Analysis of Fatty Acids by High Performance Liquid Chromatography and Electrospray Ionization-Mass Spectrometry

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Abstract

Fatty acids (FA) have been traditionally analyzed by gas chromatography (GC) as fatty acids methyl esters (FAME) and more recently using mass spectrometry (MS) detection. Since high performance liquid chromatography (HPLC) presents some advantages like the possibility to analyze them as underivatized compounds, the purpose of this work has been to investigate to which extent HPLC-MS can be a replacement or a complement technique to GC-MS.

A direct infusion (DI)-MS and an HPLC-MS method to analyze FAs were developed. Fragment diagnostic ions used for structure elucidation, are usually obtained when FAMEs are analyzed by GC-MS with electron ionization. When FAs were analyzed by HPLC-MS with electrospray ionization, this technique gave almost no fragmentation and no adducts even with collision induced. HPLC-MS therefore provides information about the molecular mass, which is often missing in GC-MS. A limitation found with HPLC-MS is that it was not possible distinguish between some isomers, which for quantification purposes limit the use of the technique to cases where no separation of isomers is needed. It was also noticed that fatty acids of different chain length have different ionization efficiencies and these depends in some extent on the mobile phase used.

Chromatographic selectivity, efficiency and retention were also investigated applying HPLC-MS. These parameters can be explained by Purnell and van Deemter equations in isocratic and isothermal chromatography. Since the retention factor (k) and number of theoretical plates (N) are not valid concepts in programmed chromatography, equivalent chain length (ECL) and peaks per carbon (PPC) were the parameters used to explain selectivity and efficiency, respectively, by HPLC with gradient elution.

The variability of ECL with different chromatographic conditions (methanol, acetonitrile, acetone or tetrahydrofuran in the mobile phase, temperature and gradient time) was studied, applying factorial design and response surface methodology to build models to predict ECL. Root mean squared errors for predictions (RMSE) were below 0.04 for all the solvents analyzed, which resulted in less than 10% of a peak width. It

was also found that ECL varies with the selection of the solvent and to some degree with the temperature, and that gradient time (steepness of the gradient) has almost no effect. Partial least square regression (PLSR) was also applied to build models to predict ECL based on the chemical structure of the molecule and based on GC retention data. Again, good prediction models were found with errors that were a fraction of a peak width.

The PPC concept was used as a measure of efficiency and is defined as the inverse of peak width in retention index units. The highest efficiency was obtained when methanol was used as solvent. Efficiency can be improved by decreasing column temperature or increasing gradient time, which results in higher time of analysis. The maximum value for PPC obtained by HPLC-MS was around 7.

List of abbreviations

ACN	Acetonitrile
ACO	Acetone
APCI	Atmospheric Pressure Chemical Ionization
CCD	Central Composite Design
ce	Collision Energy
CI	Chemical Ionization
DI	Direct Infusion
ECL	Equivalent Chain Length
ECN	Equivalent Chain Number
EI	Electron Ionization
EIC	Extracted Ion Chromatogram
ESI	Electrospray Ionization
FA	Fatty Acids
FAME	Fatty Acid Methyl Esters
FCL	Fractional Chain Length
FID	Flame Ionization Detector
GC	Gas Chromatography
H	Height of a theoretical plate
HPLC	High Performance Liquid Chromatography
IS	Internal Standard
k	Retention Factor
LC	Liquid Chromatography
LV	Latent Variable
m/*z*	Mass-to-charge Ratio
MeOH	Methanol
MLR	Multiple Linear Regression
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
N	Number of Theoretical Plates
PC	Principal Component

PCA	Principal Component Analysis
PLSR	Partial Least Square Regression
PPC	Peaks per Carbon
PUFA	Polyunsaturated Fatty Acid
R^2	Coefficient of determination
RI	Retention index
RMSE	Root Mean Square Error
RMSEC	Root Mean Square Error of Calibration
RMSECV	Root Mean Square Error of Cross Validation
RP	Reverse Phase
R_S	Chromatographic Resolution
SIM	Selected Ion Monitoring
SN	Separation Number
THF	Tetrahydrofuran
TIC	Total Ion Current Chromatogram
t_M	Hold-up time
t_R	Retention time
t'_R	Adjusted Retention time
w_b	Peak width at baseline
α	Separation Factor

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1. Introduction

1.1. Fatty acids

Fatty acids (FAs) are the major component of lipids, one of the three main nutrients, and are usually ingested in large quantities in the form of triglycerides or phospholipids. They generate energy and are also the principal component of the biological membranes providing integrity, fluidity, permeability and the possibility of interacting with enzymes. In addition to their importance as energy source, fatty acids have multiple physiological functions and even some adverse effects. For example, saturated fatty acids and trans-fatty acids are known to significantly increase coronary heart disease. In contrast, ingestion of omega-3 fatty acids is effective in preventing this disease. Polyunsaturated fatty acids (PUFAs) are also known to cause different physiological responses depending on the position of double bonds in the molecule. For instance, γ-linolenic acid (18:3 *n*-6) is known to show anticancer activity, whereas α-linolenic acid (18:3 *n*-3) has been reported to reduce the risk of heart disease [1,2].

