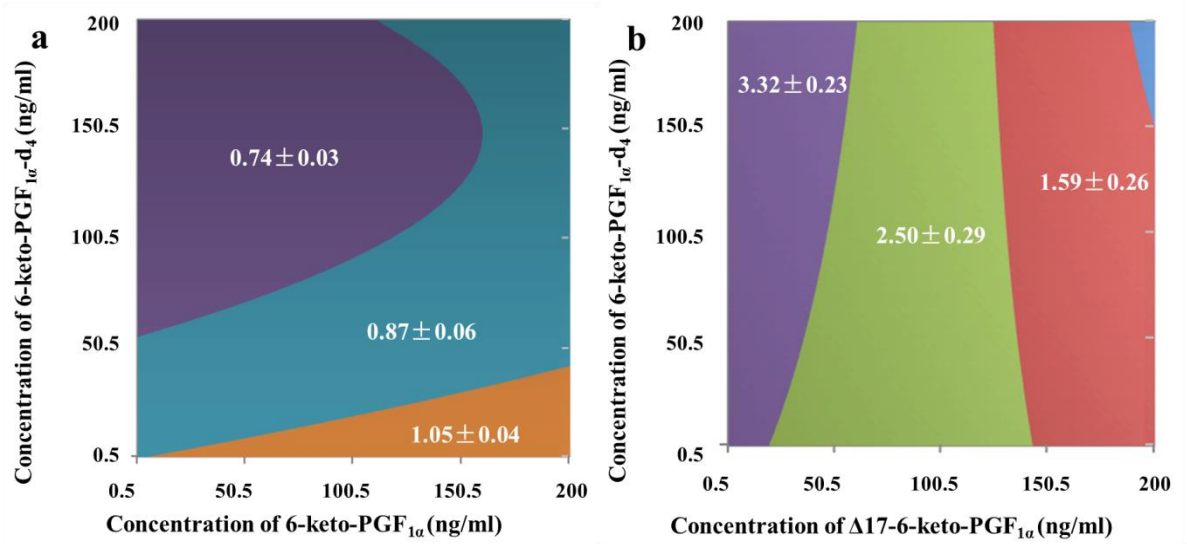


Figure 2.17 Behavior of RF as a function of **(a)** 6-keto-$PGF_{1\alpha}$ and 6-keto-$PGF_{1\alpha}$-d_4; **(b)** Δ17-6-keto-$PGF_{1\alpha}$ and 6-keto-$PGF_{1\alpha}$-d_4 in cL-15 medium, values in the figures represent the mean ± standard deviation of RFs

Similar regression models were derived for RvD_1 and RvD_2 where RF values were expressed as functions of RvD_1 or RvD_2, their common internal standard (RvD_2-d_5) and the interaction between them **(Eq. 2.19** and **Eq. 2.20)**. The second-order terms were not significant in both cases. For RvD_1, the RF was constant at concentration levels below 20 ng/ml of RvD_2-d_5 (**Figure 2.18a**). However, at this particular level the chromatographic peak exhibited a noisy baseline. For RvD_2, the RF was constant (1.88±0.14) in the range of 40-110 ng/ml of RvD_2-d_5 (**Figure 2.18b**). It was decided that 41 ng/ml of RvD_2-d_5 represent an optimal concentration level for the analysis of RvD_1 and RvD_2 generated by fish cells in cL-15 medium.

$$RF = 1.03 + 6.57 \times 10^{-5}[RvD_1] + 2.89 \times 10^{-3}[RvD_2 - d_5] - 2.47 \times 10^{-5}[RvD_1][RvD_2 - d_5] \quad (2.19)$$

$$RF = 1.29 + 4.70 \times 10^{-3}[RvD_2] + 7.27 \times 10^{-3}[RvD_2 - d_5] - 5.27 \times 10^{-5}[RvD_2][RvD_2 - d_5] \quad (2.20)$$

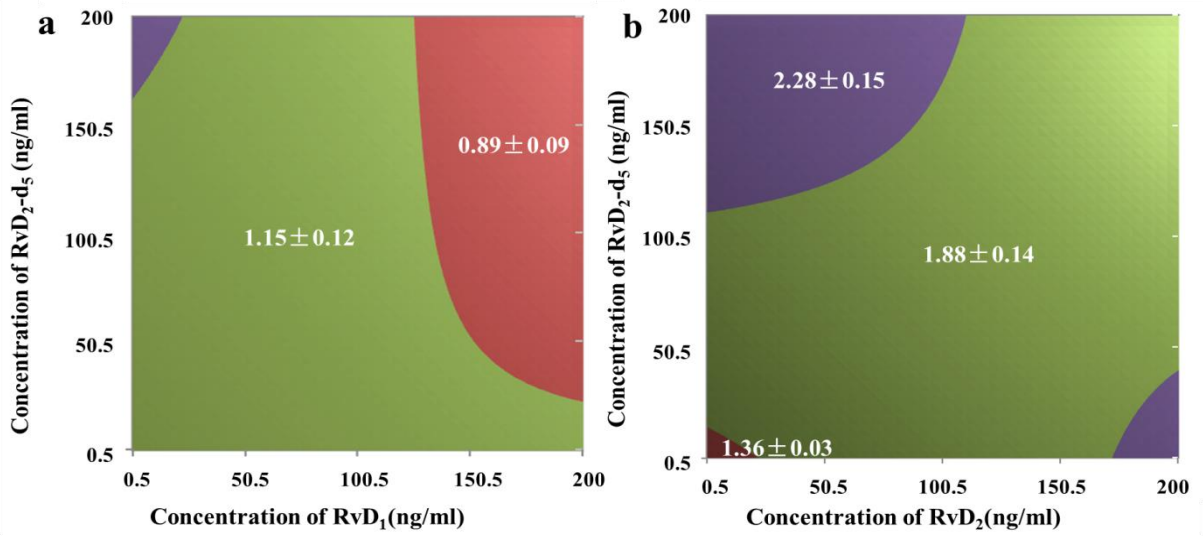


Figure 2.18 Behavior of RF as a function of **(a)** RvD_1 and RvD_2-d_5; **(b)** RvD_2 and RvD_2-d_5 in cL-15 medium, values in the figures represent the mean ± standard deviation of RFs.

Table 2.4 Statistical validation results of the RF models for selecting optimal levels of internal standards associated to the analysis of different eicosanoids generated by fish cells in cL-15 medium

	PGE_3	PGE_2	LTB_4	6-keto-$PGF_{1\alpha}$	Δ-17-6-keto-$PGF_{1\alpha}$	RvD_1	RvD_2
Residual variance	198.461(16)	0.389(17)	2.715(17)	0.600(14)	10.636(14)	0.696(14)	1.376(14)
Pure error variance	197.085(14)	0.324(14)	2.157(14)	0.587(12)	10.019(12)	0.634(12)	1.056(12)
Lack-of-fit variance	1.375(2)	0.066(3)	0.558(3)	0.013(2)	0.617(2)	0.062(2)	0.320(2)
$F_{estimated}$	0.049	0.945	1.206	0.137	0.369	0.591	1.819
$F_{theoretical}$	3.739	3.344	3.344	3.885	3.885	3.885	3.885

2.3.2 Method validation

Method validation is a process that defines the analytical characteristics of a method. Usually, once a new method is developed, its validation is necessary to confirm its precision and reliability.

After selecting the optimal internal standard concentrations, the developed SPE-HPLC-MS/MS method described in **Section 2.1** was submitted to analytical validation. The validation parameters considered in the present study were: selectivity, LOD, LOQ, linearity, analytical range, precision and recovery.

2.3.2.1 Selectivity

According to IUPAC, the term selectivity is defined as "the extent to which a method can determine a particular analyte in a complex mixture without interference from other components in

the mixture" [216]. The definition in question implies that the signal should be only due to the occurrence of analyte. This is considered the most important parameter in a method validation.

One advantage of chromatography is its separation ability. Compounds are separated and eluted in different retention times which can guarantee the selectivity. However, many analytes from the same category have similar retention times such as PGE_2 and PGE_3. In this case, to guarantee the selectivity, mass spectra are needed.

The developed SPE-HPLC-MS/MS method was highly selective as showed in the different EICs for leukotrienes (**Figure 2.2a-Figure 2.2b** and **Figure 2.10a-Figure 2.10b**), prostaglandins (**Figure 2.3a-Figure 2.3c** and **Figure 2.11a-Figure 2.11c)**), prostacyclyns (**Figure 2.4a-Figure 2.4c** and **Figure 2.12a-Figure 2.12c)**) and resolvins (**Figure 2.5a-Figure 2.5b** and **Figure 2.13a-Figure 2.13b)**) in both media, EBM-2 medium and cL-15 medium.

2.3.2.2 Limit of detection (LOD) and limit of quantification (LOQ)

LOD is the smallest concentration of analyte that can be detected with statistically significant certainty while the LOQ is the smallest concentration of analyte that can be quantified with an acceptable accuracy and precision. They can be determined by a) visual inspection of a recorded signal; b) the slope of a calibration curve; and c) the signal to noise ratio of a blank sample [216].

In the present thesis, the last approach (c) was used to determine the LOD and LOQ as follows: blank samples are injected in pentaplicate (for each type of medium), the corresponding EICs of the studied eicosanoids are obtained, their areas at specific retention times are monitored and the standard deviations for each type of blank medium (σ_{blank}) calculated. The LOD and LOQ are defined by the concentrations of spiked medium solutions that are able to produce analytical signals equivalent to $3\times\sigma_{blank}$ and $6\times\sigma_{blank}$. The LOD and LOQ values determined experimentally are shown in **Table 2.5** and **Table 2.6.**

2.3.2.3 Linearity and analytical range

Linearity is the ability of an analytical method to provide an analytical response proportional to the concentration or the amount of analyte within a specified range. Whereas, the range of an analytical method is defined as the interval between LOQ and upper concentration (amounts) of analyte in the sample for which it has been demonstrated that the analytical procedure has an

acceptable level of precision, accuracy and linearity. Mathematically, linearity is expressed as **Eq. 2.21**:

$$y = a\,(x) + b \qquad (2.21)$$

where *y*, in the present case, is the analyte/internal standard signal ratio, *x* is the analytical concentration and *a* and *b* are the slope an intercept of the calibration function respectively.

2.3.2.4 Precision

Precision is the closeness of agreement between measured values obtained by replicate measurements on the same or similar objects under specified conditions. Repeatability, intermediate precision and reproducibility are three terms associated with precision.

Repeatability is the precision of results obtained in the same measurement conditions (analyst, laboratory, instrument, etc.) over a short period of time.

Intermediate precision is the precision of results obtained in a given laboratory over an extended period of time.

Reproducibility is the precision of results obtained by changing one or more measurement conditions over a short or an extended period of time.

