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Abstract

Eicosanoids – oxidative derivatives from arachidonic acid – represent biologically active lipid mediators in inflammatory processes. Different analytical methods treat eicosanoids analysis. Among which reverse phase liquid chromatography figures as the appropriate method for eicosanoids profiling. RP-HPLC for eicosanoids analysis is often conducted on C18 columns. Some studies focused on profiling one family of eicosanoids, other considered all eicosanoids families. In both cases, co-elution remained a major issue and detection in mass spectrometry partially resolves this problem. In fact, the mass transitions used to monitor eicosanoids species are not specific enough and many isobars can be listed. For this, optimizing the RP-HPLC separation remains important. Based on the parameter F_s – deriving from hydrophobic-subtraction model – and radar plots, we chose columns with different selectivity. The hydrophobic-subtraction model guided our interpretation of molecular interactions between eicosanoids and stationary phases. We founded our approach for selectivity optimization on peak capacity per minute and time needed values. Herein, we screened seven stationary phases and evaluated their chromatographic performances in RP-HPLC. Stationary phases presented different chemistry, type of silica, length, and particles size. Superficially porous particles columns registered better chromatographic profiles than classical stationary phases; and columns with embedded polar group did not serve our purpose. The stationary phase Accucore C30 – even being the least retentive – revealed the best selectivity, efficiency and recorded the shorter duration for eicosanoids analysis.

Keywords: Accucore C30, eicosanoids, hydrophobic-subtraction model, selectivity, peak capacity per minute, time needed.

1 Introduction

Eicosanoids are highly active biological lipids. They are involved in many pharmacological and physiopathological processes. In mammals, eicosanoids mediate a wide array of biological processes and diseases such as atherosclerosis, diabetes, Alzheimer, cancer, etc. [1]. Literature mentions three eicosanoids precursors, which are Arachidonic Acid (AA), Eicosapentaenoic Acid (EPA) and Di-Homo Gamma Linolenic Acid (DGLA). These precursors are implicated in the synthesis of three subfamilies of eicosanoids as following: prostaglandins, thromboxanes and leukotrienes [2]. Pathways giving rise to eicosanoids are involved in inflammatory processes. For decades, non-steroidal anti-inflammatory drugs such as acetylsalicylic acid and ibuprofen have been used to inhibit prostanoids formation in order to treat inflammatory diseases [3]. Eicosanoids occur at low concentrations – picomolar and nanomolar – in biological matrices and are highly active molecules [4]. They present a limited stability since they are lipid mediators in response to specific biological processes. Eicosanoids play opposite or redundant roles, most often leading to a targeted response, which is the result of a whole cascade of cellular signaling. The global balance between different species of polyunsaturated fatty acids (PUFA) seems to modulate various biological processes. Due to their inconstant presence, their low quantity in biological fluids, and the multiplicity of eicosanoids species in different subfamilies, their analysis remains an analytical challenge.

Among several conducted approaches, literature describes the immunoenzymatic assays for eicosanoids quantification [5]. Immunoenzymatic assays are limited in the availability, specificity and selectivity of antibodies [4, 6–8]. In addition, oxidation occurs in biological processes and complicates eicosanoids quantification. VanRollins and VanderNoot used capillary electrophoresis to quantify EETs/DHETs regioisomers and stereoisomers and they underlined selectivity problems between structurally close species [9]. Gas chromatography coupled to tandem mass spectrometry (GC-MS/MS) was also extensively performed [4] and allowed, after a derivatization step, to reach a detection limit at the pg/mL level. Because of the three labile functions of eicosanoids and the difficulty to use the same derivatization agent [10], these previously described techniques are nowadays forsaken in favor of liquid chromatography. High performance liquid chromatography (HPLC) methods coupled with tandem mass spectrometry permitted to identify and quantify eicosanoids with considerable time saving, better sensitivity, specificity and above all covering a wide range of mediators. Despite the advances in liquid chromatography, selectivity problems persist between structurally close eicosanoids such as mono species of prostaglandins, hydroxyeicosatetraenoic acid (HETE). Eicosanoid studies in RP-HPLC treated quantitative or qualitative profiling of one subfamily of eicosanoids (for

example prostanoids); regioisomers separation optimizing (for EET and DiHETE); and sample preparation [11, 12]. To shorten analysis time, Ubhayasekera, *et al.* proposed a novel approach for eicosanoids analysis in SFC-MS/MS on five AA metabolites [13]. However, Berkecz, *et al.* concluded in a comparison study that UHPLC/MS offers a better separation and higher sensitivity compared to UHPSFC/MS for oxylipins analysis in human plasma [14]. Detection of eicosanoids is frequently carried out in mass spectrometry [1, 15]. Rarely, other detectors are encountered such as fluorescence [16] and UV through a derivatization with a reagent which absorbs light at a higher wavelength [17]. Mass transitions used to monitor eicosanoids species are not specific enough and many isobars can be listed. Besides isobars, ion suppression phenomena occurs also and complicates quantification. Hence optimizing the RP-HPLC dimension remains important. Herein, we focused on stationary phase's selection to optimize chromatographic profiling of eicosanoids. In order to increase chromatographic efficiency and selectivity , the development of stationary phases experienced a great rise and a wide range of stationary phases is currently available. Among which C18 columns are the most used for eicosanoids analysis according to literature, and columns with embedded polar groups (EPG) [18] and partially porous particles [2] performed the best chromatographic profiles. Faced by this diversity , the choice of the appropriate stationary phase is a key step of chromatographic method development [19].

To develop our eicosanoids separation we carefully selected a restricted set of stationary phases from which different retention properties were expected, we also selected specific species of eicosanoids involved in atherosclerosis. Eicosanoids mediators have opposite and redundant properties. It is the overall balance between various oxygenated species which modulates inflammatory processes. For understanding their roles as potential biomarkers for disease diagnosis or prognosis, it is important to carefully choose the species for the method reliability. Thus, eicosanoids which were chosen to elucidate disease pathways must be related to metabolmics scheme.

In order to explain the retention mechanism in RP-HPLC, numerous theories have been developed. Among them, the solvophobic theory describes the phenomenon responsible for solute retention as a reversible association process between the hydrocarbonaceous ligand anchored to the surface and the solute molecule [20]. However, solvophobic theory does not allow to elucidate the total interactions involved in RP-HPLC separation. Other models tended to explain retention in RP-HPLC among the hydrophobic subtraction model [21]. Herein, seven RP-HPLC stationary phases were selected on the basis of predicted selectivity according to hydrophobic-subtraction model and radar plots illustrations. Chromatographic performances

such as selectivity and other descriptors including time needed and peak capacity per minute (both explained in the theory / calculation section) were also evaluated.

2 Theory / Calculation

We referred to HPLC database [22] and picked up predicted values to characterize the selectivity of the seven stationary phases. The parameter F_s – deriving from hydrophobic-subtraction model – permitted to choose columns with different selectivity. Time needed and peak capacity per minute guided selectivity optimization.

2.1 Peak capacity per minute

Resolution describes only the separation performance of two consecutive peaks and does not allow describing at fair value the performance of the whole separation. Horvath and Lipsky described peak capacity in 1967 as the most common criterion to measure chromatographic separation capacity [23].

In 2005, Neue defined peak capacity P_c as the number of peaks that can be separated within a retention window ranking from t_1 to t_2 [24]. The peak width represents four times the value of the standard deviation of a peak (4σ). Peak capacity can be expressed in the following integral form:

$$\mathbf{P_c = 1 + \int_{t_0}^{t_r} \frac{1}{4\sigma} dt} \quad \textbf{(1)}$$

Where t_r is the retention time of the solute and t_0 is the hold-up time, or unretained time [24].

Other equations derive from equation (1) and consider variables which influence chromatographic behavior whether in isocratic or gradient elution modes. Numerous variables such as retention mechanisms, structure of molecules and stationary phases influence the width of the peak. Thereby, all factors – which contribute to spread or compress peak – should be considered to determine the quality of separation in gradient elution. Assuming that the peaks width in the chromatogram is similar, the integration and simplification of equation (1) yield to [24]:

$$\mathbf{P_c = 1 + \frac{t_g}{\left(\frac{1}{n}\right)\sum_1^n \omega}} \quad \textbf{(2)}$$

where t_g is the gradient run time, n is the number of selected peaks and w the width of each peak – selected for the calculation – in the chromatogram.

For the calculation, selected peaks need to be representative of the distribution of the peak width throughout the chromatogram. Neue [24] simply considers peak capacity as the gradient run time divided by the average peak width.

In order to normalize different gradient elution modes between linear gradient and optimized gradient for each stationary phase, we defined an additional parameter of peak capacity per minute. This parameter allowed us to normalize data and enabled a better comparison between stationary phases. Thus, peak capacity per minute is the measure of the number of peaks that can be contained in one-minute elution time window.

$$\mathbf{P_c/_{min}} = \frac{\mathbf{P_c}}{\mathbf{t_g}} \quad \textbf{(3)}$$

By combining equation (2) and (3), we deduced equation (4) as following:

$$\mathbf{P_c/_{min}} = \frac{1+\frac{\mathbf{t_g}}{\left(\frac{1}{\mathbf{n}}\right)\sum_{\mathbf{1}}^{\mathbf{n}}\boldsymbol{\omega}}}{\mathbf{t_g}} = \frac{1}{t_g} + \frac{1}{\left(\frac{1}{\mathbf{n}}\right)\sum_{\mathbf{1}}^{\mathbf{n}}\boldsymbol{\omega}} \quad \textbf{(4)}$$

2.2 Time needed

Besides peak capacity, time needed is another important criterion to consider in chromatography. To optimize selectivity in high-pressure liquid chromatography, P.J. Schoenmakers defined the time needed ($[t_{ne}]_{f,d}$) as the minimal time required for separation of complex mixture with a minimal selectivity [25]. The minimum time required ($[t_{ne}]_{f,d}$) depends of the retention factor and the minimum selectivity as follows:

$$[\mathbf{t_{ne}}]_{\mathbf{f,d}} = \frac{\mathbf{1+k_\omega}}{\mathbf{S^2_{min}}} \quad \textbf{(5)}$$

Where f = constant flow rate; d = constant particle diameter

k_ω = retention factor of the last peak in the chromatogram,

and S_{min} = selectivity of the critical pair of peaks within the chromatogram.

3 Experimental

3.1 Chemicals and columns

3.1.1 Chemicals

Water and isopropanol HPLC-MS grade were purchased respectively from VWR and Biosolve. Acetonitrile and methanol both HPLC grade from Sigma-Aldrich were used for HPLC analysis. Molecules including eicosanoids, related oxidized and two eicosanoids precursors were purchased from Cayman chemicals (Ann Arbor, MI, USA). The study includes the following twenty six molecules: 6-keto PGF1α , PGI2 , TXB1, TXB2, PGE3 , PGF2α, PGE2, PGD2, LXA4, (±)14(15)-DiHETE , LTB4 , LTD4, (±)5(6)-DiHETE, (±)5-HEPE, (±)20-HDHA, (±)9-HODE, (±)15-HETE, (±)12-HETE , (±)11-HETE , (±)8-HETE, (±)5-HETE, (±)8(9)-EET, Carbocyclic Thromboxane A2 or CTA2, (±)14(15)-EET, Eicosapentaenoic Acid or EPA, Arachidonic Acid or AA. It is important to note that two mixtures were preconceived by Cayman Chemistry: Primary Eicosanoid HPLC Mixture (6-keto PGF1α; TXB1; PGF2α; PGE2; PGD2) and (±) HETE mixture ((±)15-HETE; (±)12-HETE ; (±)11-HETE ; (±)8-HETE; (±)5-HETE). Eicosanoids' structures are summarized in Electronic Supplementary Material 1, ESM_1, table 1.