1.2. Fatty acid structure and nomenclature

Fatty acids consist of a carboxylic group connected to a carbon chain, which may be saturated or unsaturated, and may contain carbon branches as well as other functional groups (**Figure 1**). However, the majority of fatty acids in nature have unbranched carbon chains with 4 to 24 carbons, 0 to 6 double bonds, and no other functional groups. Double bonds in polyunsaturated fatty acids (PUFA) usually have cis geometry and are typically separated by a single methylene group. FAs with odd-numbered carbon chains are present only in small quantities in most organisms, and carbon chains longer than C24 can be present in marine lipids in minor amounts [3,4]. FAs are named by the number of carbons followed by the number of double bonds. For example, stearic acid is denoted C18:0 or 18:0 which means that it contains 18 carbons and no unsaturation. Double bond positions may be specified from either end of the molecule. Double bond positions given from the methyl end of the carbon chain are referred to by '*n*' or by '*ω*'. Alternatively, the double bond position can be described by the distance from the carbonyl group as 'Δ'.

The polarity of fatty acids covers a wide range. For instance, the biologically most important fatty acids, from 16 to 26 carbons have log P values between 6.96 and 12.06, where P is the partitioning ratio between 1-octanol and water [5].

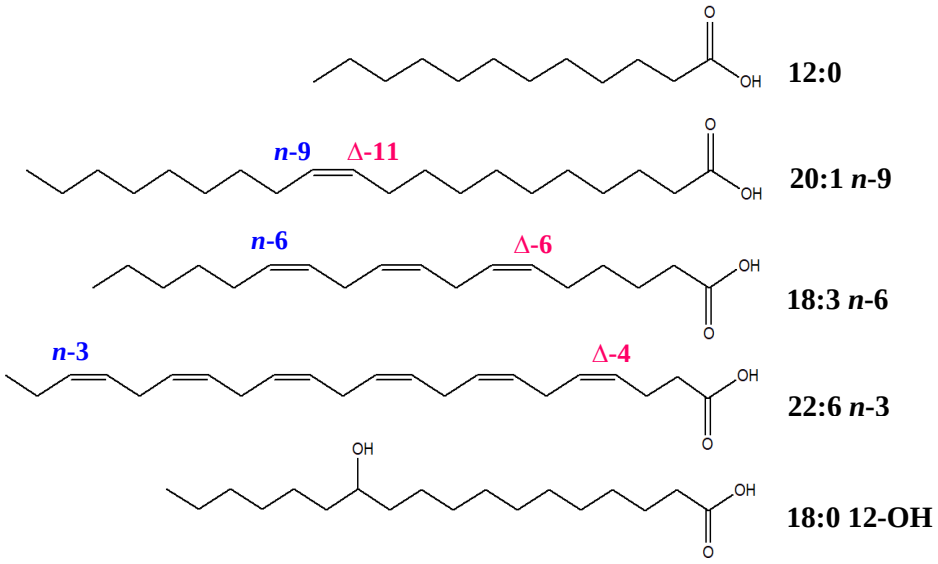

Figure 1 - Fatty acid structure and nomenclature.

1.3. Analysis of fatty acids

Several analytical methods have been developed to investigate lipids, including thin-layer chromatography, gas chromatography (GC) and liquid chromatography (LC), and because of the complexity of this family of compounds, mass spectrometry (MS) has become the leading technology for lipidomic analysis, due to its high sensitivity, specificity and dynamic range [6].

Fatty acids have been traditionally analysed by GC in the form of Fatty Acids Methyl Esters (FAMEs) using Flame Ionization Detection (FID) [7] and more recently, with MS detection. Derivatization of fatty acids where they are converted to methyl esters is a time-consuming process and there are risks of re-arrangement in some structures, leaving doubt whether the esters formed represent the structure of the original fatty acids. Even more important is that after conversion to FAME, GC does not distinguish between fatty acids from different lipid classes, so it only gives a picture of the total fatty acid composition unless a pre-separation of the lipid classes is performed. It had also been reported that the most serious inaccuracies in GC analyses of FAMEs result from losses during esterification or injection. Moreover, the GC-MS analysis of low

volatile, very-long-chain fatty acids with high molecular weight is a problem, even after fatty acid methyl ester derivatization [8]. In addition to all of this, although there are a large number of commercially available columns made especially for the analysis of FAMEs, they can be easily overloaded with sample, which may decrease resolution and quantitation capabilities [9].

More recently, LC–MS has become in an increasingly used technique for FA analysis. High Performance Liquid Chromatography (HPLC) allows analysing fatty acids as underivatized compounds, or converted to a large number of different derivatives. Electrospray Ionization (ESI) in combination with tandem mass spectrometry have offered an alternative way to ionize and detect non-volatile and heat-sensitive FAs [10].