In this thesis, the relative standard deviation of triplicate spiked medium solutions at low, medium and high concentration levels of specific eicosanoids (50, 100 and 200 ng/ml respectively) are used to determine the precision of the method. The results (**Tables 2.5-2.6**) revealed acceptable levels of precision for EBM-2 medium (0.2-27.3%) and cL-15 medium (3.5-30.7%). The precision results fulfilled the requirements for biological samples (≤30%)[217].

2.3.2.5 Recovery

Recovery is a parameter to measure accuracy which is defined as the closeness of agreement between a measured value and true value. The ratio between the measured to nominal concentration is referred as recovery and it is generally expressed in percentage units (%).

In the present thesis, the recovery studies were carried out by adding known amounts of the various analytes into blank media (EBM-2 and cL-15) to make concentrations of 50, 100 and 200 ng/ml of the various eicosanoids. Every level of concentration was prepared in triplicate. The recoveries of different analytes varied from 57.1% to 127.2 % for EBM-2 medium and from 63.9% to 128.4% for cL-15 medium. A statistical analysis for the wide recovery range observed for PGE_3

and LTB_4 in EBM-2 medium was performed by obtaining the z values and computing the normal distribution function. The results (**Appendix I**) allow to conclude that in those ranges (for PGE_3 and LTB_4), the concentrations will lie between ±2σ of the normal curve and consequently they are considered acceptable ranges. It is important to mention that these results were discussed with an expert in biological cell culture experiments who considered that these recovery ranges were very strict when compared to the wider levels of variation expected in cell experiments.

Table 2.5 Method validation parameters in EBM-2 medium

	LOD (ng/ml)	LOQ (ng/ml)	Recovery (%)	Range (ng/ml)	Precision (%)
PGE_2	0.1	0.1	81.0-120.9	0.1-200	1.4-16.3
PGE_3	0.1	0.1	60.9-120.9	0.1-200	2.0-13.6
LTB_4	0.1	0.1	57.1-118.2	0.1-200	11.2-20.7
6-keto-$PGF_{1\alpha}$	0.1	0.6	91.4-108.8	0.6-200	6.2-14.4
Δ17-6-keto-$PGF_{1\alpha}$	0.1	0.6	88.9-127.2	0.6-200	8.4-24.1
RvD_1	0.1	0.1	87.6-124.5	0.1-200	0.2-22.2
RvD_2	0.1	0.1	83.2-109.6	0.1-200	1.4-5.7

Table 2.6 Method validation parameters for cL-15 medium

	LOD (ng/ml)	LOQ (ng/ml)	Recovery (%)	Range (ng/ml)	Precision (%)
PGE_2	0.1	0.1	63.9-128.4	0.1-200	4.5-30.7
PGE_3	0.6	8.0	93.6-124.5	8-200	5.4-19.5
LTB_4	0.1	0.3	67.0-115.1	0.3-200	10.4-28.0
6-keto-$PGF_{1\alpha}$	0.1	0.1	96.8-115.2	0.1-200	3.5-18.4
Δ17-6-keto-$PGF_{1\alpha}$	2.0	8.0	88.6-125.7	8-200	9.6-21.3
RvD_1	0.6	1.0	97.7-118.2	1-200	10.5-28.1
RvD_2	0.3	0.6	91.4-112.0	0.6-200	9.1-26.2

2.4 Conclusions

In this chapter, a new SPE-HPLC-MS/MS method was developed to simultaneously analyze seven eicosanoids (PGE_2, PGE_3, LTB_4, 6-keto-$PGF_{1\alpha}$, Δ17-6-keto-$PGF_{1\alpha}$, RvD_1 and RvD_2) in cL-15 medium and EBM-2 medium. The optimal concentrations of internal standards were selected by an experimental design and different validation parameters are also defined. The method has proved to be selective, precise, accurate and suitable for the analysis of eicosanoids generated by human cells in EBM-2 medium and fish cells in cL-15 medium.

3. Application of the method to Salmon liver cells

3.1 Experimental

3.1.1 Reagents

Complete cL-15 medium was prepared by mixing Leibowitch L-15 medium with 1% glutamax, 1% antibiotika and 10% FBS.

1 M $CaCl_2$ and perfusion buffer containing 1.4 M NaCl, 0.067M KCl and 0.09M 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 1M $CaCl_2$ and 100 mg collagenase were added. Both of buffers containing EDTA and collagenase should be freshly prepared.

3.1.2 Isolation of the cells

The fish were anaesthetized by metacaine (MS222, 0.5 gram/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 (collagenase dissolved in stock perfusion buffer as describe before). The isolated cells were harvested in 10 ml 10% phosphate-buffered saline buffer (PBS buffer: 0.002M KH_2PO_4, 0.02M Na_2HPO_4, 0.03M KCl and 0.14M NaCl, pH 7.4), filtrated through a 100 μm mesh cell strainer and washed twice in the PBS buffer, re-suspended in cL-15 medium before the viability of the isolated cells was assessed. All centrifugations were done by 50 g for 5 min. The viability of the liver cells was above 90% (range: 90.8 to 94.4%). The isolations of cells were done with sterile equipment and buffers.

3.1.3 Cell culture

The culture plate was first conditioned by adding 1% Laminin (500 μL Laminin in 50 mL PBS) 1920 μL/well and kept overnight. Once the cells were obtained, these solutions were removed and 1.67×10^6 liver

cells were added to each well. According to the 2^4- full factorial design (**Table 3.1**), different combinations of fatty acids were added. At each experimental point, triplicate was done.

When the signal is positive (+), 0.5 mL medium containing the corresponding fatty acid with a concentration of 46 μM was added. Different fatty acids were first attached to FBS and then complete cL-15 medium was prepared using this FBS instead of normal one. When the signal is negative (-), 0.5 mL normal complete cL-15 medium was added. At 16^{th} experimental point, besides 2 ml normal complete medium, 25 μL of ethanol were also added.

After incubating 24 h at 9 ℃ in an incubator device (Sanyo Electric CO., Ltd., Osaka, Japan), the medium was collected carefully without disturbing the cells attached to the bottom of the plate and stored at -80 ℃ until the analysis by HPLC-MS/MS

Table 3.1 2^4- full factorial design

	LA	AA	EPA	DHA
1	+	+	+	+
2	+	+	+	-
3	+	+	-	+
4	+	+	-	-
5	+	-	+	+
6	+	-	+	-
7	+	-	-	+
8	+	-	-	-
9	-	+	+	+
10	-	+	+	-
11	-	+	-	+
12	-	+	-	-
13	-	-	+	+
14	-	-	+	-
15	-	-	-	+
16	-	-	-	-

3.2 Statistical analysis

3.2.1 Multifactor ANOVA

Usually, analysis of variance (ANOVA) is used to detect significant factors. When there is only one factor influencing the experimental response, one-way ANOVA can be used. However, when several variables are affecting the experimental response, multifactor ANOVA is applied. In this section, the

multifactor ANOVA theory is explained by using as an example a 2^4- full factorial design (**Table 3.2**).

Table 3.2 Example of a 2^4 -factorial design used for the explanation of multifactor ANOVA

NO.	Code	Responses			Mean Response	Mean in each group
1	+	$X_{1\text{-}1}$	$X_{1\text{-}2}$	$X_{1\text{-}3}$	$\bar{X}_1$	
.						
.						$\bar{X}_+$
.						
8	+	$X_{8\text{-}1}$	$X_{8\text{-}2}$	$X_{8\text{-}3}$	$\bar{X}_8$	
9	-	$X_{9\text{-}1}$	$X_{9\text{-}2}$	$X_{9\text{-}3}$	$\bar{X}_9$	
.		.	.	.	.	
.		.	.	.	.	$\bar{X}_-$
.		.	.	.	.	
16	-	$X_{16\text{-}1}$	$X_{16\text{-}2}$	$X_{16\text{-}3}$	$\bar{X}_{16}$	
					Global mean	$\bar{X}$

In a 2^4- factorial design, 16 experimental points are set and measured preferably in triplicate. From the measured responses, the following values are calculated: mean response of each triplicate ($\bar{X}_i$), mean response when the corresponding factor is negative ($\bar{X}_-$), mean response when the factor is positive ($\bar{X}_+$) and mean response of all the experiments ($\bar{X}$).

Then, mean square of effect and mean square of error can be calculated according to **Eq. 3.1** and **Eq. 3.2**, respectively. F-value is defined as the ratio between MS_{effect} and MS_{error} (**Eq. 3.3**).

$$MS_{effect} = \frac{\left[\sum(\bar{X}-\overline{X_+})^2+\sum(\bar{X}-\overline{X_-})^2\right](\mathrm{N}\times\mathrm{K})}{2-1} \tag{3.1}$$

$$MS_{error} = \frac{\sum(\bar{x_i}-X_{i-j})^2}{\mathrm{P}-\mathrm{R}} \tag{3.2}$$

$$\mathrm{F} = \frac{MS_{effect}}{MS_{error}} \tag{3.3}$$

Where N is the number of experimental points in each group, K is the number of the replicates, P is the total number of experiments, and R is the total number of experimental points.

Thereafter, the corresponding confidence level related to F-value can be calculated in Excel 2010. Usually, if the confidence level is higher than 95% (p-value lower than 0.05), the factor is considered statistically significant.

3.2.2 Fisher's Least Significant Difference (LSD) test

LSD is a test that can compare the mean of two groups. In this test, the square root of the mean square error from ANOVA is considered to be a pooled standard deviation. Considering the sample size of the two groups being compared, a standard error of difference between those two means is calculated. LSD is defined as the product of this standard error multiplied by a t-value in a confidence level (**Eq. 3.4**).

$$\mathrm{LSD} = t_{v,\alpha}\sqrt{MS_{error}\frac{2}{N}} \tag{3.4}$$

Where N is the number of observations in each group, v is the degrees of freedom and it is equal to 2N-2, α is the significance level which is often used as 0.05. MS_{error} is the mean square of error, and it is calculated as the way in one-way ANOVA like **Eq. 3.5**.

$$MS_{error} = \frac{\left(\overline{X_+} - X_{ij}\right)^2 + \left(\overline{X_-} - X_{ij}\right)^2}{2N-2} \tag{3.5}$$

The symbols in **Eq. 3.5** have the same meaning as defined in section 3.2.1.

3.2.3 Statistical software

Statgraphics Centurion XV version was used for computing numerically and graphically the significance of the factors by means of the multifactor analysis of variance (ANOVA) and Fisher's least significance options respectively.

3.3 Results and discussion

The developed and validated SPE-HPLC-MS/MS was successfully used for the simultaneous determination of prostaglandins (PGE_1, PGE_2), prostacyclins (6-keto-$PGF_{1\alpha}$, Δ17-6-keto-$PGF_{1\alpha}$), leukotriene (LTB_4) and resolvins (RvD_1, RvD_2) generated by salmon liver cells in cL-15 medium. The production of prostaglandins, prostacyclins and leukotriene were effectively confirmed by the new method (**Table 3.3**). Unfortunately, the resolvins were not detected in the analyzed samples.