3.1.2 Stationary phases

Various column chemistries (C18, C30, and Polar-RP) were tested as listed in table 1. Accucore C30 and Hypersil Gold C18 were purchased from ThermoFisher Scientific (Langerwehe, Germany). ThermoFisher Scientific lent us Accucore C18 and Accucore Polar Premium columns. Cortecs Shield RP18 was kindly provided by its manufacturer Waters (Milford, PA, USA). Zorbax Bonus RP was purchased from Agilent technologies (Little Fall, NJ, USA) and Nucleodur C18 Isis was purchased from Machery Nagel (Düren, Germany). The main characteristics of the silica particles and the chemistry of stationary phases are detailed in Table 1.

3.2 Sample preparation

All standards were evaporated under a stream of nitrogen and solubilized in methanol of HPLC grade to have a concentration of 50 µg/mL, then stored at -80°C. In addition to the preconceived

mixture (HPLC mixture and (±) HETE mixture), another mixture was prepared at a concentration of 50 µg/mL and gathered eicosanoid standards and eicosanoid precursors (AA and EPA).

3.3 Chromatographic conditions

Samples were analyzed by an UHPLC system (Thermofisher Scientific Ultimate 3000) coupled with a Charged Aerosol Detector (Corona-CAD®) equipped with a nitrogen generator. Corona-CAD® parameters were set as follow: gas pressure: 35 Psi; total flow = 1.53; flow ratio = 0.39; electrometer heater = 35°C; corona voltage =2.34 kV; Icor = 1,00Ua; IonT = 20,4 V; range = 100 pA. To compare the columns chromatographic performances for eicosanoids separation in RP-HPLC, two different approaches were investigated. First, we evaluated a linear gradient from 0% to 95% solvent B in 26 min (see Electronic Supplementary Material 2, ESM_2 table1). Secondly, the system solvent and gradient was optimized to ensure the best separation for each column. All eluents such as mobile phase A and mobile phase B were degassed prior to their usage. In linear gradient, phase A consisted in Water/Acetonitrile/Formic acid (69.96:30:0.04, v/v/v) and phase B in Acetonitrile/ Formic Acid (99.96:0.04, v/v). All methods were developed with chromatographic conditions as shown in **see Electronic Supplementary Material, ESM_2, table 3**, including stationary phases and solvents composition. Temperature was set at 25°C and injection volume was set to 1 µL with a flow rate of 0.5 mL/min. Temperature and pH were also optimized.

Table 1 The characteristics of the seven columns used for the development of eicosanoids separation by RP-HPLC

Supplier	***Thermofisher Scientific***	***Thermofisher Scientific***	***Thermofisher Scientific***	***Waters***	***Agilent***	***Machery Nagel***	***Thermofisher Scientific***
Columns	***Partially porous silica***				***Fully porous silica***		
	Accucore™ C30	Accucore™ C18	Accucore™ C18 Polar	Cortecs Shield RP 18	Zorbax BONUS-RP	Nucleodur C18 Isis	Hypersil Gold C18
Packing Material	Solid core particle. C30	Solid core particle. C18	Solid core particle. C18. Embedded amide group	Solid core particle. C18. Embedded carbamate group	Bonus-RP with an embedded amide linkage in the C14-alkyl chain	C18. Ultrapure Silica	C18. Ultrapure Silica
Pore size (Å)	150	80	150	90	80	110	175
Particule size (µm)	2.6	2.6	2.6	1 .6	3.5	1.8	3
Inner diameter (mm)	2.1	2.1	2.1	2.1	2.1	2.0	2.1
Length (mm)	100	100	100	100	50	50	50
Surface area (m^2/g)	90	130	90	100	180	340	220
Carbone load (%)	5	9	9	6.4	9.5	20	11
End capped	Yes	Yes	Yes	Yes	Triple	Yes	Yes

Table 2 The predicted values in hydrophobic-subtraction model to characterize stationary phases' selectivity [22]

Name	**Silica type**	**H**	**S***	**A**	**B**	**C (pH 2.8)**	**C (pH 7.0)**
Accucore C30	B	0.978	-0.02	-0.143	-0.002	0.321	0.462
Accucore C18	B	1.09	0.054	0.055	-0.04	0.072	0.095
Accucore Polar Premium	EP	0.871	0.103	-0.567	0.217	-0.207	0.787
Cortecs UPLC Shield RP18	EP	0.869	-0.023	-0.28	0.1	-0.19	0.096
Zorbax Bonus RP	EP	0.65	0.1	-1.04	0.37	-2.97	-1.1
Nucleodur Isis	B	1.02	0.05	-0.07	-0.02	-0.01	0.15
Hypersil GOLD	B	0.88	0	-0.01	0.03	0.16	0.47

4 Results and discussion

Eicosanoids analysis needs to combine precise, sensitive and high-throughput methods. Literature focused on eicosanoids implication in several diseases [1, 3, 26], but it is less common to find a comparison study resolving selectivity issues between eicosanoids isomers and isobars. Studies underlined numerous co-elutions [2, 6, 27]. Even if some studies covered around 170 eicosanoids [2, 6], very few eicosanoids were detected and quantified in human plasma [2, 13, 18], urine [1, 26], cells supernatants [8, 10, 27] and biological fluids [6].

4.1 Targeted eicosanoids species

As our project aims to analyze eicosanoids related to atherosclerosis, we targeted the selection on 24 different species. Some prostanoids such as PGF2α and PGI2 are vasoactive lipid mediators while 6-Keto-PGF1α is an inactive form of PGI2. As, PGI2 with low half time [28] is rarely found in biological fluid, we selected 6-Keto PGF1α as it might be a marker of the transient presence of PGI2. We have also included HETEs species, LXA4 and LTB4. Indeed, LXA4 level differs in function of disease [26] and is known in literature as leukocyte activation factor and chemotaxis effects. HODE involved in the inflammation resolution in several disease and EET – Cytochrome P450 product of AA– may potently induce arteriolar dilatation. In addition, the chosen molecules mimic a complex mixture as some present very close structures. HETEs species are isomers and isobaric compounds. PGE2 and PGD2 are isomers and isobaric molecules. TXB1 and TXB2 share the same structure and differs in one unsaturation.

4.2 Column selection systems in RP-HPLC

To perform eicosanoids separation, we selected columns with different selectivity based on column selection systems especially Hydrophobic Subtraction Model. Retention mechanism in RP-HPLC is strongly linked to phenomena at the molecular level. Molecules' structure induces particular interactions which should be considered before choosing the appropriate stationary phase. To date, HPLC database [22] report around 751 RP-HPLC columns with large differences in selectivity. Most of them subtly differ in terms of selectivity, but the difference is often sufficient to achieve a required separation [19].

Several column selection system (CSS) were developed and guided chromatographers in environmental, biological, pharmaceutical molecules separation, etc. Most of them evaluate column performance through a ratio between the retention factor of a given molecule and a

reference molecule which represents the interaction occurring during chromatographic process. Based on the ratio of k factor between polar or basic analytes and test compounds, Engelhardt column selection system (CSS) provided interest information on silanol accessibility [19]. Drawing inspiration from Engelhardt CSS, Tanaka, *et al.* developed another system which highlights additional interactions such as hydrogen bonding capacity and shape selectivity. To select a suitable column with EPG for acid or basic solutes, Layne, *et al.* CSS has been shown to be advantageous. From 26 to 8 chromatographic probes, Euerby modified Tanaka CSS and defined the column difference factor (CDF) as the euclidian distance between a given column and an *a priori* selected reference column [19]. Herein, Euerby CSS does not serve our purpose as it is difficult to predict which stationary phase can be chosen as a reference column for eicosanoids isomers and isobars separation. Indeed, the size, polarity, rigidity, planarity and unsaturation's degree owing to eicosanoids structures makes difficult the selection of a reference column.

A relevant quantitative approach for the prediction of chromatographic retention from the molecular structure appeared in 1970 and was known as quantitative structure-retention relationships (QSRRs). In this model introduced by Abraham [21], linear free energy relationships deriving from solvophobic parameters, are used to predict the chromatographic retention factors for columns. However, the model do not cover parameters that increase with retention such as: the shape selectivity, ionic interactions of the cation exchange type and π- π complexation phenomena.

Hydrophobic subtraction model (HSM) was described by Snyder and co-workers to complete Abraham model. HSM model makes consensus in the scientific community, as shown in many applications for column selectivity [19, 21, 29, 30].

We have used Hydrophobic Subtraction Model (HSM) and selected 7 columns. In our case, analyzing eicosanoids without a derivatization step can only be conducted by a universal detector or Mass spectrometry (MS). All reference compounds in CSS are volatile and cannot be detected by corona CAD®. Consequently, it is less practical to perform the comparison of column selectivity through most of CSS in the context of eicosanoids.

The columns predicted selectivity available on HPLC database [22], was practical to classify columns. All the interactions during chromatographic process are reported in the database through different selectivity according to HSM parameters. Indeed, this model was described to choose a column of very different selectivity and to select replacement columns that will give the same separation. Overlapping peaks oblige to change or improve column selectivity. The strength and weakness of HSM model is that the columns are ranked versus 67

chromatographic probes which are representative of the different solute-stationary phase interactions. Other studies reduced the test compounds to 18 and decreased the redundancy of some solutes in HSM [31]. In our case, eicosanoids exhibit a very few structure variability. The close structure of eicosanoids steered our approach towards a thoughtful choice of columns with different selectivity. To take into account the close structure and select columns with different selectivity, we used *Fs* parameter [29]. This permitted to reduce the number of chosen columns while covering different selectivities. We kept in mind that the Euclidean distance F_s expresses the difference in selectivity. One should not forget that for a same F_s value, the underlying parameters might differ.

4.3 The hydrophobic-subtraction model in RP-HPLC

Retention was attributed to solvophobic or hydrophobic interactions [20]. Hydrophobic interactions and silanol activity contribute to retention but are not enough to define it. Several models tended to explain retention in RP-HPLC. Snyder, *et al.* described the hydrophobic-subtraction model – equation 6 – [21] to reflect the contributions from other types of solute–column interactions.

$$\log\alpha \equiv \log\left(\frac{k}{k_{EB}}\right) = \eta' H - \sigma' S^* + \beta' A + \alpha' B + \kappa' C \quad (6)$$

(i) (ii) (iii) (iv) (v)

The parameters H, S*, A, B, C are specific to each column: H (hydrophobicity), S* (steric resistance to insertion of bulky solute molecules into the stationary phase), A (measurement of hydrogen-bond acidity precisely attributed to non-ionized silanol activity), B (measurement of hydrogen-bond basicity), C (evaluation of cation exchange interaction between chemical groups of solute and ionized silanol). Factors η', σ', β ', α', κ ' are specific to each solute and vary with the hydrophobicity, the configuration or the spatial geometry of the molecule and the presence of acid and/or basic groups. Furthermore, it should be noted that the terms (i), (iii), (iv) and (v) express attractive interactions, which are therefore positive. Only, the term (ii) relates to a repulsive interaction, which is negative. It is important to note that all these molecular interactions define the selectivity between molecules during reverse phase separations.