1.4. Mass spectrometry

Mass spectrometry is a powerful analytical technique to identify and quantify analytes, using the mass-to-charge ratio (m/z) of ions generated from a sample. Ions are formed in an ion source and are separated according to m/z values in a mass analyzer. If the ionization of the analyte in the source produces little fragmentation, it is referred to as soft technique, and the most abundant peak in the mass spectrum (the base peak) is often the molecular ion. On the contrary, if the ion source produces extensive fragmentation, it is referred to as hard ionization, and the largest peaks in the resulting spectra are typically fragment ions. The type of ionization will depend on the analytical technique used; ionization methods are described in the following sections.

When fragment ions are formed in a separate collision cell (collision induced dissociation), they are known as product ions, and the technique applied is called tandem mass spectrometry (MS/MS). The ions that give rise to product ions are the precursor ions [11]. In a triple quadrupole analyser (QqQ) (**Figure 2**) the middle quadrupole q, acts as a collision cell where the ions are fragmented by collision with a gas before entering the third analyzer. In this way the response of the analyte decreases and the spectra is more complex but more structural information can be obtained.

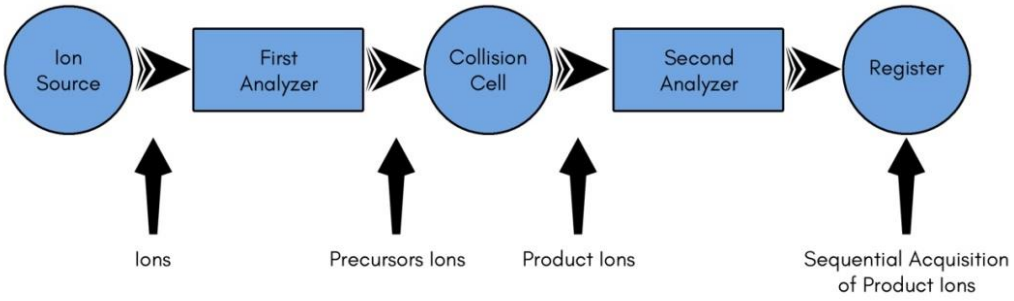

Figure 2 - Scheme of a triple quadrupole mass spectrometer

There are different acquisition modes depending on whether MS or MS/MS is applied. In MS technique, full scan mode or selected ion monitoring (SIM) can be used. In full scan, all ions formed are detected, but when high sensitivity is needed SIM mode may be preferred, where only the ions of interest are scanned. Multiple scans modes exist in MS/MS: product ion scan, precursor ion scan, neutral loss scan and selected reaction monitoring. In product ion scan mode a precursor ion is selected in the first stage, allowed to fragment in the collision cell, and then all the resultant masses are scanned in the second mass analyzer. In precursor ion scan the product ion is selected and the precursor masses are scanned in the first mass analyser. In neutral loss scan, the ions that lose a neutral fragment are scanned. Finally selected ion monitoring mode is the analogous to SIM mode in MS where both analyzers are set to a selected mass. The analysis of FAs by MS in direct infusion usually only provides information of molecular ions, therefore, MS/MS is generally applied for the sensitive and selective analysis [12].

Although direct infusion-mass spectrometry (DI-MS) can be used for the analysis of FAs, frequently, the use of chromatography is more useful. Chromatography is the most powerful tool for the separation of complex mixtures of either natural or synthetic origin, and the retention time is a parameter for identification of compounds [13]. Column separation can enrich low-abundance molecular species and exclude the interaction of many lipid species and also facilitates the identification of isomeric species with identical fragmentation patterns.

1.4.1. Gas Chromatography-Mass Spectrometry

The basic operating principle of GC involves volatilization of the sample in a heated inlet or injector, followed by separation of the components of the mixture in a specially prepared column. Only the compounds that can be vaporized without decomposition are suitable for GC analysis. Acids are among the compounds that frequently require derivatization to increase their volatility [14]. In GC, a carrier gas (the mobile phase), usually hydrogen or helium, is used to transfer the sample from the injector, through the column, and into the mass spectrometer. The mass spectrometer ionizes the gas-phase coming from the GC column.

Among the most used ionization techniques in GC-MS are electron ionization (EI) and chemical ionization (CI). In EI, the molecules in gas phase are bombarded with high-energy electrons to form radical ions. It is a hard ionization technique, producing very energetic molecular ions where a significant number will undergo fragmentation [3]. The fragmentation of the ions is used to determine the structure of an analyte. On the other hand, CI is a relatively soft ionization technique that uses a reagent gas (methane, isobutane, ammonia, etc) that is ionized by EI, and this gas is used to ionize the analytes. The most common use of CI is to produce protonated molecular cations of the analytes. This technique provides information about the molecular ions, and the mass spectra show low fragmentation. Molecular ions formed by EI are sometimes so energetic that their mass spectra do not exhibit the molecular ion peak. This is why the soft ionization techniques like CI can be considered complementary to EI because they usually provide the molecular mass of the analyte [14].