Some reports have established similar metabolic pathways (dotted circle line in **Figure 1.2**) for the isobaric resolvins RvD_1, RvD_2 (analyzed in this work) and RvD_3, RvD_4 (MW= 376). The expected base

peak generated by RvD_1 and RvD_2 (m/z 375→141) and RvD_3 and RvD_4 (m/z 375→147)[218] were extracted from the chromatograms of analyzed medium. Surprisingly, the results did not show the presence of the former resolvins (**Figure. 3.1a**). However, a significant peak for the latter resolvins (**Figure 3.1b**) with intensity similar to its internal standard was observed. These unexpected results indicated that the major metabolites of DHA in salmon liver cells are RvD_3 and RvD_4, and not RvD_1 and RvD_2 (as initially assumed in the present thesis).

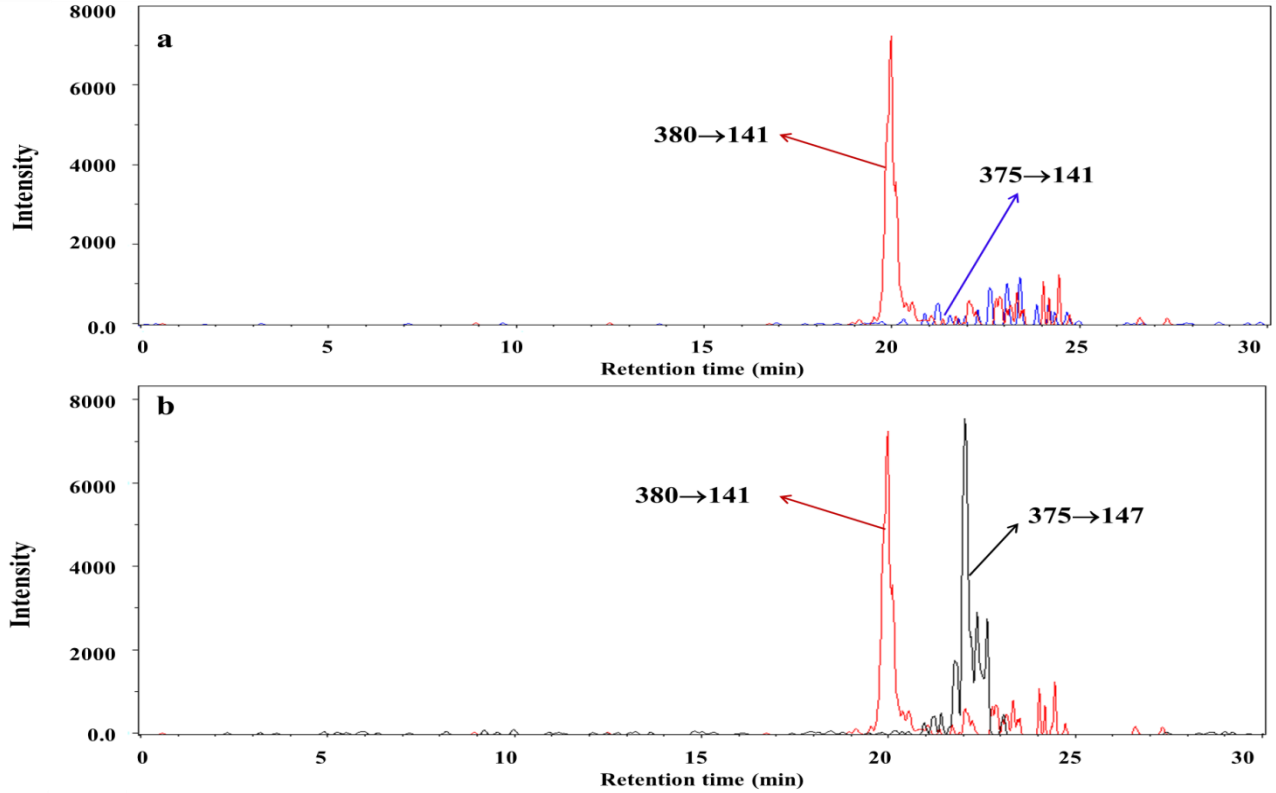


Figure 3.1 EICs of the **a)** resolvins analyzed in this work (RvD_1 and RvD_2 at m/z 375→141) and **b)** resolvins not analyzed in this work (RvD_3 and RvD_4 at m/z 375→147) with their corresponding internal standard (RvD_2-d_5 at m/z 380→141) generated by salmon liver cells in cL-15 medium.

The concentrations of other eicosanoids (PGE_2, PGE_3, LTB_4, 6-keto-$PGF_{1\alpha}$ and Δ17-6-keto-$PGF_{1\alpha}$) were determined by using their corresponding RF values and the results are shown in **Table 3.3**.

A multifactor ANOVA was applied to study the impact of different fatty acids on the production of eicosanoids by salmon liver cells and the results of this analysis are shown in **Table 3.4**. The graphical representations of the mean concentrations of the generated eicosanoids with their corresponding 95% least significant intervals are shown in **Figure 3.2-1** and **Figure 3.2-2**.

Table 3.3 Concentrations in ng/ml of various eicosanoids released in cL-15 medium by salmon liver cells after exposure to different PUFAs.

		LTB4	PGE2	6-keto-PGF$_{1\alpha}$	PGE3	Δ17-6-keto-PGF$_{1\alpha}$
LA+AA+EPA+DHA	1.1	0.390	3.175	2.786	8.669	6.581
	1.2	0.646	6.011	1.129	30.827	8.170
	1.3	0.559	4.437	2.537	18.486	4.114
LA+AA+EPA	2.1	0.882	4.645	2.264	4.525	5.891
	2.2	0.932	4.610	1.115	42.721	5.998
	2.3	0.431	4.798	1.635	5.592	3.637
LA+AA+DHA	3.1	0.675	3.493	2.304	8.819	5.163
	3.2	1.217	2.635	0.930	4.093	4.339
	3.3	1.121	3.465	2.304	12.723	3.967
LA+AA	4.1	0.351	2.074	3.181	9.808	5.049
	4.2	0.589	1.105	1.115	3.206	5.803
	4.3	0.514	3.391	1.504	15.985	7.247
LA+EPA+DHA	5.1	0.832	0.814	0.437	8.880	0.866
	5.2	1.031	1.108	0.368	2.832	0.613
	5.3	0.684	0.354	0.381	13.408	1.115
LA+EPA	6.1	0.632	0.208	0.340	3.489	1.494
	6.2	0.596	0.186	0.067	4.252	0.730
	6.3	0.663	0.345	0.257	5.837	0.329
LA+DHA	7.1	0.191	0.326	0.642	4.427	2.733
	7.2	0.441	0.864	0.315	7.315	0.963
	7.3	0.725	0.400	0.277	10.474	0.251
LA	8.2	0.272	0.226	0.392	6.313	1.033
	8.2	0.351	0.131	0.245	5.576	0.658
	8.3	0.322	0.586	0.268	4.142	0.810
AA+EPA+DHA	9.1	0.934	4.793	3.624	6.916	7.123
	9.2	0.895	1.225	1.570	3.166	4.813
	9.3	0.954	4.418	2.279	7.228	7.517
AA+EPA	10.1	0.577	3.173	3.377	13.128	10.610
	10.2	0.545	4.112	2.131	11.950	7.100
	10.3	0.687	7.032	2.738	10.290	8.734
AA+DHA	11.1	0.738	3.463	2.945	13.109	6.782
	11.2	1.011	3.015	1.208	10.120	7.248
	11.3	0.958	6.634	1.719	7.519	6.296
AA	12.1	0.379	4.149	2.509	8.693	6.426
	12.2	0.503	2.191	1.890	3.337	7.110
	12.3	0.697	5.555	2.199	5.517	8.889
EPA+DHA	13.1	0.820	0.785	0.514	2.671	0.435
	13.2	0.890	0.831	0.601	8.731	1.628
	13.3	0.890	0.389	0.309	6.987	1.623
EPA	14.1	1.017	1.296	1.077	9.827	2.791
	14.2	0.687	0.979	0.759	5.039	1.639
	14.3	0.955	0.667	1.001	5.054	3.333
DHA	15.1	0.747	1.022	0.402	8.792	3.404
	15.2	0.361	0.383	0.181	4.154	1.410
	15.3	0.877	0.340	0.539	4.395	3.933
with ETOH	16.1	0.145	0.194	0.573	3.082	0.706
	16.2	0.571	0.175	0.267	4.823	0.654
	16.3	0.654	0.260	0.558	5.906	1.732

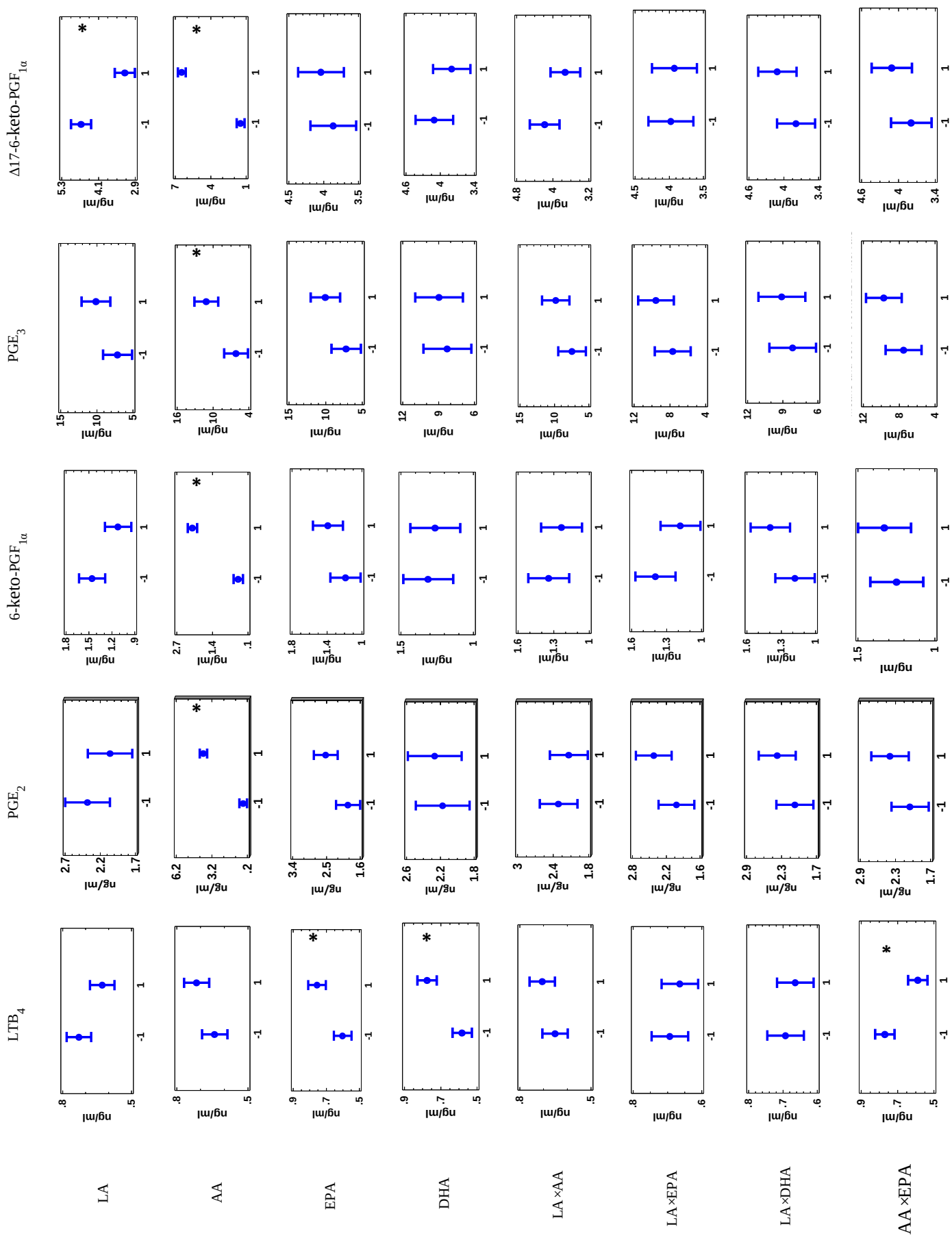


Figure 3.2-1 Mean concentration values with their 95% least significant intervals of different eicosanoids in cL-15 medium of salmon liver cells