4.3.1 Comparison of columns selectivity from HPLC database

Based on hydrophobic subtraction model, various column chemistries (C18, C30, and Polar-RP) were selected as listed in table 1. Among seven columns, three are totally porous particles columns (Hypersil Gold C18, Nucleodur Isis C18 and Zorbax Fusion RP) and four are superficially porous particles columns (Accucore C30, Accucore C18, Accucore Polar Premium, and Cortecs Shield C18). In addition, three columns are polar embedded reversed-phase columns (Accucore Polar Premium, Cortecs Shield C18 and Zorbax Fusion RP..Table 2 summarizes the predicted values of H, S*, A, B, C for each stationary phase [22]. We did not consider the C term at pH=7 since the mobile phase contains formic acid. Polar embedded reversed-phase columns – Accucore Polar Premium, Cortecs Shield C18 and Zorbax Bonus RP – show the less hydrophobicity (H values). They also engage less acid hydrogen-bond (A values) with an acceptor solute and they present the highest basicity. The embedded polar group (EPG) increases basicity. EPG are amide for Accucore Polar Premium and Zorbax Bonus RP, and carbamate for Cortecs Shield C18 (the amide basicity is higher than carbamate) [30]. In addition, both Accucore Polar Premium and Zorbax Bonus RP record the greatest steric resistance (S*).

The polar embedded reversed-phase columns are the least likely to establish cation exchange interactions with analytes at acid pH. The limited number of EPG in these columns does not allow to neutralize utterly the silanol activity at pH 7 (**C**) as demonstrated by Snyder [21]. By referring to C values for Accucore C30 (0.321) and Accucore Polar Premium (-0.207) at pH 2.8, we can expect a strong cation exchange interaction for Accucore C30: the low percentage of grafting in Accucore C30 gives access to a greater amount of free silanol and could explain this observation.

The database [22] provides the parameter F_s to compare two columns each other. F_s is defined as the distance between two columns in the five dimensional plot (H, S*, A, B and C) [29, 30]. Overall, a F_s factor < 3 indicates that these two columns are very similar. A F_s > 3 indicates that these two columns are very different [30]. But one should not neglect that underlying parameters defining F_s might differ. F_s derives from hydrophobic-subtraction model and permits to choose columns with different selectivity. It is calculated according to the following equation:

$$F_S = \sqrt{\left(12.5(H_1 - H_2)\right)^2 + \left(100(S_1^* - S_2^*)\right)^2 + \left(30(A_1 - A_2)\right)^2 + \left(143(B_1 - B_2)\right)^2 + \left(83\left(C_{2.8_1} - C_{2.8_2}\right)\right)^2} \quad \textbf{(7)}$$

Weigthing factor varies in function of molecule structure, and depends especially on values of basicity and cation exchange interactions. Eicosanoids are molecules with polar function and hydrocarboneous chain. Since eicosanoids are acid carboxylic molecules except the cysteinyl leukotrienes –which contains amino acids–, we choose the default weighting factor for F_s calculation: 12.5, 100, 30, 143, 83 are respectively the default weighting factor which represent the difference in hydrophobicity, steric interactions, hydrogen bond acidity, hydrogen bond basicity, and charge interactions at pH 2.8.

According to F_s values (Table 3), the stationary phases Accucore C18 and C30 are similar. Both display equivalent F_s values with Hypersil Gold (F_s=1.15 and F_s=1.49 respectively). In addition, Hypersil Gold exhibits a comparable chromatographic behavior with Nucleodur Isis. Hence, one would expect a similar behavior between Accucore C30 and Nucleodur Isis: this is not completely true as Accucore C30 and Nucleodur Isis record an *Fs* value >3. Besides, EPG columns exhibit high F_s values in comparison with the four previously cited columns (Accucore C18, Accucore C30, Hypersil Gold and Nucleodur Isis are non-EPG columns). Among EPG columns, Zorbax Bonus RP – a C14 column with an amide group – is expected to be significantly different from all other stationary phases. Accucore Polar and Cortecs Shield are equivalent columns (F_s=3.02).

Even if *Fs* parameter was described to characterize column selectivity, some conclusions related to this parameter deserves more thorough examination. For example, Hypersil Gold and Accucore C30 would theoretically provide the same selectivity, same observation could be underlined between Nucleodur Isis and Accucore C18. For graphical visualization in terms of selectivity between selected columns, we normalized hydrophobic subtraction model data for each column to 1. Fig.1 illustrates the radar plot of normalized data. According to this plot, Nucleodur Isis and Accucore C18 can be discriminated through factor H and A. Except cation exchange interaction value and acid hydrogen bond value, Accucore C30 and Hypersil Gold did not share the same levels of other interactions in hydrophobic subtraction model. Comparison through both previously presented examples reveal that F_s parameter alone is not sufficient to compare columns selectivity. The radar plot displayed for each column alone (Fig.1) confirms the diversity of predicted selectivity of our selected columns. In addition, the best eicosanoids profile obtained in RP-HPLC with each column (section 4.6) showed the diversity behavior of column in terms of ability to separe isomers and isobars of oxylipins. Among classical columns, Nucleodur C18 Isis – an octadecylsilyl phase with crosslinked surface modification – enables fast separation of triacylglycerol regioisomers according to

Tchapla, *et al.* [32]. We included stationary phases with EPG in our columns set. The secondary polar interactions mediated by the polar embedded reverse-phase could help to discriminate eicosanoids isomers especially for prostanoids.

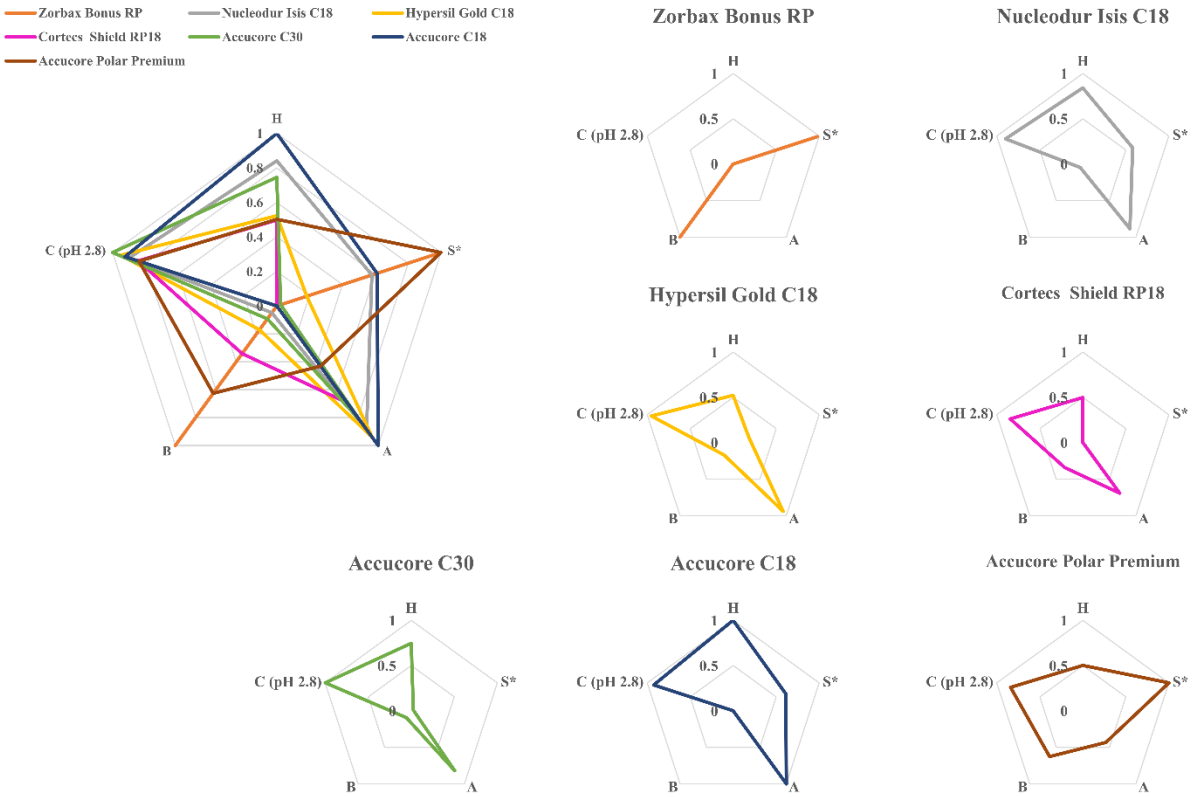

Fig.1: Radar plot representation of columns comparison based on predicted selectivity according to hydrophobic subtraction model. H: hydrophobicity, S*: spatial selectivity, A: hydrogen bond acidity, B: Basicity,and C: the degree of silanol ionization or cation-exchange capacity. Individual values are normalized to the highest value.

Table 3 *Fs* parameter calculated for the pairwise comparison of the seven tested columns*

Manufacturer	**Stationary phases**	*Accucore C30*	*Accucore C18*	*Accucore Polar*	*Cortecs Shield RP18*	*Zorbax Bonus RP*	*Nucleodur Isis C18*	*Hypersil GOLD C18*
Thermo/Hypersil	*Accucore C30*	0						
Thermo/Hypersil	*Accucore C18*	**3.61**	0					
Thermo/Hypersil	*Accucore Polar*	18.52	14.1	0				
Waters	*Cortecs Shield RP18*	11.93	6.53	**3.02**	0			
Agilent	*Zorbax Bonus RP*	472.82	415.35	322.15	335.66	0		
Macherey Nagel	*Nucleodur Isis C18*	4.90	**0.57**	9.61	**3.44**	389.57	0	
Thermo/Hypersil	*Hypersil GOLD C18*	**1.49**	**1.15**	13.27	6.55	431.58	**1.67**	0

**Fs was calculated from hydrophobic-subtraction model values for each column at pH 2.8 (HPLC database)* [22] *with equation (7). We did not consider the C term at pH=7 since the mobile phase contains formic acid.*

4.4 Retention factor and efficiency evaluation

We screened the characteristics of 7 RP-HPLC stationary phases (Table 1) which offers different selectivity (Fig.1) to define which ones are suitable for eicosanoids isomers and isobars separation. Column assessment was conducted with respect to retention, efficiency and selectivity.

4.4.1 Analysis in linear gradient mode

Four representative molecules of the overall eicosanoids profile were chosen for retention factor calculation. Retention factor (k) is calculated for PGE2, LXA4, 5HETE and EPA. Among the seven columns, Nucleodur C18 Isis phase was the most retentive (Fig.2). The retention behavior of Nucleodur C18 correlated with a high percentage of carbon grafting (20%); whereas for other columns it ranges from 5% to 11%. Accucore C30 has the lowest carbon grafting percentage (5%) and was the least retentive.