As mentioned, fatty acids are traditionally analysed as methyl ester derivatives by GC with temperature programming. Derivatization of FAs is performed to increase the volatility of the substances, to reduce dimerization in the vapor phase, to reduce adhesion to the instrumental construction materials and columns, to improve separation, and to reduce tailing [13]. Modern, commercially available fused-silica capillary columns give very good separation of FAMEs from biological samples. High polar stationary phases offer excellent separation of FAMEs but have relatively low thermal stability, resulting in long retention times for long chain FAs. Non-polar phases have

better thermal stability but lower selectivity. For many analytes, phases of intermediate polarity are the most suitable [8].

1.4.2. Liquid Chromatography-Mass Spectrometry

Liquid chromatography (LC), and especially High-Performance LC (HPLC) is the most widely used technique for the analysis of chemical mixtures and has contributed in a major way to science and everyday laboratory practice [15]. LC techniques with various detection methods have been attempted for FA analyses. However, due to the weak UV absorption and no fluorescent properties, low sensitivity is found with spectroscopic detection unless the compounds are derivatized. Thus, it is necessary a pre- or post-column derivatization of FAs, such as esterification or incorporation of appropriate and strong chromophores or fluorophores [16]. The evaporative light scattering detector (ELSD) is an alternative to UV and fluorescence that is commonly used for fatty acids and other lipids, but the poor linearity and low sensitivity with this method limit its use [17]. Coupling liquid chromatography with mass spectrometry overcome these detection difficulties and, allows to obtain rich detection information useful for both identification and quantification purposes.

Electrospray ionization (ESI) and Atmospheric Pressure Chemical Ionization (APCI) are soft ionization techniques developed to make MS suitable for LC coupling. In ESI, the effluent from the LC is passed through a narrow metal capillary where a high voltage is applied. The partial charge separation between the liquid and the capillary produces instability of the liquid that results in expulsion of charged droplets from a Taylor cone formed at the tip of the capillary (**Figure 3**). A nebulizing gas like Nitrogen helps to direct the charged droplets toward a counter electrode, as also speeds up the evaporative process. As the solvent evaporates, the droplets size decreases and the charge density increases. When the electrostatic repulsion exceeds the surface tension, the drops disintegrate into smaller subunits. Ions formed then pass through a sampling cone and extraction cones (skimmers) before entering to the high vacuum region of the mass analyser. ESI can produce negative or positive ions, depending on the sign of the applied electrical field [11,18]. ESI in positive mode shows MS spectra dominated by protonated molecular cations, $[M+H]^+$ or other positive ionic species, due to the high

tendency of lipids to form adducts with sodium, potassium and ammonium; in negative mode, the deprotonated molecular anions $[M-H]^-$ and some acetate and/or formic adducts are often observed [6].

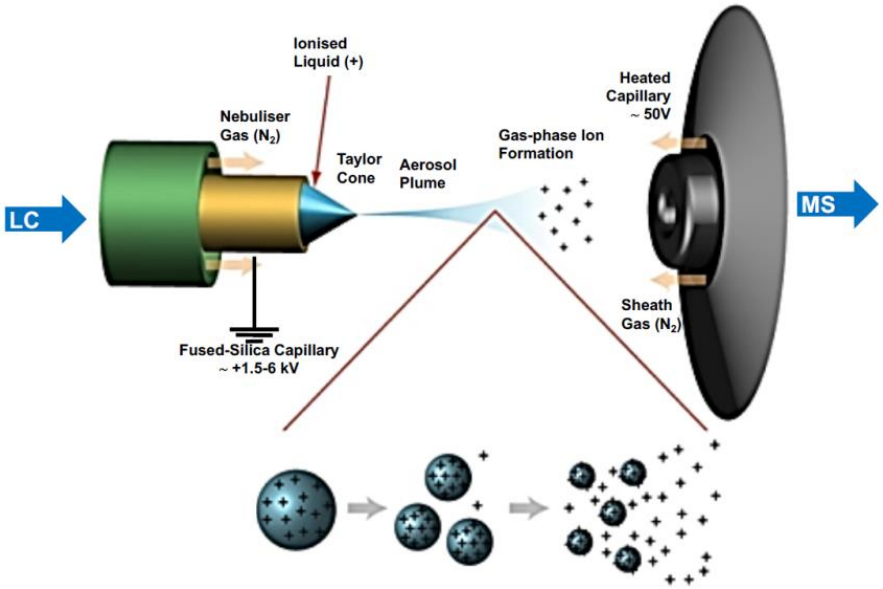

Figure 3 - Scheme of ionization process with ESI. (Lecture note, EMQAL curse, AM0912 "Fundamentals of mass spectrometry and hyphenated techniques", 2018).