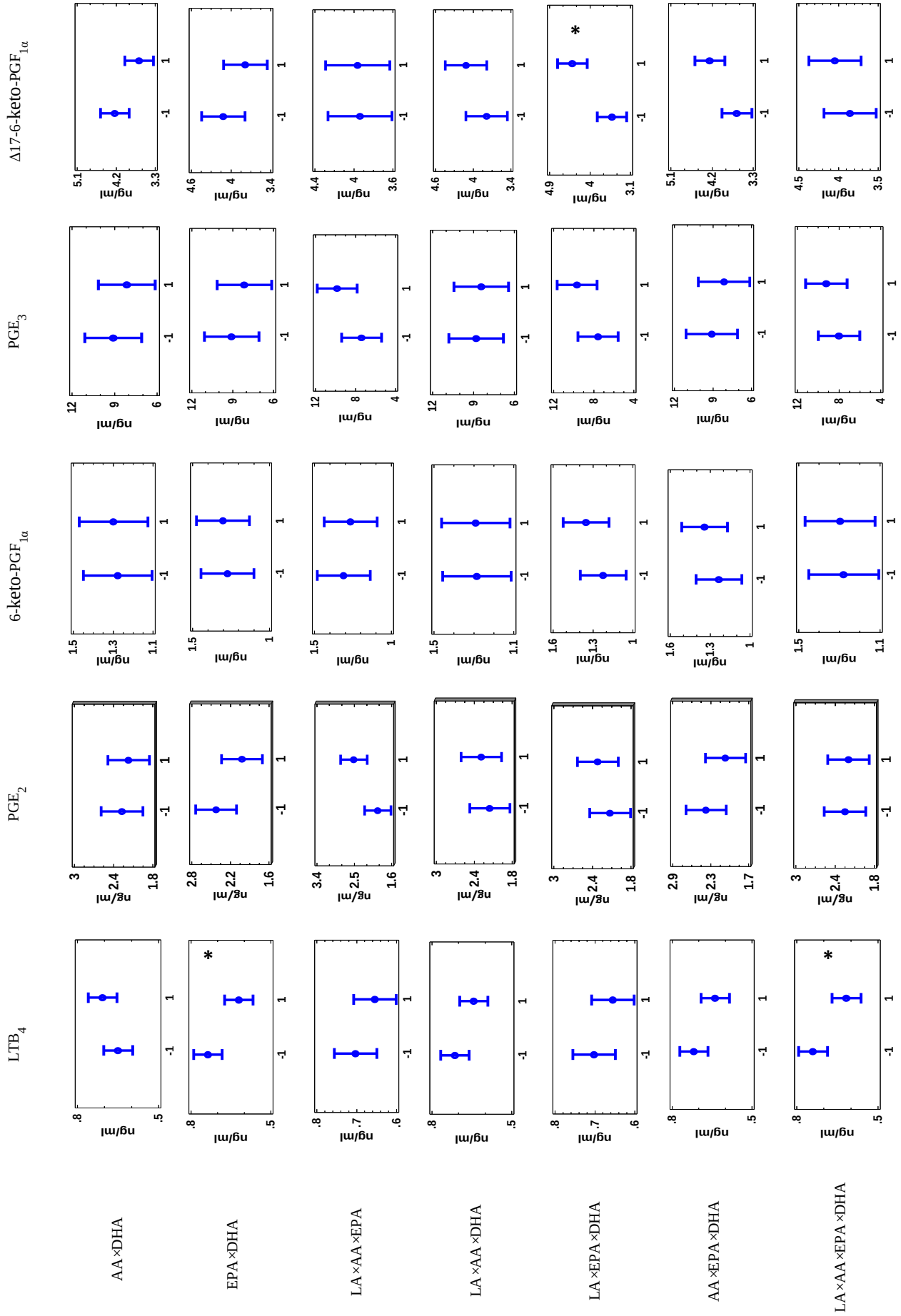


Figure 3.2-2 Mean concentration values with their 95% least significant intervals of different eicosanoids in cL-15 medium of salmon liver cells

Table 3.4 Multifactor ANOVA results with the significant effects (P- values) for the impact of PUFAs on the production of various eicosanoids by salmon liver cells.

	LTB_4	PGE_2	6-keto-$PGF_{1\alpha}$	PGE_3	Δ17-6-keto-$PGF_{1\alpha}$
LA	0.0610 ↓	0.3137 ↓	0.0529 ↓	0.1376 ↑	0.0001 ↓ *
AA	0.1551 ↑	0.0000 ↑ *	0.0000 ↑ *	0.0161 ↑ *	0.0000 ↑ *
EPA	0.0059 ↑ *	0.0659 ↑	0.2438 ↑	0.1588 ↑	0.5854 ↑
DHA	0.0008 ↑ *	0.7625 ↑	0.7779 ↓	0.7269 ↑	0.3530 ↓
LA*AA	0.3083 ↑	0.5846 ↓	0.5286 ↓	0.2366 ↑	0.1662 ↓
LA*EPA	0.5812 ↓	0.2071 ↑	0.2142 ↓	0.3471 ↑	0.8744 ↓
LA*DHA	0.5923 ↓	0.3412 ↑	0.2089 ↑	0.6391 ↑	0.3281 ↑
AA*EPA	0.0015 ↓ *	0.2830 ↑	0.6393 ↑	0.2707 ↑	0.3313 ↑
AA*DHA	0.2784 ↑	0.7481 ↓	0.9000 ↑	0.6327 ↓	0.0928 ↓
EPA*DHA	0.0338 ↓ *	0.2053 ↓	0.8630 ↑	0.6379 ↓	0.3067 ↓
LA*AA*EPA	0.3612 ↓	0.0745 ↑	0.7902 ↓	0.2246 ↑	0.9401 ↑
LA*AA*DHA	0.1846 ↓	0.6681 ↑	0.9741 ↑	0.8487 ↓	0.3168 ↑
LA*EPA*DHA	0.3740 ↓	0.5459 ↑	0.4550 ↑	0.2962 ↑	0.0102 ↑ *
AA*EPA*DHA	0.1344 ↓	0.3329 ↓	0.5276 ↑	0.6291 ↓	0.0764 ↑
LA*AA*EPA*DHA	0.0246 ↓ *	0.8641 ↓	0.9194 ↑	0.5389 ↑	0.5631 ↑

'*' indicates significant effect at confidence level of 95%

'↑' indicates the mean concentration of corresponding eicosanoid with the addition of fatty acids is higher than that without fatty acids;

'↓' indicates the mean concentration of corresponding eicosanoid with the addition of fatty acids is lower than that without fatty acids

Table 3.4 and **Figure 3.2** show that the presence of AA significantly increases the production of PGE_2, 6-keto-$PGF_{1\alpha}$, PGE_3 and Δ17-6-keto-$PGF_{1\alpha}$. The concentration of LTB_4 is also slightly increased by AA, but its effect is not significant. Increase of PGE_2, 6-keto-$PGF_{1\alpha}$ and LTB_4 indicates that during the incubation period, AA is incorporated into the cell membrane resulting in the releasing of AA into the cells and the synthesis of AA-derived eicosanoids The high levels of PGE_2 and 6-keto-$PGF_{1\alpha}$ compared to the low levels of LTB_4 produced after exposing the cells to AA, are indicative that the added ω-6 PUFA is basically enrolled in COX pathway instead of LOX pathway. The production of EPA metabolites, PGE_3 and Δ17-6-keto-$PGF_{1\alpha}$, are also increased after exposing the cells to AA. Although, it was not expected the AA could have an influence on the aforementioned EPA metabolites, some researchers has already demonstrated that the presence of AA can stimulate in some degree the production of Δ17-6-keto-$PGF_{1\alpha}$ in human endothelial cells[219]. In addition, increased levels of EPA-metabolites have also been observed in skin cells

from dogs fed n-6/n-3 diet ratios of 5/1 and 10/1[220].

Production of LTB_4 is enhanced either by the presence of EPA or DHA. This confirms that EPA and DHA are incorporated into the by-layer phospholipid cell membrane after releasing AA in a dose- and time-dependent manner[221,222]. At the first 24 h, EPA and DHA are incorporated into the cell membrane at the expense of AA which is released and converted into AA-derived eicosanoids. The significance of EPA and DHA in the production of LOX metabolites compared to COX metabolites could be explained in terms of the activation energy of both enzymes (COX and LOX). For instance, if the activation energy of LOX were lower than that of COX, then it would be expected that the reaction rate of the former enzyme would be larger than of the latter enzyme and a higher production of LOX metabolites compared to COX metabolites would be observed[223]. Unfortunately, this kind of measurements requires another type of experimental set up that was not considered at the time of designing the present experiments.

LA reduces the production of Δ17-6-keto-$PGF_{1\alpha}$ significantly (**Table 3.4, Figure 4.2).** The production of PGE_3 and all the AA-derived metabolites are slightly affected by the exposure of salmon liver cells to LA. The former and the latter revealed some non-significant increased and decreased production of eicosanoids respectively (**Table 3.4, Figure 4.2)**. It is possible that LA could form some AA (as indicated in **Figure 1.2**) which in turn replaces EPA in the cell membrane. The released EPA causes a decreasing in the levels of all the AA-metabolites due to the activation of the COX-pathways of EPA through the PGH_3 synthase. It is well-known that PGH_3 synthase is a common substrate for the production of both Δ17-6-keto-$PGF_{1\alpha}$ and PGE_3. The synthesis of Δ17-6-keto-$PGF_{1\alpha}$ is possible through a multiple step mechanism where several chemical compounds are involved while the formation of PGE_3 requires a less complex mechanism. Consequently, the observed reduction of Δ17-6-keto-$PGF_{1\alpha}$ and increase of PGE_3 could be explained as a result of spending PGH_3 synthase in the formation of latter instead of the former. Perhaps, it would be advisable to measure the levels of PGI_3 which is the precursor of Δ17-6-keto-$PGF_{1\alpha}$ to determine effectively whether or not the PGH_3 synthase has more affinity towards the production of the prostaglandin rather than the prostacyclin.