Despite the substantial difference in carbon load, Hypersil Gold C18 and Accucore C18 (columns without EPG) as well as Cortecs Shield C18 and Accucore C18 Polar (EPG columns) exhibited comparable retention performance. In addition to the carbon load, which correlated with retention performance, the EPG mediated secondary polar interactions and contributed to the retention.

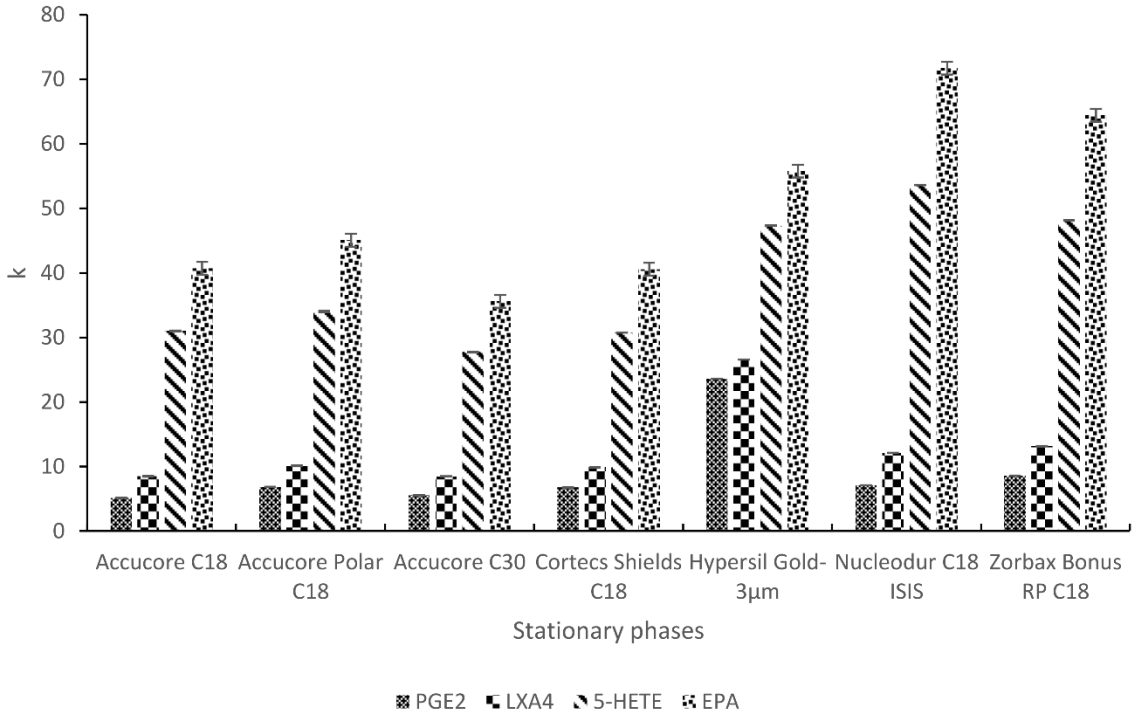

Fig.2: Average of retention factors for four eicosanoids (stationary phases evaluated in linear gradient). Mobile phase: A (Water:ACN, 70:30,v/v) containing 0.04% HCOOH; B(ACN,100,v) containing 0.04%HCOOH ; T = 25°C, average of k and standard deviation are calculated on triplicate.

4.4.2 Column separation efficiency

Among the seven RPLC columns, stationary phases with superficially porous particles provide a better efficiency compared to conventional columns (Fig.3). This is expected and is already well described in the literature [33]. Accucore C30 and Cortecs shield RP18 registered similar apparent plate heights and the best efficiency. These columns have an efficiency close to the sub-2 µm columns with a much lower pressure drop [33, 34]. The term A of Van Deemter equation for superficially porous particles was described merely as the homogenous packing and regular shapes of superficially porous particles [35, 36]. Guiochon, *et al.* [37, 38] also linked a narrow particle size distribution to high efficiency.

Accucore C30 and Accucore Polar present the same pore size (150 Å) but display different efficiency. This difference could be attributed to packaging defects causing a lack of homogeneity of the particles in the column, especially on the walls [33]. The difference in column chemistry of Accucore C30 and Accucore Polar (presence of EPG and length of grafted chains) could be another reason for this result. While superficially porous particles contributes to produce narrow peaks and allows more peaks to be separated; the nature of silica particles did not influence significantly the selectivity between isomers. Indeed, the

grafting chemistry is the main factor which contributes to isomers discrimination during chromatographic process.

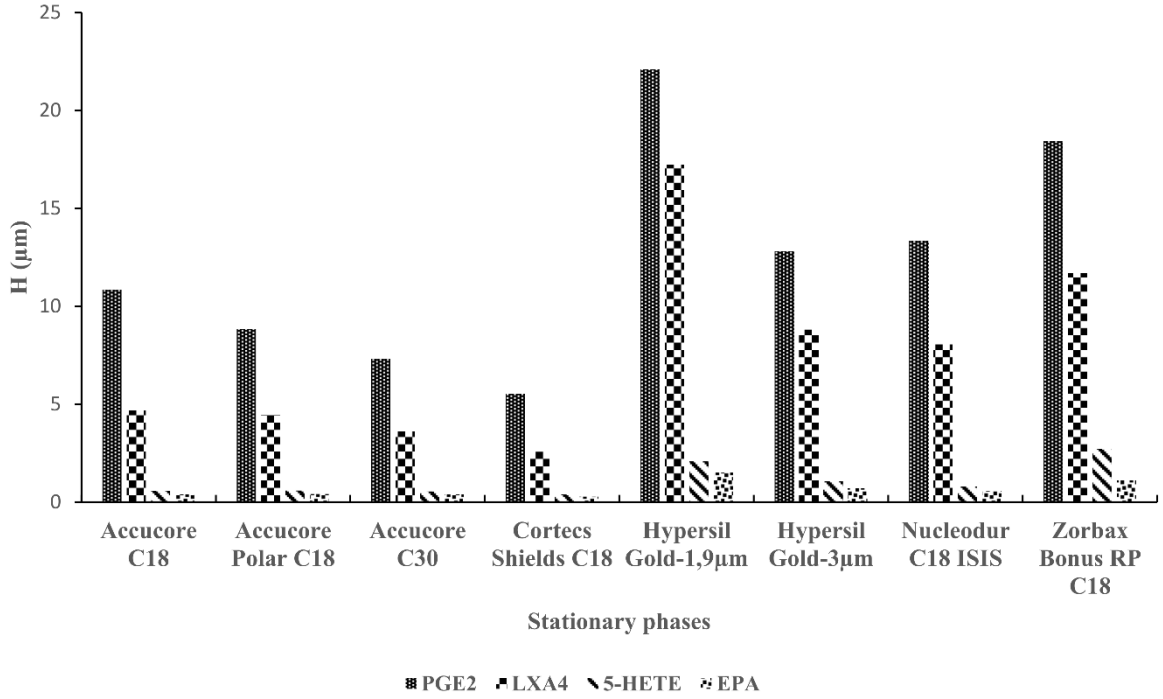

Fig.3: Efficiency assessment of seven stationary phases in the same gradient linear mode elution. Efficiency evaluation was performed through the parameter H for all columns. Mobile phase: A (Water:ACN, 70:30,v/v) containing 0.04% HCOOH; B(Acetonitrile,100,v) containing 0.04% HCOOH ; T = 25°C.

4.4.3 Differences in selectivity for eicosanoids separation

Figure 4 gathers stationary phases into two groups: on one hand, Cortecs Shield and Accucore columns (C18, Polar C18 and C30); and on the other hand, Hypersil Gold, Nucleodur Isis and Zorbax Bonus RP. Superficially porous particles columns emphasized the best selectivity: α value ranged from 1.3 to 2 (Fig.4). Accucore Polar premium and Cortecs Shield C18 – two superficially porous particles columns with EPG – exhibited a comparable selectivity and confirmed predicted F_s values previously. The similar chromatographic behavior between the latter three stationary phases is striking compared with what we presented above for F_s values. Different chromatographic behavior should have been observed. This could be explained by close interactions between prostanoids and stationary phases. A selectivity >1 between different species of prostanoids point out a difference in retention factor of molecules. These differences in retention are probably due to interactions between solutes and stationary phases and F_s values do not reflect it completely. Columns with EPG underlined two co-elutions among prostanoids (6-Keto-PGF1 alpha co-eluted with PGI2 and TXB1 co-eluted with TXB2) and other co-elutions in the full RPLC profile of eicosanoids. TXB1 and TXB2 share

the similar structures and differ in the number of unsaturation (Fig.4, structures). Unsaturation might not be susceptible to polar interactions. Based on the hydrophobic-subtraction model, the steric resistance to penetration opposed by grafts of EPG columns might be the main cause of the co-elution of TXB1 and TXB2. For our knowledge, literature never reported this information for eicosanoids. Thus, this observation confirms the predicted values of S (Table 2) for EPG columns.