APCI and ESI are similar processes since both involve the ionization at atmospheric pressure, nebulization and desolvation. However, the mode of ionization is different. In APCI the eluent coming from the LC is evaporated and the vapor passes by a needle with applied current that generates a corona discharge. Molecules coming from the mobile phase are predominantly ionized and therefore they act as a reagent gas ionizing the analyte molecules [11].

FAs have been analized by LC-MS (ESI), which is a non-derivatizing method that has advantages in terms of sensitivity, specificity and capability to analyze complex samples, where the mass spectrometric detection provides the identification of partially resolved or co-eluting peaks [19]. Although LC reduces the complexity of the eluent at any given elution time, ionization suppression effects when ESI is applied can happen. Sample matrix, coeluting compounds, and cross-talk can affect the performance of a mass detector. It has been demonstrated that the main cause of ion suppression is a change in the spray droplet solution properties caused by the presence of non-volatile or

less volatile interferences. The mass and charge of individual analytes are also important factors in the ion suppression phenomenon. All of this influence the ionization efficiency of an analyte and is often observed as a loss in response [20, 21].

1.5. The theory of chromatography

1.5.1. Ideal conditions

In chromatography, the components are distributed between two phases, one of which is stationary (stationary phase) while the other (mobile phase) moves in a defined direction. The distribution of an analyte between stationary and mobile phase is expressed by the retention factor, *k*, and is given by Equation 1:

$$k = \frac{amount\ of\ analyte\ in\ stationary\ phase}{amount\ of\ analyte\ in\ mobile\ phase} \qquad \textit{Equation 1}$$

The retention factor can be affected by column diameter, type and thickness of stationary phase and temperature. When conditions are maintained constant, like in isothermal GC and isocratic LC the retention factor is also given in terms of retention time (Equation 2 and Equation 3):

$$t'_R = t_R - t_M \qquad k = \frac{t'_R}{t_M} \qquad \textit{Equation 2 and Equation 3}$$

Where t_R is the retention time of a compound, which is the time when an analyte leave the column. The adjusted retention time (t'_R) is the time the analyte spend in the stationary phase and the holdup time or 'dead time' (t_M) is the t_R of an unretained analyte (**Figure 4 (A)**).

In chromatographic theory, the peaks are usually assumed to have perfect Gaussian shapes. Measures of resolution and efficiency normally involve the estimation of the chromatographic peak width. Peak width can be estimated in several ways as shown in **Figure 4 (B)**. The peak width at baseline (w_b) is usually defined as four standard deviations (4σ).

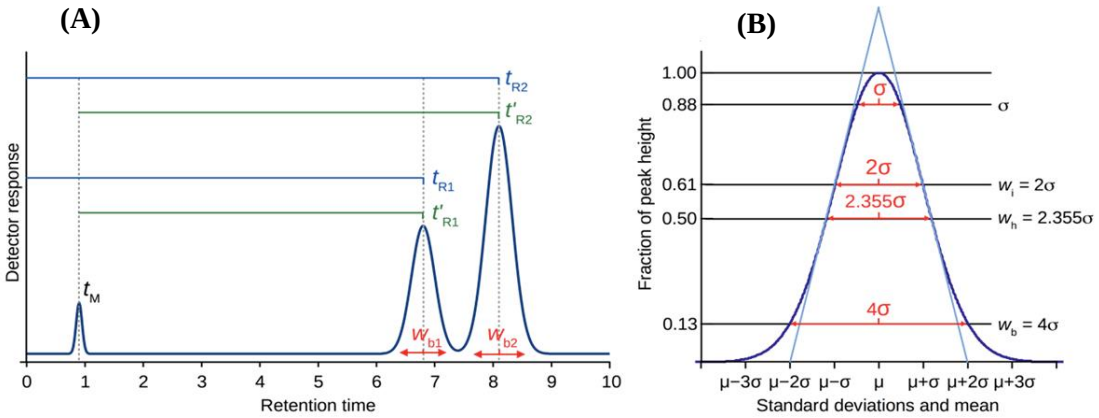

Figure 4 - (A) Representation of a chromatogram. (B) Peak width definitions.

The degree of separation between two chromatographic peaks is given by the resolution (R_S). Adequate R_S between adjacent peaks of interest is one primary goal in the development of a liquid chromatographic method [22]. The R_S between two peaks A and B is defined in Equation 4, where $t_{R(A)}$ and $t_{R(B)}$ are the retention times of A and B respectively and $w_{b(A)}$ and $w_{b(B)}$ are the peak width at the baseline of the compounds.

$$R_S = \frac{2\,(t_{R(B)} - t_{R(A)})}{w_{b(A)} + w_{b(B)}} \qquad \textit{Equation 4}$$

Two factors affect R_S between two peaks: the distance between the peak maxima and the average peak width. Thus better separation can be achieved either by increasing the distance between the peaks or by decreasing the peak width.