One interesting finding is that both AA and EPA increase the production of five out of the seven eicosanoids studied in the present investigation. However, when they are present in medium at the same concentration, their combined effect remarkably decrease the production of LTB_4 and slightly increase the production of all the COX-metabolites. The interaction EPA×DHA decreases the production of LTB_4 which is also opposite to the individual effect of EPA or DHA indicating perhaps that the ratio between

different PUFAs is a relevant aspect that should be evaluated when studying their impact on the on the production of eicosanoids[1].

Usually, high-order interactions are negligible. However, the significance of the interactions LA ×EPA ×DHA and LA ×AA ×EPA ×DHA in Table 3.4 could be related to the incorporation and releasing of PUFAs. However, these results should be confirmed by introducing more replicates or perhaps by proposing a different experimental design.

3.4 Conclusions

In this chapter, the validated SPE-HPLC-MS/MS method was used to analyze the levels of eicosanoids released in cL-15 medium by salmon liver cells exposed to different combination of PUFAs. The present results seems to confirm the observations of Calder[222] who has recently pointed out that the actions of ω-6 and ω-3 PUFAs and their derivatives on inflammatory processes involve more complex mechanisms than previously recognized. Besides the availability of PUFAs, there are many other factors affecting the amount and type of the synthesized eicosanoids.

4. Application of the method to salmon head kidney cells

4.1 Experimental

4.1.1 Reagents

NaCl-EDTA buffer was prepared by dissolving 9 g NaCl and 7 g of EDTA in 1 L of H_2O and adjusting pH to 7.2.

Stock isotonic Pecoll (SIP) was prepared by mixing 10 mL of 1.5 M NaCl with 90 mL of Percoll.

From NaCl-EDTA buffer and SIP, 1.06 g/ml (21 ml SIP and 29 ml Buffer) and 1.08 g/ml (29 SIP and 21 Buffer) of Percoll gradients were prepared and then 10 mL of 1.06 gradient was added slowly to 10 mL of 1.08 gradient making sure these two phases were not mixed. The mixed Percoll gradients were used for cell isolation.

4.1.2 Isolation of cells

For each fish, the head kidneys were directly sampled and added PBS and then squeezed through a 40 uM Falcon cell strainer. The cells were transferred to tubes and centrifuged in a Hettich Zentrifugen, 320 R, at 400 g, 5 min, and 4 ℃. Cell pellets were resuspended in PBS and layered carefully on top of equal amounts of diluted Percoll in densities 1.08 g/mL and 1.06 g/mL. The tubes were centrifuged at 800 g, 30 min, at room temperature. The cell layer in the interface containing the head kidney leukocytes was collected and the cells were pelleted by centrifugation, 400 g, 4 ℃, and 5 min. An additional washing step in PBS was performed. In addition the cells were counted.

4.1.3 Cell culture

1.72×10^6 head kidney cells were added to each well and different combinations of fatty acids were added according to 2^4 -factorial design as the same with liver cells. These cultures were also incubated at 9 ℃ in the incubator device. After 24h, suspensions were first centrifuged at 3200 rpm, 6 ℃ for 6 min and then the supernatant were collected carefully and stored at -80 ℃ until the analysis by HPLC-MS/MS.

Table 4.1 Concentrations in ng/ml of various eicosanoids released in cL-15 medium by salmon head kidney cells after exposure to different PUFAs.

		LTB4	PGE2	6-keto-$PGF_{1\alpha}$	PGE3	Δ17-6-keto-$PGF_{1\alpha}$
LA+AA+EPA+DHA	1.1	1.032	0.725	11.495	0.305	31.152
	1.2	0.902	0.924	17.876	0.584	3.294
	1.3	2.558	0.858	0.019	0.931	6.303
LA+AA+EPA	2.1	2.078	0.838	1.015	0.534	2.748
	2.2	1.712	0.763	1.066	0.486	4.864
	2.3	2.709	1.024	2.853	1.301	19.079
LA+AA+DHA	3.1	1.296	1.309	1.066	0.966	1.895
	3.2	1.157	0.896	0.023	1.010	0.061
	3.3	2.086	1.393	1.149	1.124	1.034
LA+AA	4.1	0.990	0.855	0.786	0.399	1.710
	4.2	0.545	1.308	0.044	0.528	0.082
	4.3	0.991	1.095	0.089	1.196	0.106
LA+EPA+DHA	5.1	0.477	0.045	1.443	0.916	2.677
	5.2	0.306	0.037	2.317	0.390	3.518
	5.3	0.792	0.166	9.929	1.677	5.746
LA+EPA	6.1	0.224	0.051	2.793	0.465	3.295
	6.2	0.296	0.037	2.012	0.357	5.822
	6.3	0.647	0.954	16.391	13.736	19.739
LA+DHA	7.1	0.260	0.022	5.821	0.467	3.693
	7.2	0.266	0.019	3.298	0.691	2.651
	7.3	0.182	0.051	6.880	1.053	1.852
LA	8.2	0.025	0.012	1.778	0.256	1.186
	8.2	0.031	0.019	3.160	0.215	4.748
	8.3	0.832	0.164	3.434	1.410	3.460
AA+EPA+DHA	9.1	2.057	1.025	10.560	0.531	27.114
	9.2	0.260	0.022	16.146	0.467	39.729
	9.3	2.883	0.885	53.727	1.214	68.589
AA+EPA	10.1	1.241	0.812	17.846	0.329	52.346
	10.2	1.103	0.993	11.275	0.408	33.132
	10.3	3.296	1.036	29.956	1.624	28.281
AA+DHA	11.1	1.101	1.004	21.904	0.372	35.263
	11.2	0.850	0.836	21.170	0.298	38.661
	11.3	2.963	1.376	32.968	1.693	54.500
AA	12.1	1.187	1.423	25.834	0.408	61.174
	12.2	0.465	1.525	8.921	0.952	42.691
	12.3	2.517	1.272	48.918	1.852	53.586
EPA+DHA	13.1	0.313	0.034	1.546	0.134	7.897
	13.2	0.348	0.030	2.996	0.328	6.509
	13.3	0.191	0.093	3.680	1.122	6.359
EPA	14.1	0.395	0.055	1.451	0.338	8.481
	14.2	0.237	0.056	1.403	0.267	9.566
	14.3	0.411	0.142	3.445	1.694	15.453
DHA	15.1	0.021	0.022	1.229	0.204	3.731
	15.2	0.039	0.020	2.370	0.254	2.707
	15.3	0.141	0.152	7.661	0.910	6.970
with ETOH	16.1	0.055	0.028	4.001	0.318	6.816
	16.2	0.075	0.026	4.182	0.242	4.011
	16.3	0.482	0.242	8.850	2.203	9.253

4.2 Results and discussion

The studied resolvins released in cL-15 medium by salmon head kidney cells were also not detected (similar results to the previous section by using salmon liver cells). The results showed in **Table 4.2 and Figure 4.1** reveal that LA reduces the production of Δ17-6-keto-$PGF_{1\alpha}$ and 6-keto-$PGF_{1\alpha}$ significantly. The production of PGE_3 is slightly increased while the remaining eicosanoids are not affected. The observed production trends for Δ17-6-keto-$PGF_{1\alpha}$ and PGE_3 have been already explained in the previous chapter, while the decreasing levels 6-keto-$PGF_{1\alpha}$ could be interpreted as a significant reduction of AA which is incorporated into the cell membrane with the subsequent releasing of EPA. The results for AA (**Table 4.2**, **Figure 4.1**) are similar to those reported for salmon liver cells (**Table 3.4**, **Figure 3.2**) except that the production of PGE_3 is unaffected.

Table 4.2 Multifactor ANOVA results with the significant effects (P- values) for the impact of PUFAs on the production of various eicosanoids by salmon head kidney cells.

	LTB_4	PGE_2	6-keto-$PGF_{1\alpha}$	PGE_3	Δ17-6-keto-$PGF_{1\alpha}$
LA	0.9599 ↑	0.7789 ↑	0.0004 ↓ *	0.3642 ↑	0.0000 ↓ *
AA	0.0000 ↑ *	0.0000 ↑ *	0.0006 ↑ *	0.4726 ↓	0.0000 ↑ *
EPA	0.1062 ↑	0.0392 ↓ *	0.9020 ↑	0.4312 ↑	0.2591 ↑
DHA	0.9897 ↑	0.0934 ↓	0.5685 ↑	0.3270 ↓	0.6282 ↓
LA*AA	0.4676 ↓	0.5826 ↓	0.0001 ↓ *	0.3095 ↓	0.0000 ↓ *
LA*EPA	0.6420 ↑	0.2190 ↑	0.2319 ↑	0.3361 ↑	0.1042 ↑
LA*DHA	0.9119 ↑	0.3791 ↑	0.7980 ↑	0.5867 ↓	0.6980 ↑
AA*EPA	0.4732 ↑	0.0024 ↓ *	0.8203 ↑	0.2811 ↓	0.7676 ↓
AA*DHA	0.8872 ↑	0.7144 ↓	0.4912 ↑	0.3642 ↑	0.4614 ↑
EPA*DHA	0.3625 ↓	0.5207 ↓	0.4768 ↑	0.3957 ↓	0.4958 ↑
LA*AA*EPA	0.6617 ↑	0.9302 ↓	0.6655 ↑	0.3305 ↓	0.1211 ↑
LA*AA*DHA	0.8142 ↓	0.0882 ↑	0.8972 ↑	0.4523 ↑	0.7294 ↑
LA*EPA*DHA	0.6072 ↓	0.4435 ↓	0.6472 ↓	0.2978 ↓	0.4414 ↓
AA*EPA*DHA	0.2158 ↓	0.8435 ↑	0.3445 ↑	0.4592 ↑	0.1695 ↑
LA*AA*EPA*DHA	0.5063 ↓	0.9285 ↑	0.7862 ↑	0.4610 ↑	0.6472 ↓

'*' indicates significant effect at confidence level of 95%

' ↑ ' indicates the mean concentration of corresponding eicosanoid with the addition of fatty acids is higher than that without fatty acids;

' ↓ ' indicates the mean concentration of corresponding eicosanoid with the addition of fatty acids is lower than that without fatty acids