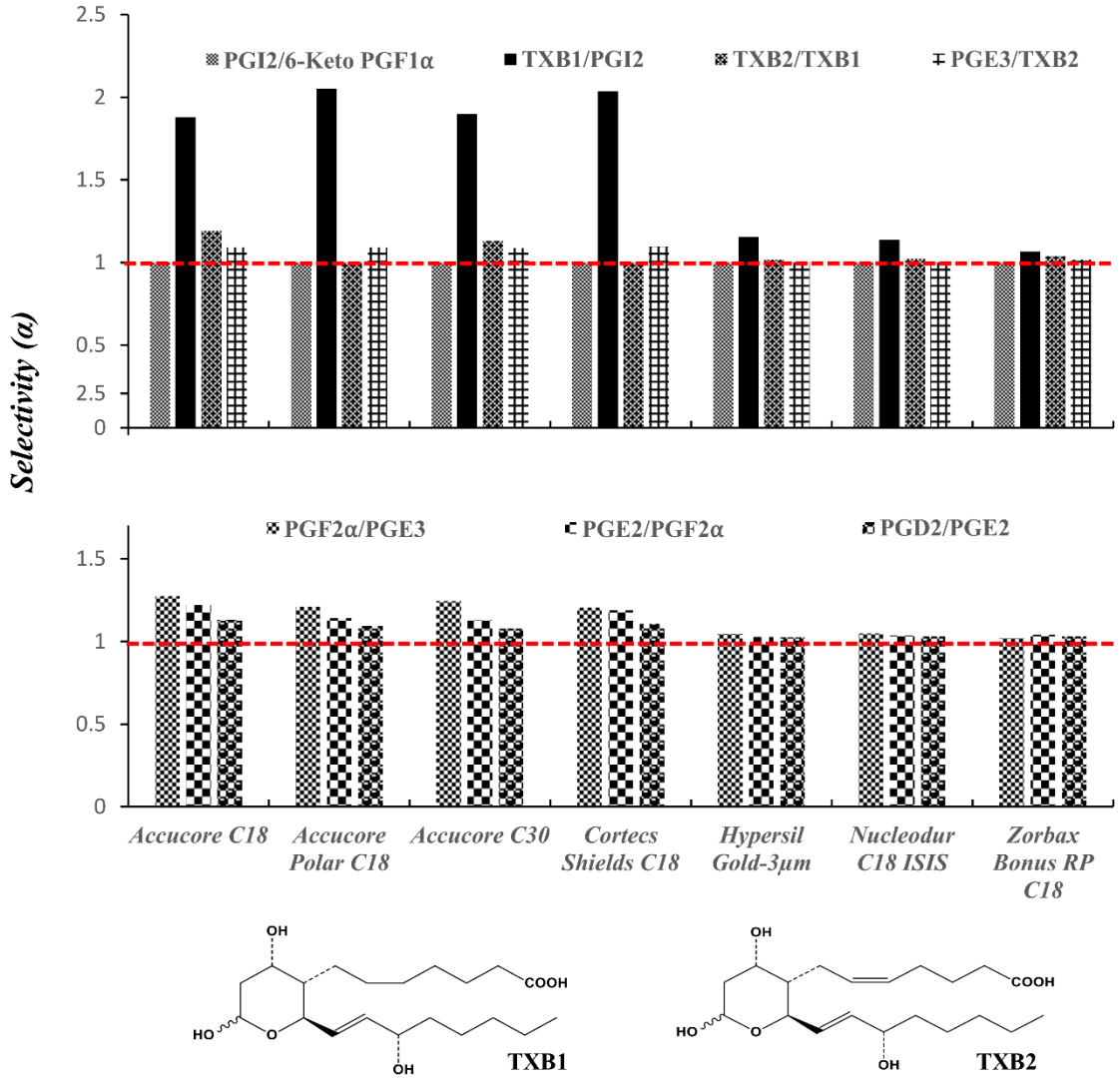

Fig.4: Column selectivity evaluated through prostanoids separation. Phase mobile: A (Water:ACN, 70:30,v/v) containing 0.04% HCOOH; B(Acetonitrile,100,v) containing 0.04%HCOOH ; T = 25°C.

Accucore C30 and Accucore C18 showed one co-elution between 6-keto PGF1α and PGI2. Superficially porous particles columns without EPG registered less co-elutions, come after superficially porous particles EPG columns and then classical columns. Steric selectivity and functional selectivity explain the columns' performances. Herein, we can underline that the functional selectivity characterized EPG columns.

To illustrate that the steric selectivity leading to TXB1 and TXB2 co-elution and as eicosanoids derive from C20 PUFA, we present in Fig.5 the possible molecular interactions between three PUFA (C18:1, C18:2 and C20:3) and reverse phase columns. As shown, each unsaturation of PUFA could induce a reduction of the docking area between the solute and the stationary phase and reduce retention. In fact, the number and the position of unsaturation would imply particular spatial geometry of the molecules while eluting. This geometry contributes to the resistance to penetration during the solute-stationary phase interaction. This phenomenon makes us wonder if the high grafting percentage would be a limiting factor for the penetration of polyunsaturated solutes? The previous question requires another investigation to understand the correlation between the polyunsaturated compounds and the carbon load in columns. Gilroy, *et al*. reported that S increases with increasing ligand length and concentration, and decreases with larger pore diameters [29]. Thus, these findings support our hypothesis on a possible relation between steric resistance and high grafting percentage.

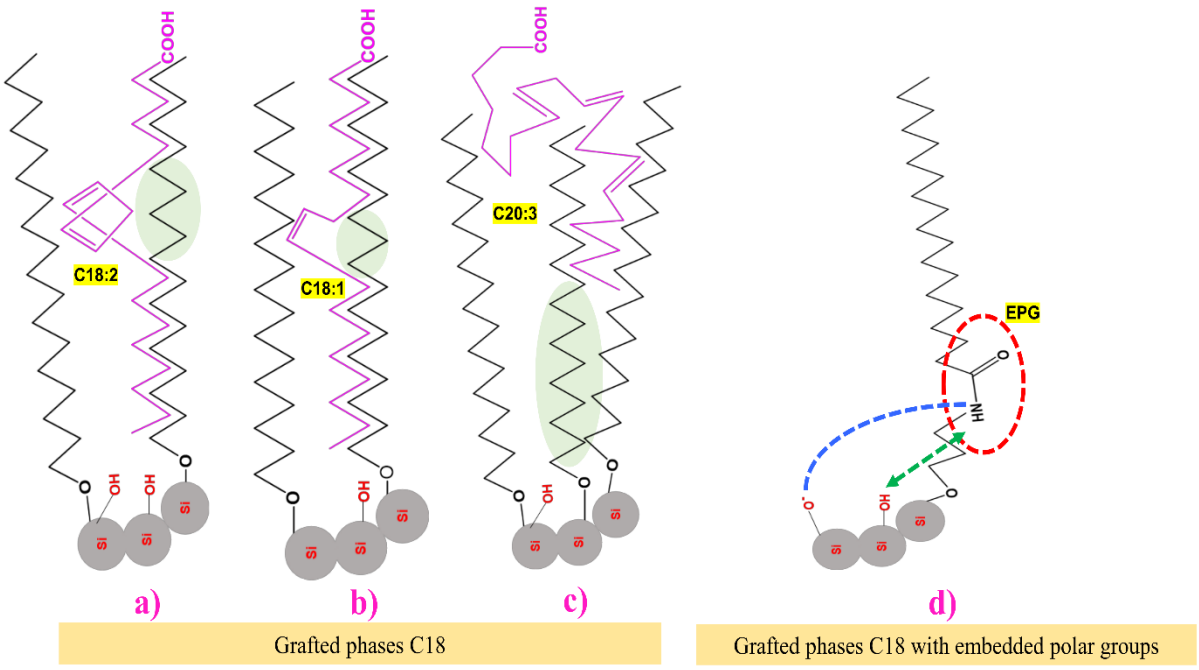

Fig.5: Retention mechanism of polyunsaturated fatty acids, steric resistance to penetration and secondary polar interactions on a C18 phase and a C18 phase with EPG. a, b and c show the influence of the unsaturations for polyunsaturated fatty acids on their retention through reverse phase stationary phase. The green arrows indicate the hydrogen bond between a silanol and the carboxyl group of the solute (a donor or acceptor group such as the carbamate of EPG). The blue arrows show the ionic interactions (cation exchange) between an ionized silanol and a group of either the stationary phase or solute. d is an illustration of a C18 grafted phase with EPG, which is susceptible to various interactions such as hydrogen bonding or ion exchange. The molecular interactions between three PUFA (C18:1 for oleic acid, C18:2 for linoleic acid, and C20:3 for homo-gamma-linolenic acid) and reverse phase columns during liquid chromatography separations are illustrated. As shown, each unsaturation of PUFA induces a reduction of the docking area between the solute and the stationary phase, thus implying a reduction in retention. In fact, the number and the position of the unsaturations would imply a particular geometry of the molecule resulting in a resistance to penetration during the solute-stationary phase interaction.

Indeed, literature highlights the solute penetration for triglycerides in the grafts of reverse phase columns [39, 40]. Analysis of the retention factors logarithm for triglycerides according to the number of carbon showed a derivation in slope from a certain number of carbon. The discontinuity in the linearity of the plot has been explained by Martin, *et al.* [39] as a change in conformation of the molecules during their penetration into the grafts of the stationary phases. Due to steric hindrance, the glyceric head is arranged in opposite to the direction of penetration. NMR and X-Ray spectroscopic studies of triglycerides structural conformations [41] showed that the two end chains were oriented in the same direction and the middle chain was in the opposite direction. Therefore, partial penetration occurs due to the steric hindrance and the presence of double bonds [39]. Consequently, we can deduce that multiple conformations of eicosanoids could occur during penetration within the grafts. Altogether, efficient separation of eicosanoids regioisomers or stereoisomers could be improved with a sufficient steric recognition by the grafts. Other interactions including hydrogen or ion exchange occur during a chromatographic separation (Fig.5.d). It concerns EPG columns especially. The outstanding selectivity of EPG columns as described [18] would come from the specific interactions such as hydrogen bond and ion exchange compared to columns without EPG. These results accord with the literature [42, 43].

4.5 Peak capacity and time needed

Two other chromatographic separation criteria – time needed and peak capacity – were applied to eicosanoids analysis and helped to discriminate our columns. Time needed and peak capacity descriptors permitted to evaluate selectivity and helped to measure the separation over the entire chromatographic domain. Peak capacity per minute illustrates the number of peaks that can be separated within a retention window and is a common criterion to measure chromatographic separation capacity. Indeed, time needed is a criterion to optimize selectivity.

4.5.1 Peak capacity

Peak capacity calculation is appropriate if the peak width is representative of all peaks in the chromatogram. All peak widths in our chromatographic profile were similar except 6-Keto PGF1α.

Results in Fig.6 exhibit a greatest peak capacity for optimized gradient compared to linear gradient. Accucore C30 and Cortecs Shield C18 revealed the highest peak capacity. This is expected as superficially porous particles properties improve the peak capacity.

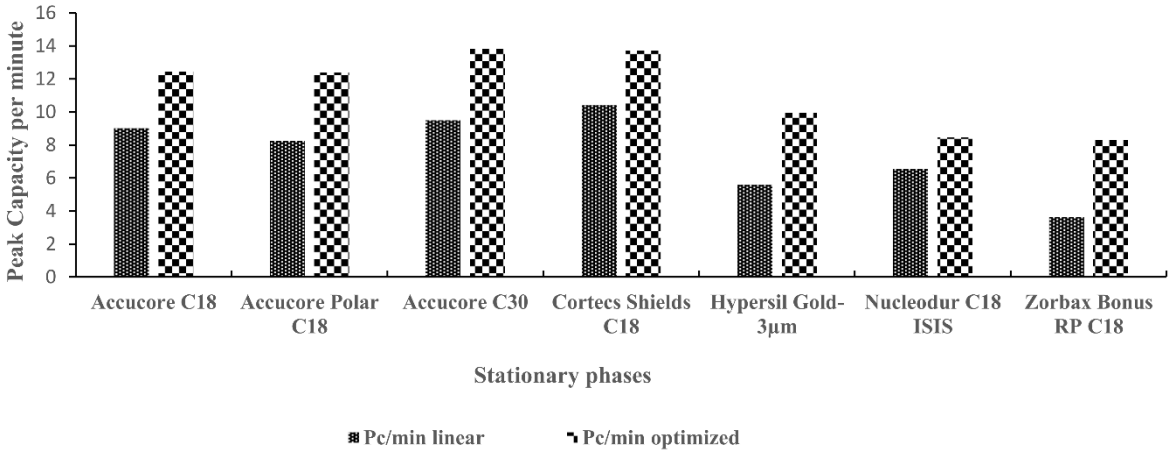

Fig.6: Peak capacity per minute for the seven stationary phases. Linear gradient (mobile phase: A (Water:ACN, 70:30,v/v) containing 0.04% HCOOH; B(Acetonitrile,100,v) containing 0.04%HCOOH ; T = 25°C ; V injec = 1µL ; Flow rate = 0.5 mL/min). For more details on optimized gradient conditions, see **Electronic Supplementary Material, ESM_2 table 2 and table 3.**

4.5.2 The time needed (t_{ne})

According to theorical calculation of time needed, Accucore C30 emerged as the fastest (15 min) for eicosanoids separation followed by Accucore C18 (18min). Zorbax Bonus-RP and Nucleodur Isis C18 stood out with a long-time analysis in optimized gradient (respectively 31 and 48 min: Fig.7).