- ***Selectivity and efficiency in ideal conditions***

The selectivity or relative retention between two peaks is a function of the t_R and can be expressed by the separation factor α:

$$\alpha = \frac{k_B}{k_A} = \frac{t'_{R(B)}}{t'_{R(A)}} \qquad \textit{Equation 5}$$

From Equation 4 it can be seen that the R_S can be increased by increasing the difference in retention between the compounds, which means by increasing α. On the other hand, R_S can also be increased by narrowing the peak width. Efficiency is related to the peak width and is traditionally reported by the number of theoretical plates (N), and the

height equivalent to a theoretical plate (H). The theoretical plates can be seen as discrete sections of a column where a partitioning of the analytes between the stationary and the mobile phase occur [23]. The plate height is dependent on the column length (L) and the plate number N. The smaller the height, the greater the number of plates and thus higher is the efficiency per column meter. Equation 6 and Equation 7 explain these concepts.

$$N = 16\left(\frac{t_R}{w_b}\right)^2 \qquad H = \frac{L}{N}$$ *Equation 6 and Equation 7*

N is only meaningful as long as chromatographic conditions are kept constant during the run (mobile phase composition and temperature). In isothermal GC or isocratic LC, the three factors leading to chromatographic separations: efficiency, selectivity and retention are summarized in the Purnell equation [24]:

$$R_s = \frac{\sqrt{N_2}}{4} \cdot \left[\frac{\alpha - 1}{\alpha}\right] \cdot \left[\frac{k_2}{k_2 + 1}\right]$$ *Equation 8*

Efficiency Selectivity Retention

In order to increase resolution any of the three terms can be improved. The resolution increases proportionally with $\sqrt{N}$, and N increase proportionally with L. Thus increasing the length of the column will increase efficiency. Improving R_S through k_2 is efficient only when k_2 is low. Improving selectivity (increasing α) by changing the MP composition (LC) or the chromatographic column is often the best choice to improve resolution.

- ***Band broadening and van Deemter equation***

Band broadening is a phenomenon that reduces the efficiency of the chromatographic separation and is caused by three main factors: multiple paths, longitudinal diffusion and resistance to mass transfer.

Multiple paths (A): This term refers to the column packing, where different paths with slightly different lengths exist. Solute molecules following these different paths will elute at different retention times. Small column particles and homogeneous column packaging will reduce this factor. The multiple path effect is independent of the mobile phase velocity.

Longitudinal diffusion (B): Molecules, which are constantly in motion in the mobile phase, will gradually spread out because of diffusion. The faster the elution of a compound the less will the peak be broadened by this effect. This effect is inversely proportional to the mobile phase velocity.

Resistance to mass transfer (C): The exchange of a molecule between the mobile and the stationary phase takes time, and for a molecule to move from one phase to the other, it must first diffuse to the interface between the two phases. While some molecules are trapped in the stationary phase the molecules in the mobile phase will move further down the column, contributing to band broadening. Increasing the flow velocity increases the contribution to spread by resistance to mass transfer.

The van Deemter equation put all the terms together as a function of the mobile phase velocity (Equation 9) [25]:

$$H = A + \frac{B}{u} + Cu \qquad \textit{Equation 9}$$

Where A, B and C are the three terms contributing to band broadening mentioned above and u is the mobile phase velocity. In LC the column flow rate is proportional to the mobile phase velocity. The effects of the three terms are illustrated in **Figure 5**. The optimal mobile phase velocity is found where $A + B/u + C \cdot u$ has a local minimum, meaning that the derivative is 0 and is given by Equation 10:

$$u_{opt} = \sqrt{\frac{B}{C}} \qquad \textit{Equation 10}$$

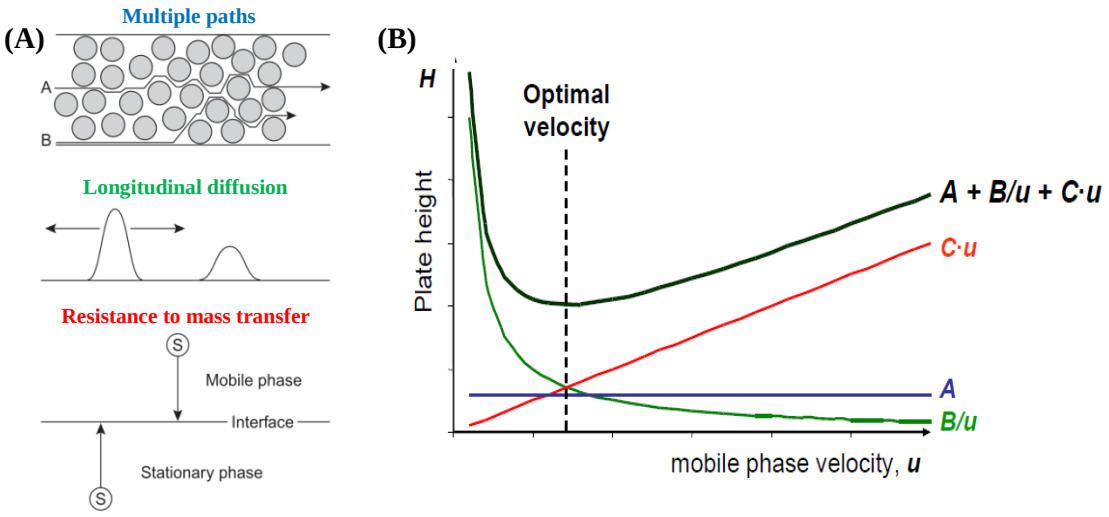

Figure 5 - (A) Schematic illustration of the three effects contributing to band broadening. (B) The van Deemter curve.

1.5.2. Non-ideal conditions

Of two different mobile phases, the one that gives the lowest retention factors, k, has the highest *solvent strength* (also referred to as *mobile phase strength*) [26]. Due to the wide range of polarities of FAs, choosing a high solvent strength will give poor separation of the least retained compounds, because the last factor of the Purnell equation (Equation 8) become too small. Choosing a low solvent strength may give very high retention factors, and therefore very high retention times, for the most retained compounds. The solution is to use gradually increasing mobile phase strength. This is referred to as solvent programming or gradient elution. In reversed phase LC, increasing solvent strength is achieved by decreasing the polarity of the mobile phase. In GC, the equivalent to gradient elution is temperature programming because temperature has the same effect as mobile phase strength in LC. Since the retention factor (k) varies when the chromatographic conditions are not constant like in programmed chromatography, the equations depending directly or indirectly on k are no longer valid. In these cases selectivity and efficiency must be redefined.

- ***Selectivity in non-ideal conditions***

Retention index (RI) based on homologous series of reference compounds are often applied in GC for identification of analytes. The Kovats' indices (KI), which is based on the n-alkanes, are well established to report retention index of organic compounds. In isothermal GC, a linear relationship exists between log t'_R and the number of carbons in members of a homologous series.

$$RI_x = 100\left[\frac{log\ t'_{R(x)} - log\ t'_{R(z)}}{log\ t'_{R(z+1)} - log\ t'_{R(z)}} + z\right] \qquad \textit{Equation 11}$$

The RI of a compound under constant chromatographic conditions can be calculated with Equation 11, where *x* is the compound of interest, *z* is the n-alkane with *z* carbon atoms eluting before the compound of interest and *z+1* is the n-alkane with *z+1* carbons eluting after the compound of interest [27].

The KI was developed for isothermal GC but has later been extended to programmed chromatography [28]. In programmed conditions the linear relationship between log t'_R and RI is not valid, and a new relationship must be established using the van den Dool and Kratz method represented by Equation 12, where *n* is the difference in the carbon number of the two *n*-alkanes used as a reference while the other terms are the same as in Equation 11, [29, 30, 31].

$$RI_x = 100\left[n\frac{t_{R(x)} - t_{R(z)}}{t_{R(z+n)} - t_{R(z)}} + z\right] \qquad \textit{Equation 12}$$

Particularly, in the analysis of fatty acids methyl esters (FAME), equivalent chain lengths (ECL) are the dominating retention index system, where the retention of a compound is described relative to the saturated straight chain FAMEs used as reference compounds [29]. Its calculation is analogous to the calculation of RI where a modification of the Van den Dool and Kratz equation can be used [28] (Equation 13). By definition 18:0 has an ECL value of 18, 20:0 has an ECL value of 20, etc [32].

$$ECL_x = n\ \frac{t_{R(x)} - t_{R(z)}}{t_{R(z+n)} - t_{R(z)}} + z \qquad \textit{Equation 13}$$

$t_{R(x)}$ is the retention time of a compound *x*, $t_{R(z)}$ is the retention time of a saturated straight chain FAME eluting before *x* and *z* is the number of carbons in the fatty acid

chain, $t_{R(z+1)}$ is the retention time of a saturated straight chain FAME eluting after *x* and *n* is the difference in carbons between the two reference FAMEs.

The fractional chain length (FCL) is another concept to express the retention of fatty acids. Is defined as the difference between the ECL value of the actual FAME and the ECL value of the unbranched saturated molecule with the same number of carbons [32]. Equation 14 shows this concept:

$$FCL_x = ECL_x - ECL_z \qquad \textit{Equation 14}$$

where *x* is the compound of interest and *z* is the saturated fatty acid with the same number of carbons.