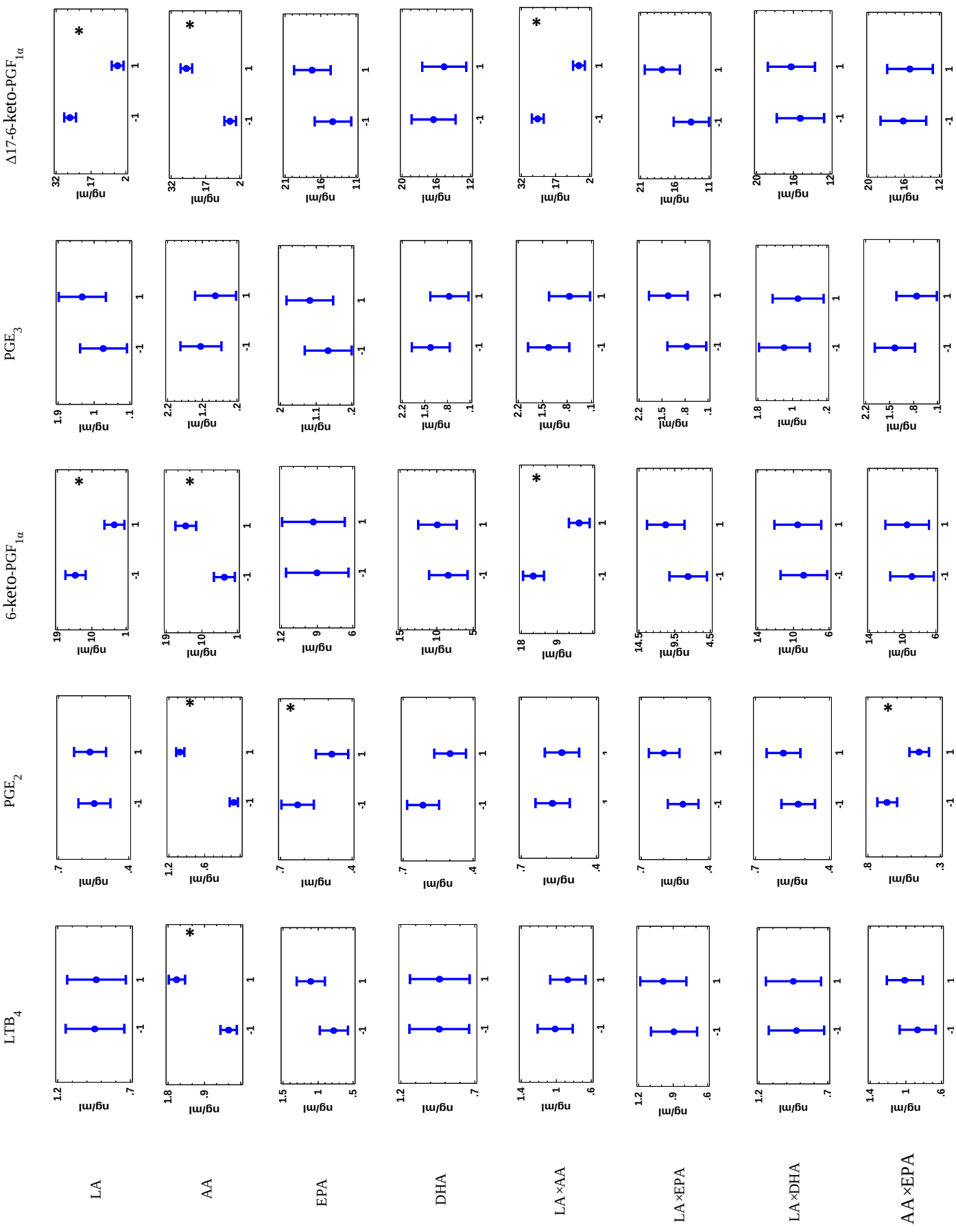


Figure 4.1-1 Mean concentration values with their 95% least significant intervals of different eicosanoids in cL-15 medium of salmon head kidney cells

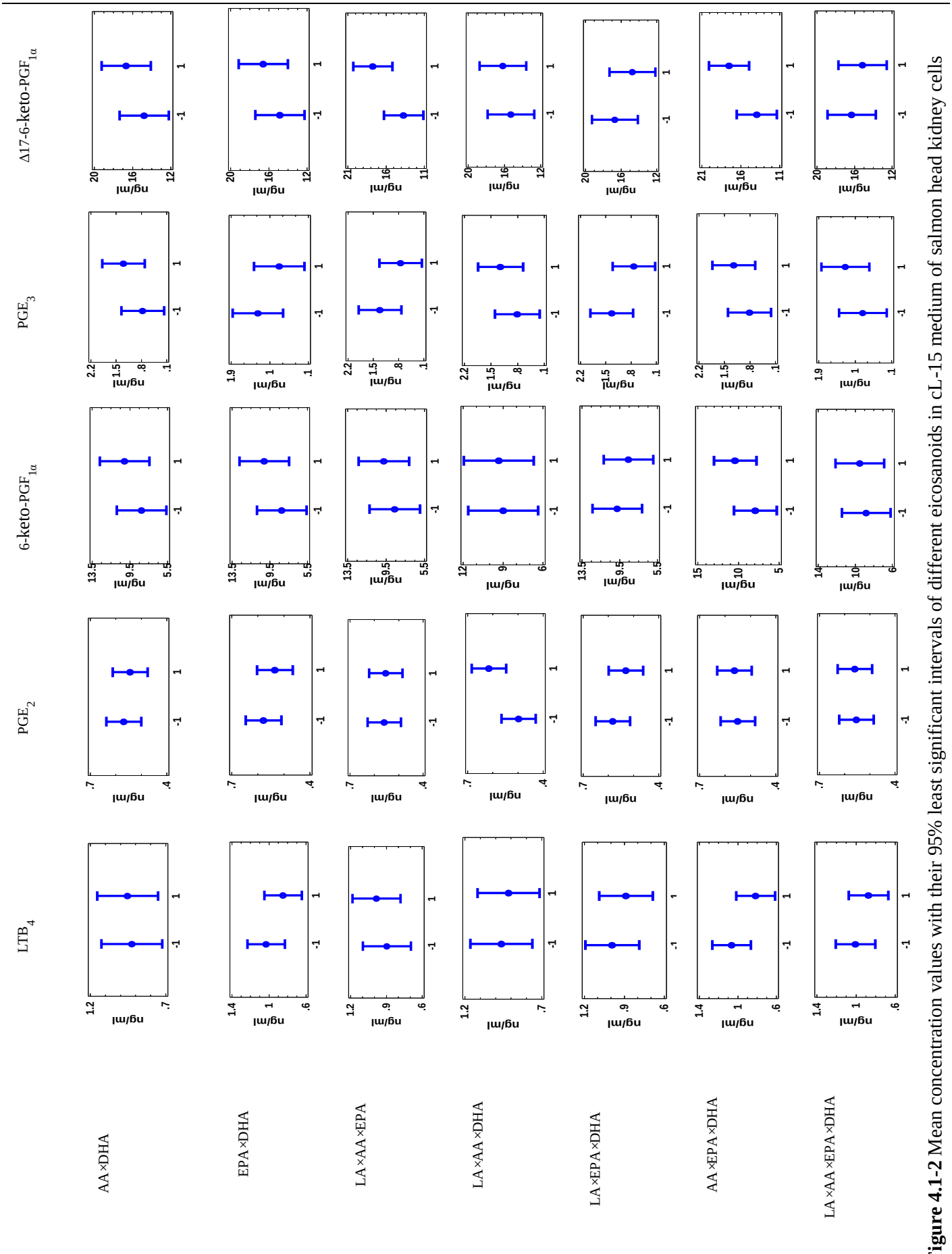


Figure 4.1-2 Mean concentration values with their 95% least significant intervals of different eicosanoids in cL-15 medium of salmon head kidney cells

The addition of EPA or DHA does not reveal any significant effect on the production of LTB_4, PGE_3, 6-keto-$PGF_{1\alpha}$ and Δ17-6-keto-$PGF_{1\alpha}$. However, the production of PGE_2 is markedly reduced by either EPA or DHA, possibly due to the released of AA from the membrane which in term is engaged in the LOX pathway rather than the COX. This observation seems to be supported by the slightly increased levels of LTB_4 after the addition of EPA.

The combined effect LA×AA is similar to the effect of pure LA, while the effect of AA×EPA is similar to the effect of pure EPA. The observed similarity could suggest similar production mechanisms.

4.3 Conclusions

In general, the results after exposing salmon head kidney cells to pure or combined LA, AA, EPA and DHA were similar to those reported for salmon liver cells. The main differences with respect to the previous chapter were the decreased production of PGE_2 (**Table 4.2**, **Figure 4.1**) after exposing the salmon head kidney cells to pure EPA. These observations could indicate that the two types of fish cells has the similar metabolism of PUFAs.

5. Application of the method to human umbilical vein endothelial cells

In the previous two chapters, the method is applied to two types of fish cells. There is slight difference for the effect of PUFAs on the production of eicosanoids in different types of cells, but in general, they are almost the same. In this chapter, the type of cells is changed from fish to human, and the effects of PUFAs on human cells are investigated.

5.1 Experimental

5.1.1 Reagents

Complete EBM-2 medium containing EBM^{TM}-2 basal medium supplemented with 0.1 % Heparin, 0.1 % R^3-IGF-1, 0.1 % ascorbic acid, 0.04 % Hydrocortisone, 0.4 % h-FGF-B, 0.1 % h-EGF, 0.1 % GA-1000 and 2 % FBS.

5.1.2 Cell culture

Human umbilical vein endothelial cells (HUVEC) were bought from Sigma-Aldrich and incubated in culture plate with complete EBM-2 medium. Once the number of cells reached 10^5 cells/well, the medium was removed. Different fatty acids, which were first attached to FBS and then dissolved in EBM-2 medium, were added according to 2^4-full factorial design. Cultures were incubated at 37 ℃ in humidified atmosphere of 95% air and 5% CO_2. After 24 h, the supernatant were collected and stored at -80 ℃ until the analysis by HPLC-MS/MS.

5.2 Results and discussion

Resolvins are also not detected in HUVEC. **Table 5.1** shows the concentrations of the other five eicosanoids. The results of multi-factor ANOVA and LSD test are shown in **Table 5.2** and **Figure 5.1** respectively.