Experimental results accorded with calculations. Results revealed that Accucore C30 separated all eicosanoids and PUFA in 6.7 minutes and the longest analysis took 9.8 minutes for Nucleodur C18. Accucore C30 was the fastest and the most selective column. Even if Accucore C30 and Cortecs Shield C18 had comparable peak capacity per minute in optimized gradient (Fig.8), Accucore C30 remained the best because of the minimal t_{ne} among all columns.

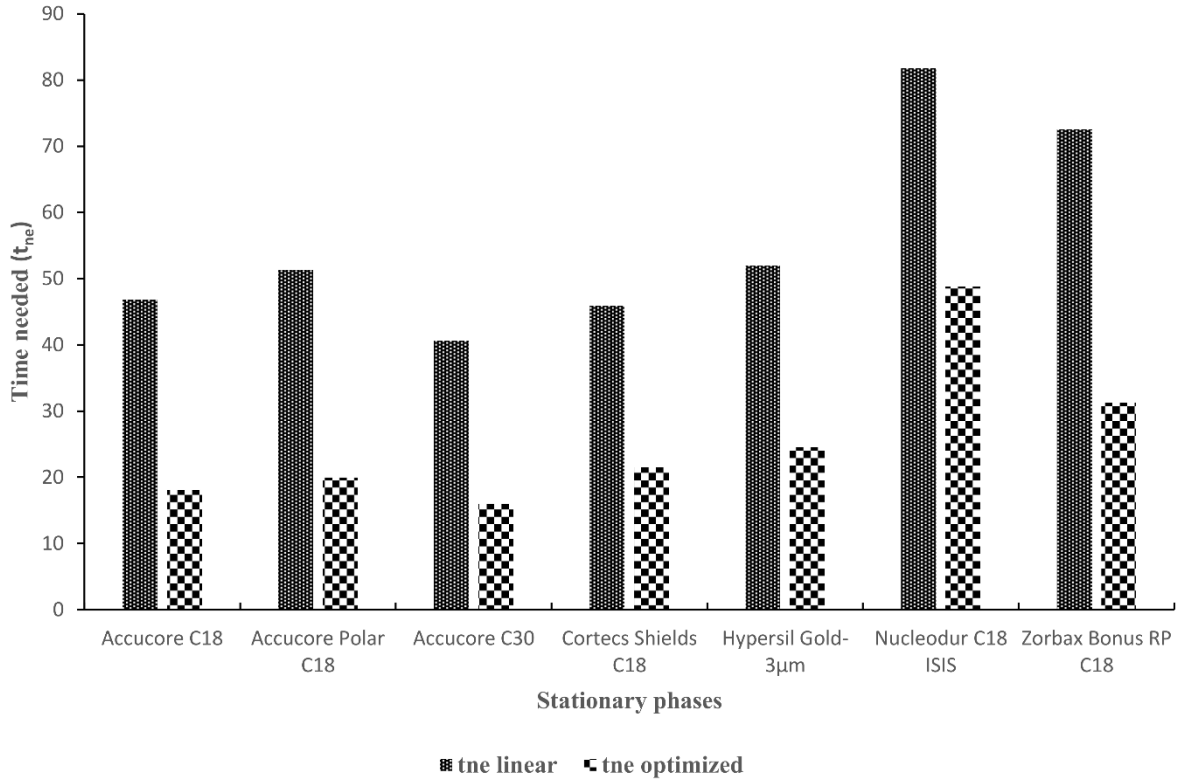

Fig.7: Evaluation of time needed for eicosanoids mixture separation within seven stationary phases. Linear gradient (Mobile phase: A (water:ACN, 70:30,v/v) containing 0.04% HCOOH; B(Acetonitrile,100,v) containing 0.04%HCOOH ; T = 25°C ; V injec = 1µL ; Flow rate = 0.5mL/min). For optimized gradient, see **Electronic Supplementary Material, ESM_2 table 2 and table 3.**

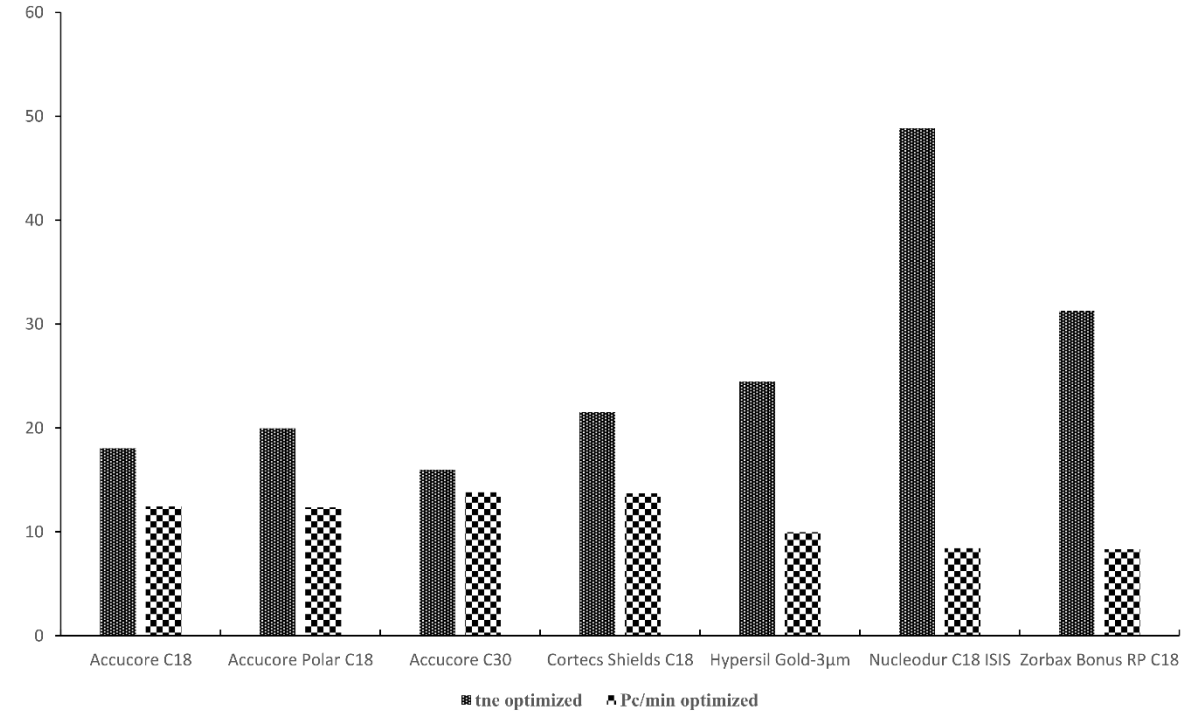

Fig.8: Summary of tne and peak capacity per minute for all stationary phases in optimized gradient. For Optimized gradient and chromatographic conditions, see **Electronic Supplementary Material, ESM_2 table 2 and table 3.**

4.6 Investigation of columns' dependent chromatographic profiles

Analysis of chromatographic profiles – obtained in optimized gradient for each column – underlines differences in selectivity. Classical columns (Fig.9.e, f, g) separated prostanoids with a resolution close to 1 and showed a co-elution of oxidized derivatives such as HETE and HODE. Eicosanoids profiling took between 8 to 10 minutes. For all fully porous particles column (Fig.9.e, f and g), several co-elutions emerged: thromboxanes (surrounded in dotted blue color), HETEs (encircled in dotted pink) , and EETs (encircled in dotted brown). Superficially porous particles stationary phases except Cortecs Shield C18 offered a profiling of 7 minutes and presented satisfactory selectivity for PGs and HETEs. We also noted some differences in elution: superficially porous particles EPG columns are unable to discriminate thromboxanes (TXB1 and TXB2) and epoxy-eicosatetraenoic acid ((±)14(15)-EET and (±)8(9)-EET). Conferring to the terms A, B, C of the hydrophobic subtraction model, co-elution observed for EPG columns could be due to secondary polar interactions between the EPG (and / or silanols) and eicosanoids.

In a target study of eicosanoids in the context of atherosclerosis conducted on 18 eicosanoids and comparing various column chemistries (C8, C18, PFP, Polar-RP), Rago, *et al.*[18] selected Synergi Fusion-RP column which offered the best overall selectivity and resolution for the panel of compounds. Synergi Fusion-RP column is a C18 column with ether linked phenyl as EPG which mediates secondary polar interactions with polar groups of oxylipins [18]. Our study conducted on 24 eicosanoids illustrated a better selectivity compared to [18] between 14,15-EET and 8,9-EET, 15-HETE and 8-HETE (subsection 4.7.2).

We calculated *Fs* value for Synergi Fusion-RP column. With a value of 2.74, this stationary phase presented a predicted selectivity equivalent to Hypersil Gold C18 and Nucleodur Isis C18.

Berkecz and co-wokers conducted a comparative study for profiling 64 oxylipins with BEH C18 column to evaluate the potential of both Ultra High Performance Liquid Chromatography (UHPLC) and Ultra High Performance Supercritic Fluid Chromatography (UHPSFC) [14]. Overall, the evaluation of isobaric and isomeric species selectivity by UHPSFC and UHPLC coupled to MS, revealed a better sensitivity for UHPLC [14]. However, some co-elutions persisted and some species recorded a lesser selectivity with BEH C18 column.