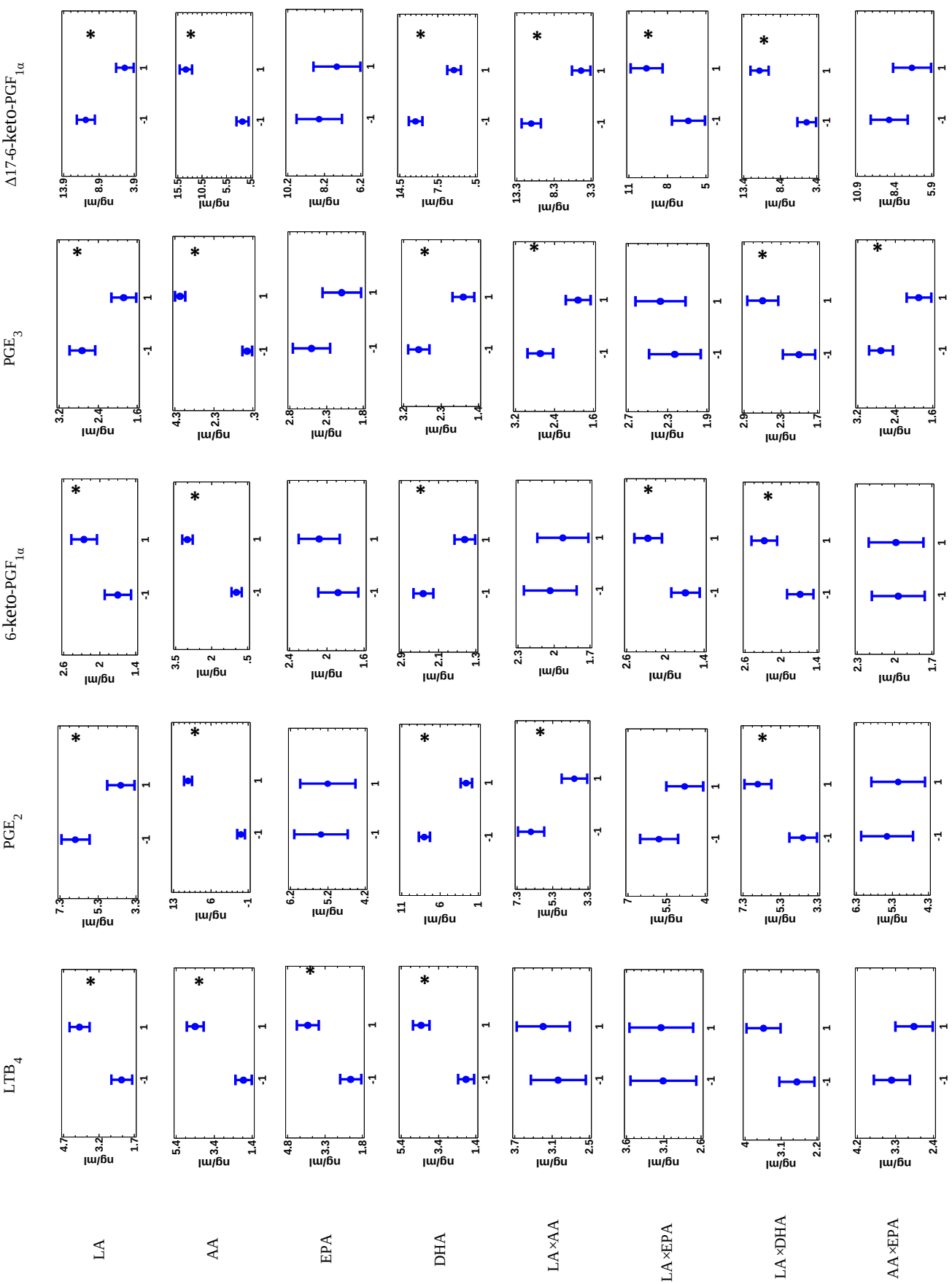


Figure 5.1-1 Mean concentration values with their 95% least significant intervals of different eicosanoids in EBM-2 medium of HUVEC

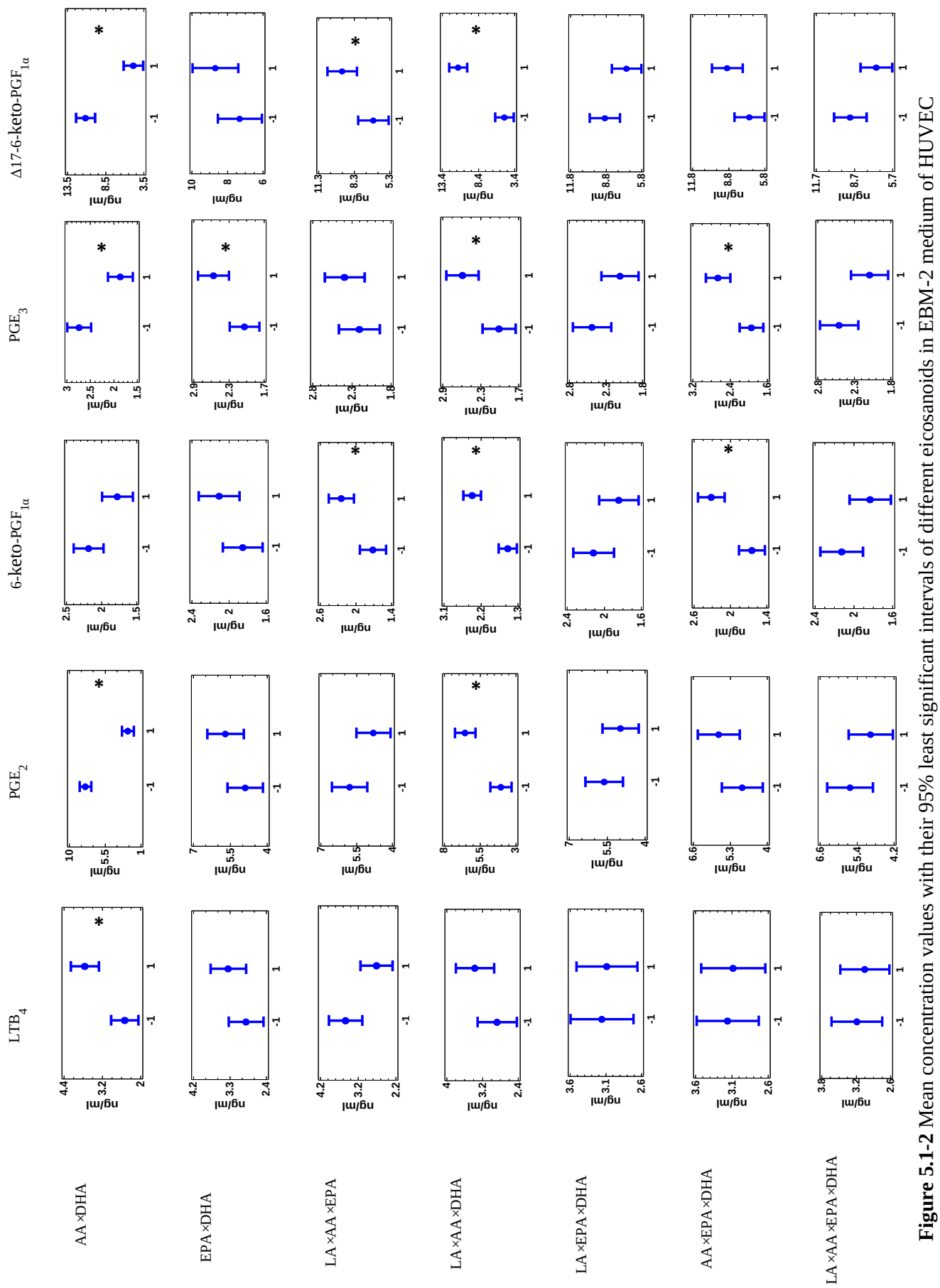


Figure 5.1-2 Mean concentration values with their 95% least significant intervals of different eicosanoids in EBM-2 medium of HUVEC

Table 5.1 Concentrations in ng/ml of various eicosanoids released in EBM-2 medium by HUVEC after exposure to different PUFAs

		LTB4	PGE2	6-keto-$PGF_{1\alpha}$	PGE3	Δ17-6-keto-$PGF_{1\alpha}$
LA+AA+EPA+DHA	1.1	11.190	4.510	2.797	2.845	9.449
	1.2	9.115	3.023	5.187	2.332	6.670
	1.3	4.281	3.885	4.503	2.605	10.204
LA+AA+EPA	2.1	2.778	9.870	4.787	2.858	15.033
	2.2	3.010	8.738	4.069	3.056	9.749
	2.3	3.315	9.592	2.052	2.859	5.099
LA+AA+DHA	3.1	8.509	6.369	1.661	2.804	7.264
	3.2	9.517	5.613	2.422	2.548	3.267
	3.3	4.875	5.973	3.326	3.766	7.590
LA+AA	4.1	1.610	17.206	2.653	5.659	5.263
	4.2	4.177	13.345	3.327	4.242	5.796
	4.3	2.567	6.076	2.004	2.883	6.611
LA+EPA+DHA	5.1	4.887	0.254	0.742	0.652	1.110
	5.2	4.931	0.296	0.605	0.648	2.964
	5.3	5.809	0.308	1.157	0.343	4.387
LA+EPA	6.1	6.393	0.775	2.721	0.889	3.184
	6.2	1.200	0.318	1.627	0.964	3.549
	6.3	1.801	0.426	1.573	0.779	4.838
LA+DHA	7.1	0.936	0.173	0.466	0.319	0.946
	7.2	1.965	0.151	0.805	0.341	1.824
	7.3	1.633	0.166	1.710	0.385	2.843
LA	8.2	0.771	0.354	1.649	0.315	1.166
	8.2	0.595	0.399	0.956	0.331	2.167
	8.3	0.457	0.376	1.424	0.465	3.927
AA+EPA+DHA	9.1	6.110	4.151	1.436	2.968	5.500
	9.2	5.548	9.890	1.943	4.074	6.853
	9.3					
AA+EPA	10.1	4.081	20.468	2.689	2.951	26.638
	10.2	1.147	20.077	3.193	6.307	31.578
	10.3	3.535	19.056	3.019	4.530	17.796
AA+DHA	11.1	3.153	1.087	1.261	2.102	8.591
	11.2	3.581	1.467	0.719	3.692	6.776
	11.3	3.053	5.116	1.286	3.306	7.040
AA	12.1	0.856	18.788	5.248	10.791	56.405
	12.2	2.237	29.011	6.776	7.396	34.969
	12.3	1.393	16.155	4.027	6.430	31.214
EPA+DHA	13.1	1.846	0.243	0.293	0.857	0.511
	13.2	3.225	0.317	0.465	0.616	0.914
	13.3	2.142	0.273	0.326	0.372	0.698
EPA	14.1	1.677	0.427	0.660	0.900	1.603
	14.2	0.738	0.379	1.407	1.277	1.698
	14.3	0.999	0.360	0.967	0.948	3.778
DHA	15.1	0.290	0.255	0.245	0.492	0.636
	15.2	0.534	0.203	1.118	0.343	2.290
	15.3	0.824	0.165	0.616	0.355	1.460
with ETOH	16.1	0.177	0.163	0.346	0.273	1.660
	16.2	0.154	0.211	0.389	0.411	1.812
	16.3	0.207	0.304	0.574	0.458	1.710

Table 5.2 Multifactor ANOVA results with the significant effects (P- values) for the impact of PUFAs on the production of various eicosanoids by HUVEC

	LTB_4	PGE_2	6-keto-$PGF_{1\alpha}$	PGE_3	Δ17-6-keto-$PGF_{1\alpha}$
LA	0.0002 ↑ *	0.0022 ↓ *	0.0127 ↑ *	0.0021 ↓ *	0.0001 ↓ *
AA	0.0000 ↑ *	0.0000 ↑ *	0.0000 ↑ *	0.0000 ↑ *	0.0000 ↑ *
EPA	0.0003 ↑ *	0.7969 ↓	0.3454 ↑	0.1125 ↓	0.4411 ↓
DHA	0.0000 ↑ *	0.0000 ↓ *	0.0002 ↓ *	0.0002 ↓ *	0.0000 ↓ *
LA*AA	0.5716 ↑	0.0018 ↓ *	0.6251 ↓	0.0044 ↓ *	0.0000 ↓ *
LA*EPA	0.9474 ↑	0.1743 ↓	0.0105 ↑ *	0.5701 ↑	0.0131 ↑ *
LA*DHA	0.0594 ↑	0.0019 ↑ *	0.0082 ↑ *	0.0259 ↑ *	0.0000 ↑ *
AA*EPA	0.2245 ↓	0.6711 ↓	0.9364 ↑	0.0032 ↓ *	0.2255 ↓
AA*DHA	0.0060 ↑ *	0.0000 ↓ *	0.0628 ↓	0.0016 ↓ *	0.0000 ↓ *
EPA*DHA	0.3099 ↑	0.2703 ↑	0.2407 ↑	0.0453 ↑ *	0.2755 ↑
LA*AA*EPA	0.0628 ↓	0.1723 ↓	0.0181 ↑ *	0.4623 ↑	0.0415 ↑ *
LA*AA*DHA	0.2514 ↑	0.0014 ↑ *	0.0003 ↑ *	0.0296 ↑ *	0.0000 ↑ *
LA*EPA*DHA	0.8730 ↓	0.3721 ↓	0.2097 ↓	0.1412 ↓	0.1496 ↓
AA*EPA*DHA	0.8574 ↓	0.2560 ↑	0.0036 ↑ *	0.0082 ↑ *	0.1470 ↑
LA*AA*EPA*DHA	0.7480 ↓	0.3568 ↓	0.1776 ↓	0.1104 ↓	0.1073 ↓

'*' indicates significant effect at confidence level of 95%

' ↑ ' indicates the mean concentration of corresponding eicosanoid with the addition of fatty acids is higher than that without fatty acids;

' ↓ ' indicates the mean concentration of corresponding eicosanoid with the addition of fatty acids is lower than that without fatty acids

In general, HUVEC are more sensitive than fish cells. Hence, the results revealed more significant effects. LA significantly decreases the production of EPA-derived eicosanoids and PGE_2, while it increases the production of LTB_4 and 6-keto-$PGF_{1\alpha}$. This result may indicate that during the incubation period, LA is incorporated into cell membranes releasing some ω-6 fatty acids and consequently the production of EPA-derived eicosanoids is reduced and AA-derived eicosanoids enhanced. However, the production of PGE_2 which is one of COX product of AA is decreased indicating that prostacyclin may be the major product of COX in this case.

The effects of AA and EPA have the similar behavior with those in fish cells. DHA remarkably increases the levels of eicosanoids from LOX pathway and significantly reduces the levels of eicosanoids from COX. These observations could indicate that DHA is incorporated into cell membrane during the incubation period by releasing AA which in turn is mainly enrolled in the LOX pathway.

The interactions LA×DHA, EPA×DHA and LA×AA×DHA increase the production of PGE_2, PGE_3, LTB_4, 6-keto-$PGF_{1\alpha}$, Δ17-6-keto-$PGF_{1\alpha}$. It is evident that these interaction terms exhibit a behavior similar to single AA. The terms AA×DHA, LA×AA exhibit a similar behavior to pure DHA, increase the production of LTB_4 and decrease the production of PGE_2, PGE_3, 6-keto-$PGF_{1\alpha}$ and Δ17-6-keto-$PGF_{1\alpha}$. These similarities for the combined and pure PUFAs could indicate a similar metabolic fate. The interactions LA×EPA and LA×AA×EPA bring about a mainly markedly increased on the production of the two ω-3 and ω-6 derived prostacyclins (Δ17-6-keto-$PGF_{1\alpha}$ and 6-keto-$PGF_{1\alpha}$ respectively).

5.3 Conclusions

It is evident from the results portrayed in the present section that human cells behave differently from salmon cells when exposed to the same set of pure or combined PUFAs. Considering that the analyzed salmon cells (head kidney and liver) exhibited a similar eicosanoid production behavior which was different from human cells, then it is possible to conclude that the metabolic fate of PUFAs is mainly dictated by the type of organism .

6. Conclusions and further prospectives

In this thesis, a new SPE-HPLC-MS/MS method was firstly developed to determine simultaneously prostaglandins (PGE_2, PGE_3), leukotriene (LTB_4), protacyclins (6-keto-$PGF_{1\alpha}$, Δ17-6-keto-$PGF_{1\alpha}$) and resolvins (RvD_1 and RvD_2) released in cL-15 and EBM-2 medium by fish and human cells respectively. The proposed method was optimized systematically and validated. The optimal concentrations of the various internal standards, defined as those where the response factors remain constant in the whole analytical range were determined using a Doehlert design.

The method validation carried out by using the optimal concentrations of internal standards showed that the developed SPE-HPLC-MS/MS method was highly selective, precise, accurate with acceptable LOD (0.1-2 ng/ml) and LOQ (0.1-8 ng/ml).

The validated method was successfully applied to analyze the levels of eicosanoids released into different culture media by fish and human cells exposed to different combination of PUFAs.

It must be said that this work is the first approach to compare the production of eicosanoids released into media by fish and human cells.

The change trend of each eicosanoids in different types of cells are summarized in **Table 6.1**. In general, AA, one of the most studied PUFAs in the current literature due to its implication in various pathological processes, has the tendency to increase the production of PGE_2, PGE_3, LTB_4, 6-keto-$PGF_{1\alpha}$, Δ17-6-keto-$PGF_{1\alpha}$, and the addition of EPA markedly increased the production of LTB_4 in both fish and human cells. The behaviour of LA was similar towards the production of prostacyclin in fish cells. However, its behaviour was different when human cells were analyzed, indicating that the type and production of eicosanoids are affected by both the avaiablity of fatty acids and type of cells.

Finally, it must be said that several improvements could be introduced in future research aiming at study the impact of PUFAs on the production of eicosanoids. Firstly, it would be convenient to analyze RvD_3 and RvD_4 (detected in the present work but their identity not confirmed) considering that RvD_1 and RvD_2 were not released into the media by any types of the studied cells. Secondly, the cells could be incubated for a longer period of times since the incorporation and releasing of PUFAs in and from cell membranes is time-dependent. Thirdly, the ratio between different ω-3 and ω-6 PUFAs should be explored considering that the incorporation of PUFAs in the cell membrane is dose-dependent.

Table 6.1 Summary of the variation of different eicosanoids generated by fish liver cell (LC), fish head kidney cell (HKC) and HUVEC

	LTB_4			PGE_2			6-keto-$PGF_{1\alpha}$			PGE_3			Δ17-6-keto-$PGF_{1\alpha}$		
	LC	HKC	HUVEC	LC	HKC	HUVEC	LC	HKC	HUVEC	LC	HKC	HUVEC	LC	HKC	HUVEC
LA	↓	–	↑*	↓	–	↓*	↓	↓*	↑*	↑	↑	↓*	↓*	↓*	↓*
AA	↑	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↑*	↓	↑*	↑*	↑*	↑*
EPA	↑*	↑	↑*	↑	↓*	–	↑	–	↑	↑	↑	↓	↑	↑	↓
DHA	↑*	–	↑*	–	↓	↓*	–	↑	↓*	–	↓	↓*	↓	↓	↓*
LA×AA	↑	↓	↑	↓	↓	↓*	↓	↓*	↓	↑	↓	↓*	↓	↓*	↓*
LA×EPA	↓	↑	–	↑	↑	↓	↓	↑	↑*	↑	↑	↑	–	↑	↑*
LA×DHA	↓	–	↑	↑	↑	↑*	↑	–	↑*	↑	↓	↑*	↑	–	↑*
AA×EPA	↓*	↑	↓	↑	↓*	–	↑	–	↑	↑	↓	↓*	↑	–	↓
AA×DHA	↑	–	↑*	–	–	↓*	–	↑	↓	↓	↑	↓*	↓	↑	↓*
EPA×DHA	↓*	↓	↑	↓	↓	↑	–	↑	↑	↓	↓	↑*	↓	↑	↑
LA×AA×EPA	↓	↑	↓	↑	-	↓	–	↑	↑*	↑	↓	↑	–	↑	↑*
LA×AA×DHA	↓	–	↑	↑	↑	↑*	–	–	↑*	–	↑	↑*	↑	-	↑*
LA×EPA×DHA	↓	↓	–	↑	↓	↓	↑	↓	↓	↑	↓	↓	↑*	↓	↓
AA×EPA×DHA	↓	↓	–	↓	-	↑	↑	↑	↑*	↓	↑	↑*	↑	↑	↑
LA×AA×EPA×DHA	↓*	↓	–	–	–	↓	–	–	↓	↑	↑	↓	↑	↓	↓

'*' indicates significant effect at confidence level of 95%

'↑' indicates the addition of corresponding PUFAS increases the concentration of corresponding eicosanoid;

'↓' indicates the addition of corresponding PUFAs reduces the concentration of corresponding eicosanoid;

'-' indicates P-values in multifactor ANOVA is higher than 0.7 which means there is no obvious changes

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Appendix I

Statistical analysis to validate the recovery range (60-120%) for PGE_3 and LTB_4 when the nominal concentration is 50 ng/ml

$$z = \frac{x-u}{\sigma} \qquad f(z) = \frac{1}{\sqrt{2\pi}} e^{-\frac{z^2}{2}}$$

% recovery	expected concentrations(x) (ng/ml)	z-values	f(z)
120	60	1.65	0.10
18	59	1.54	0.12
16	58	1.43	0.14
114	57	1.32	0.17
112	56	1.21	0.19
110	55	1.10	0.22
108	54	0.99	0.24
106	53	0.88	0.27
104	52	0.77	0.30
102	51	0.66	0.32
100	50	0.55	0.34
98	49	0.44	0.36
96	48	0.33	0.38
94	47	0.22	0.39
92	46	0.11	0.40
88	44	-0.11	0.40
86	43	-0.22	0.39
84	42	-0.33	0.38
82	41	-0.44	0.36
80	40	-0.55	0.34
78	39	-0.66	0.32
76	38	-0.77	0.30
74	37	-0.88	0.27
72	36	-0.99	0.24
70	35	-1.10	0.22
68	34	-1.21	0.19
66	33	-1.32	0.17
64	32	-1.43	0.14
62	31	-1.54	0.12
60	30	-1.65	0.10
Mean (μ)	**45**		
Standard deviation (σ)	**9**