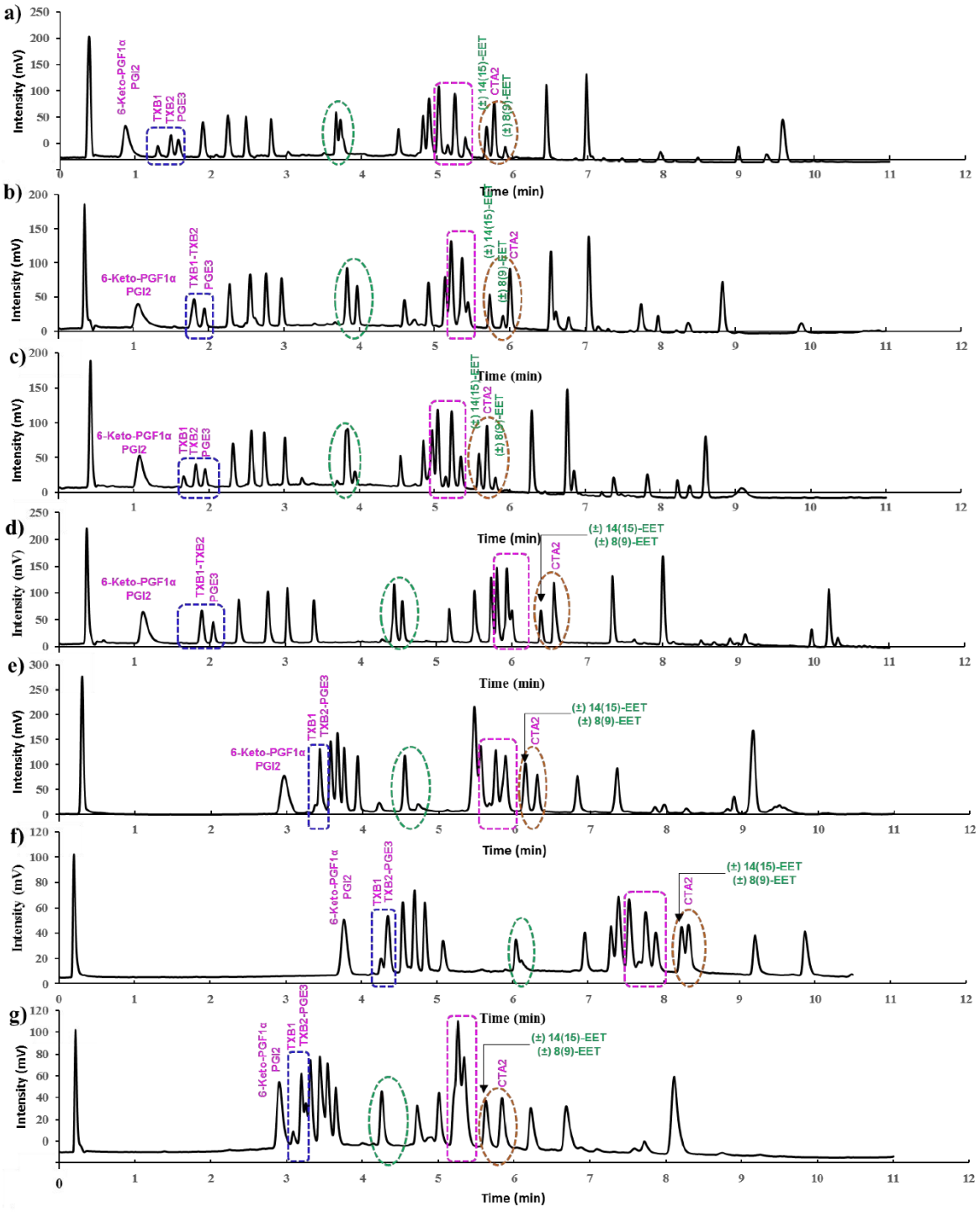

Fig.9: Chromatograms obtained in optimized gradient for each stationary phases. a) Accucore C18; b) Accucore Polar Premium c) Accucore C30; d) Cortecs Shield C18; e) Hypersil Gold C18-3µm; f) Nucleodur Isis C18; g) Zorbax Bonus RP. For mobile phases composition as well as details optimized gradients, refer to **Electronic Supplementary Material, ESM_2, table 2**. T = 25 ° C; V injected = 1µL; flow rate = 0.5mL/min.

Based on our results, Accucore C30 enabled to overcome these co-elutions. For example, 14,15-DiHETE and 5,6-DiHETE are separated with an appropriate selectivity (subsection 4.7.2), but are co-eluted according to Berkecz, *et al.* study. Similar selectivity between the

HETEs isomers can be pointed out between Accucore C30 profile registered in 6 minutes (subsection 4.7.2) and Berkecz, *et al.* UHPLC profile acquired in 12 minutes.

As expected, the trifunctionally bonded BEH particles of the C18 column gets closer – according to calculated *Fs* parameter – to non-EPG columns (Accucore C30, Accucore C18, HypersilGold C18 and Nucleodur Isis C18) than columns incorporating a polar group in their grafting (Accucore Polar Premium, Cortecs Shield RP18 and Zorbax Fusion RP).

Based on the chemistry of Accucore C30 which presented the best selectivity in our study and results from Acquity UPLC BEH C18 [14] and Synergi Fusion-RP [18] columns, we can deduce that the use of EPG stationary phases is not determinant to resolve the separation of eicosanoids with closely related structures. In our study, Accucore C30 – even being the least retentive – displayed the best selectivity, efficiency and the shortest time for atherosclerosis related eicosanoids analysis.

4.7 The influence of pH and temperature on eicosanoids separation

Regarding our above mentioned results, we only considered the Accucore C30 to determine the optimal values of pH and temperature.

4.7.1 The impact of pH on eicosanoids separation

Different percentages of formic acid (0%, 0.02%, 0.04%, 0.06% and 0.08%) in the mobile phase are tested. As shown in Fig.10.a (blue), the formic acid seems essential for prostanoids elution. According to literature, hydroxyeicosatetraenoic acid (HETEs) and oxidized products are eluted independently of the mobile phase pH but some leukotrienes elution varies with pH [44, 45]. Leukotrienes elution (Fig.10) confirmed pH impact and is in accordance with Powell, *et al.* observations [17]. Leukotrienes co-eluted beyond 0.08 % HCOOH (Fig.10.d). Indeed, peptido-leukotriene such as LTC4, LTE4, LTF4 and especially LTD4 have their amino group protonated and then induce additional interactions anion exchange with silanols in acid pH. According to Mathews, *et al.*, the most hydrophobic eicosanoids such as HETEs / EETs require only a high proportion of organic solvent to ensure good resolution and efficiency [45]. Based on our results, we considered a pH of 3 – corresponding to 0.04% formic acid – for eicosanoids analysis.

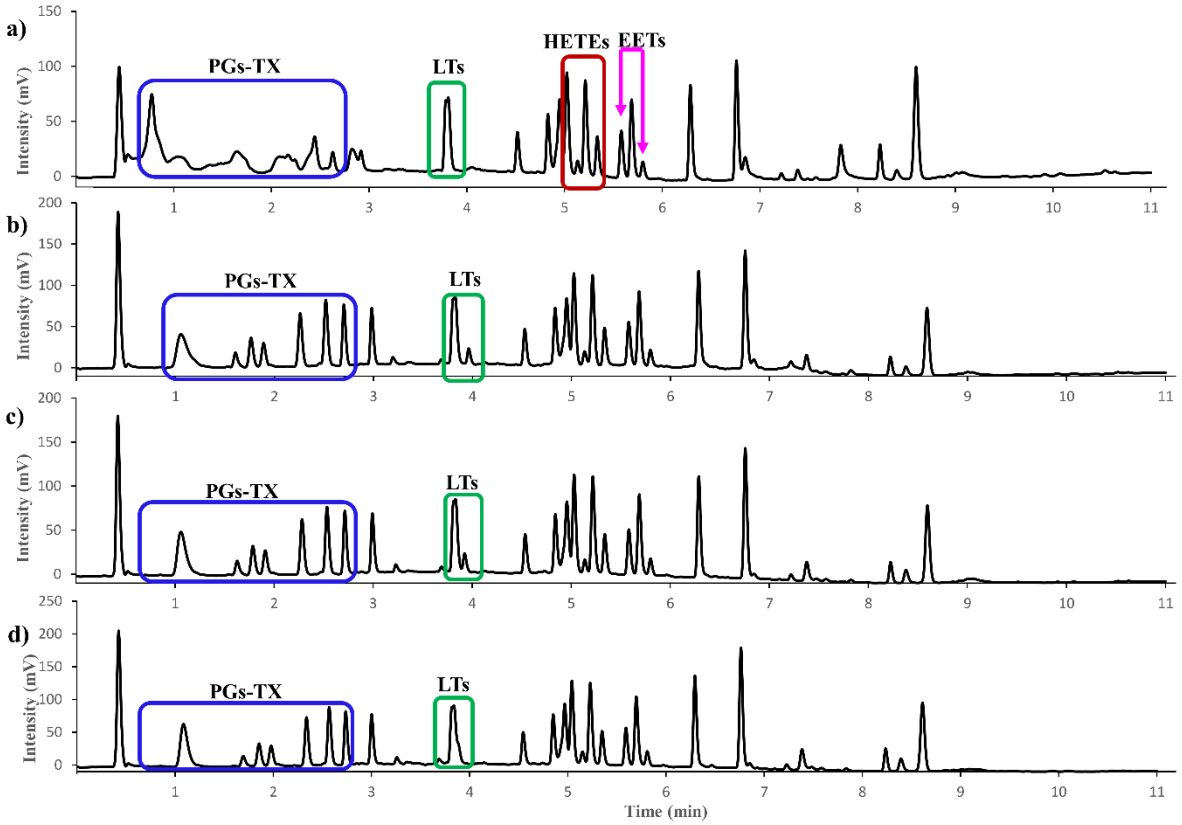

Fig.10: Influence of pH on eicosanoids separation through Accucore C30. Mobile phase: A (Water:ACN, 70:30,v/v), B (Acetonitrile,100,v) containing each a) 0% HCOOH; b) 0.02% HCOOH; c) 0.04% HCOOH; d) 0.08% HCOOH; at T = 25°C; with a V injec = 1µL; and a flow rate of 0.5 mL/min. PGs-TX: Prostaglandins-Thromboxanes; LT: Leukotrienes.

4.7.2 The influence of temperature on eicosanoids separations

Temperature increase was not enough to discriminate 6-keto PGF1α and PGI2 regardless the stationary phase in this study (Fig.11-orange). In contrast, an increase in temperature improved the asymmetry and tailing factor of this peak. This co-elution relates more to the secondary polar interactions: the tailing peak of 6-keto PGF1α and PGI2 results from a hydrogen bonding interaction between a donor hydrogen (from silanols) and the ketone or oxygen of hydroxyle group (from 6-keto PGF1α and PGI2). In addition, it is important to note that PGI2 is an unstable cyclooxygenase metabolite at neutral or acid pH which hydrolizes rapidly to 6-keto PGF1α when exposed to air. Resolution between TXB2 and PGE3 decreased beyond 30°C (Fig.11-blue) and co-eluted at 45 °C. During the development, it was very difficult to perform the separation of TXB1-TXB2-PGE3 and LTB4-14,15-DiHETE-5,6-DiHETE. Fig.12 displays how selectivity varies between some isomers such as TXB1 and TXB2 on one hand; and 5,6-DiHETE and 14,15-DiHETE on the other hand. Concerning TXB1, TXB2 and PGE3, the selectivity between TXB2 and PGE3 decreased while increasing temperature gradually. This observation confirms the steric selectivity which permits to discriminate TXB1, TXB2 and PGE3. Considering LTB4 and 14,15-DiHETE, selectivity

increased from 20°C (fig.12). In sum, temperature influences the steric recognition for some molecules and should be set in function. As the selectivity improvement between isomers was one goal in our study as well as profiling 24 eicosanoids, we selected the temperature of 20°C as a compromise for eicosanoids separation.

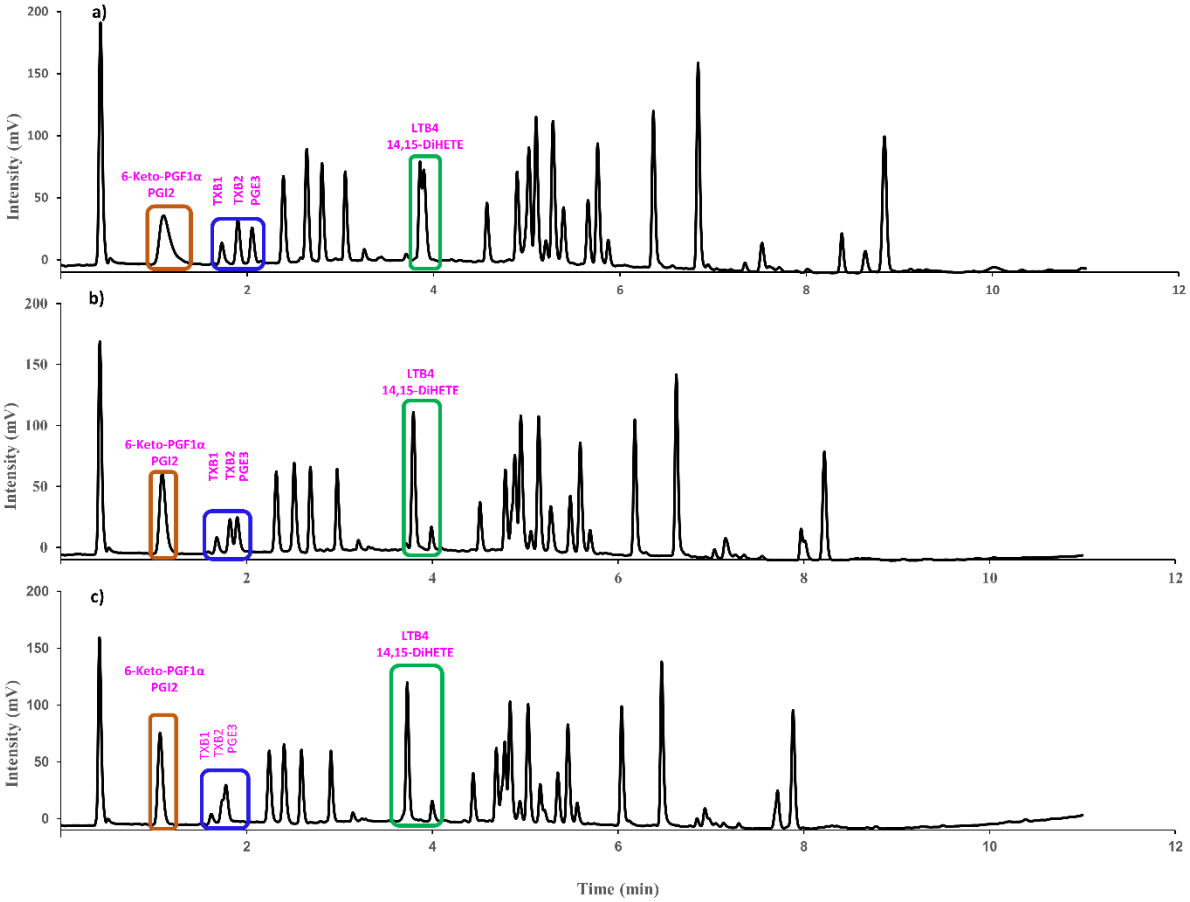

Fig.11: Influence of temperature on eicosanoids retention. Stationary phase : Accucore C30 (100mm x 2.1mm x 2.6µm) ; Mobile phase: A (water:ACN, 70:30,v/v) containing 0.04% HCOOH; B(Acetonitrile,100,v) containing 0.04%HCOOH ; a) T = 20°C ; b) T=35°C ; c) T= 45°C ; V injec = 1µL ; Flow rate = 0.5 mL/min.

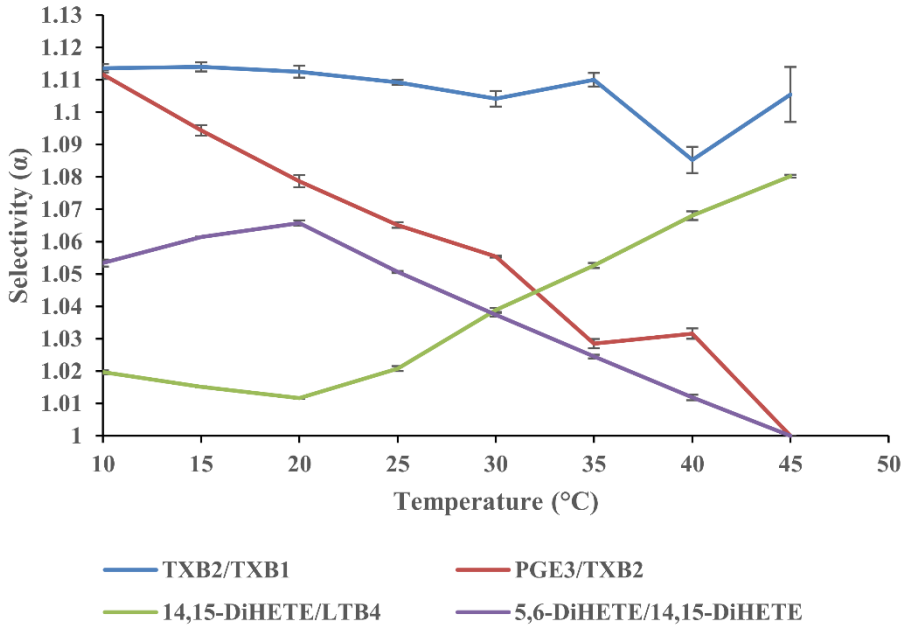

Fig. 12: Influence of temperature on selectivity between some isomers of eicosanoids during RP-HPLC profiling. Separation was performed from the 26 molecular species of eicosanoids and PUFA in triplicate for each temperature (10°C, 15°C, 20°C, 25°C, 30°C, 35°C, 40°C and 45°C), average and standard deviation are presented for each temperature. Stationary phase: Accucore C30 (100mm x 2.1mm x 2.6µm); Mobile phase: A (water:ACN, 70:30,v/v) containing 0.04% HCOOH; B(Acetonitrile,100,v) containing 0.04%HCOOH; V injec = 1µL ; Flow rate = 0.5 mL/min.

Regardless of the optimization conditions (mobile phase composition, temperature, pH), the first peak of the chromatogram corresponded to a co-elution of 6-Keto PGF1α and PGI2. Fig.13 displays the best chromatographic profile of 26 molecular species on Accucore C30 at pH=3 and T=20°C. Accucore C30 displayed the best selectivity, efficiency and procured the fastest acquisition time among the seven-screened columns. The most polar eicosanoids such as prostaglandins and thromboxanes eluted early with a good selectivity. Leukotrienes and related oxidized eicosanoids eluted towards the end of the gradient with a higher proportion of organic phase.

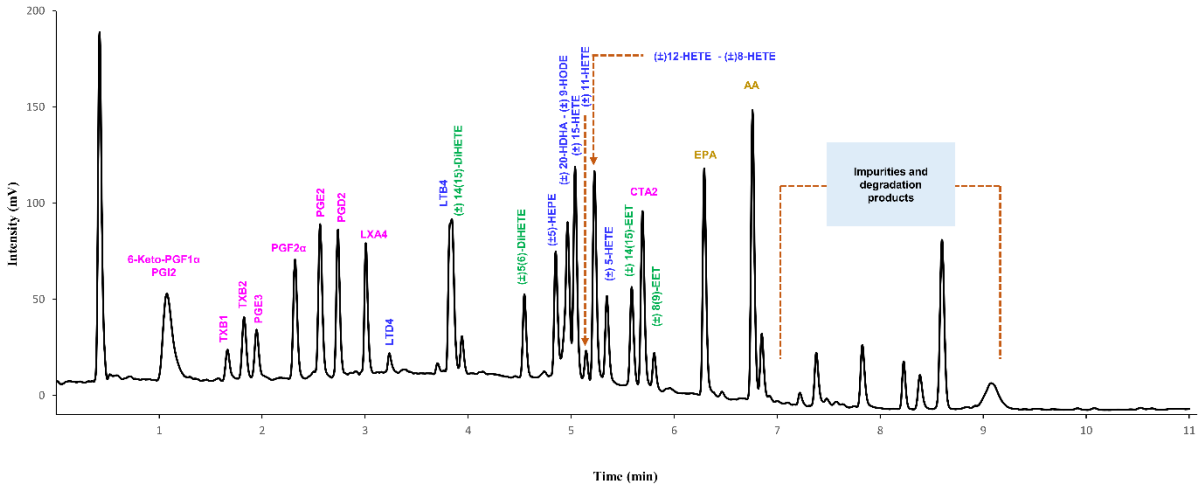

Fig.13: Profiling of 24 eicosanoids and 2 polyunsaturated fatty acids by reverse phase ultra-high performance liquid chromatography coupled to corona-CAD® detector. Stationary phase: Accucore C30 (100 x 2.1 mm x 2.6µm); Mobile

phase: A (water: acetonitrile, 70:30, v/v); B (Acetonitrile, 100, v) containing 0.04% HCOOH each, at 20°C with V injec = 1µL and a flow rate of 0.5 mL/min.

5 Conclusion:

Eicosanoids occur at traces level in biological samples and present many isomers and isobars. Therefore, chromatographic performances in terms of selectivity should be improved to resolve co-elutions and to permit their quantification in complex matrices (i.e.: cellular matrices). Various column selection systems have been reported for chromatographic characterization of stationary phases. It is difficult to decide on the procedure to select an appropriate column especially when analyzing close structure molecules such as eicosanoids.

In the present study, hydrophobic subtraction model helped us to select columns with different predicted selectivity. In this context, the complementary between F_s parameter and radar plots for columns performance discrimination was highlighted. Chromatographic performance descriptors guided us to select the suitable stationary phase for atherosclerosis related eicosanoids analysis. Analysis on Accucore C30 – the least retentive column with a t_{ne} of 15 minutes – lasted around 7 minutes. Accucore C30 displayed the best selectivity, efficiency and the shorter time for eicosanoids analysis, even being the least retentive. The present study demonstrated the interest of using Accucore columns for lipid analysis – herein the different subfamilies of eicosanoids.

Reverse phase liquid chromatography has been previously reported as an appropriate method for eicosanoids analysis even though the separation of regioisomers remained an unresolved issue [2, 18, 27]. Anticipating new developments in core-shell and/or sub-2µm stationary phases chemistry as well as the possibilities offered by SFC in addition to HPLC, significant improvements in structurally close molecules separation can be expected. For example, using a 2-PIC stationary phase, Ubhayasekera, et al. [13], published a rapid SFC-MS method able to quantify 4 prostanoids and 1 leukotriene with a run time of 3 minutes and a satisfactory, selectivity. However, our study confirmed the difficulty of separating eicosanoids isomers involved in physiopathological process. In addition, it demonstrated the imperative need to select RP-HPLC stationary phases with different selectivity. This approach is particularly appropriate because it allows rapid selection of the appropriate column and permits to reach the desired selectivity for particular molecules such as eicosanoids. The present study demonstrated the interest of using Accucore columns for lipid analysis – herein the different sub-families of eicosanoids.

6 Acknowledgements

This work took place thanks to a funding by a PhD scholarship from the French Ministry of Higher Education, Research and Innovation (MESRI). The authors also thank the representatives of Thermo Fisher Scientific and Waters for the gracious loan of their columns.

7 Conflict of interest

The authors declare that they have no conflict of interest.

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