- ***Efficiency in non-ideal conditions***

Plate number and plate high are no longer applicable concepts when the chromatographic conditions are not constant. In 1963, two similar expressions were first described: the separation number and the effective peak number [33]. The separation number (SN) express the number of peaks that can be separated in the space between two consecutive members of a homologous series [34]. The separation number can be calculated from Equation 15, where $t_{R(z)}$ and $t_{R(z+1)}$ are the retention time of two members of the homologous series with *z* and *z+1* carbons respectively, and $w_{h(z)}$ and $w_{h(z+1)}$ are the respective peaks widths at half peak heights.

$$SN = \frac{t_{R(z+1)} - t_{R(z)}}{w_{h(z+1)} - w_{h(z)}} + 1 \qquad \textit{Equation 15}$$

A high separation number always means better efficiency. However, a SN of zero does not mean zero efficiency. Because SN is defined as the number of peaks that can be separated between two members of a homologous series, the two homologs are still separated when SN is zero, which means that there is some separation efficiency, this can bring problems for calculations and modelling. An alternative to separation number is the peaks per carbon (PPC) concept, a measure that is zero when there is zero separation between the homologs, and that calculates the number of theoretically resolved peaks with a resolution of 1. Thus PPC is defined as the number of peaks that can be separated with chromatographic resolution equal to 1 per compound in a

homologous series, and it can be calculated from Equation 16 where w_b is the peak width at baseline [24].

$$PPC = \frac{t_{R(z+1)} - t_{R(z)}}{0.5(w_{b(z+1)} - w_{b(z)})} \qquad \textit{Equation 16}$$

Since measures of efficiency in non-ideal conditions are based on a homologous series of compounds, there is a link between efficiency and retention indices if they are based on the same series of homologs. If both retention and peak widths are measured in retention indices scale instead of t_Rs, PPC can be calculated as shown in Equation 17 where $w_{b,ECL}$ is the peak width at baseline expressed in retention index units [24].

$$PPC = \frac{1}{w_{b,ECL}} \qquad \textit{Equation 17}$$

Resolution, peaks per carbon and equivalent chain length are related by the following equation:

$$R_S = \Delta ECL \cdot PPC \qquad \textit{Equation 18}$$

where ΔECL is the difference in ECL between the two peaks. Because H is not valid under programmed conditions, since N is not a valid measure, the van Deemter equation is not strictly valid. However, it is possible to replace H with other meaningful values representing the inverse of the separation efficiency, such as 1/SN or 1/PPC as shown in Equation 19.

$$\frac{1}{PPC} = A + \frac{B}{u} + Cu \qquad \textit{Equation 19}$$

This means that peak width in retention index units can be used instead of H to evaluate efficiency. The effects of A, B and C are the same in programmed chromatography as in isothermal and isocratic chromatography. Therefore, conditions that are good in isocratic/isothermal chromatography will be good also in programmed chromatography.

1.6. Use of RI for Identification

RIs are traditionally applied for identifications of analytes in GC. Compounds can be tentatively identified from historical and tabulated data achieved on similar stationary phases. Positive identification of FAME needs comprehensive information including both standard mass spectra and GC RIs on standard phases. For example, the mass spectra for many isomeric methyl esters are highly similar, therefore GC and GC/MS identification of FAMEs needs the use of RI [35]. More recently RIs have been introduced in reverse phase LC-MS in metabolomics analysis to convert the t_R to a more stable retention variable. RIs show better reproducibility than t_R, since RIs are relatively invariant to analytical conditions, such as column dimensions, gradients and other instrumental parameters [36].

Accurate prediction of retention indices may be valuable for identification of unknown compounds not available as standards. Models that predict RIs may be an effective tool for elimination of incorrect tentative identifications. Prediction of RIs can be also used for optimization of elution patterns and prediction of chromatographic overlaps, which occur frequently in complex samples, being possible to test if a given compound will be resolved or hidden under other peaks [23, 33, 37]. Accurate prediction of ECL-values in GC is more challenging with temperature-programmed chromatography than with isothermal chromatography, especially when using stationary phases with properties that depend on temperature. The same occur in LC with gradient elution where the mobile phase is continuously changing. Analytical conditions, such as temperature will also have some influence [38]. The dependence of ECL values on analytical conditions can sometimes limit the possibility of using these indices for identification of unknown compounds. Nevertheless, retention patterns can be modified by changing chromatographic conditions, and overlapping peaks can often be resolved [39]. In this way, more unique retention data used for identification can be achieved by comparing the ECL values obtained at different chromatographic conditions.

1.7. Retention patterns on Liquid Chromatography

FA analyses by LC are usually done in reverse phase (RP) mode, typically with C18 or C8 columns and mobile phases with solvents like acetonitrile or methanol as apolar modifiers. In RP-LC the equivalent carbon number (ECN) has been used as a rough estimate to predict elution order. ECN is calculated as the total carbon number (CN) of the fatty acyls minus two times the number of double bonds (DB) [40, 41]:

$$ECN = CN - 2\,DB \qquad \textit{Equation 20}$$

The changes in retention with increasing ECN have been studied, and in isocratic RP-LC exists a linear relationship between log *k* and the ECN. Thus, FAs within the same ECN group, like 16:0, 18:1 and 20:2, will have similar *k* and may be challenging to separate [42, 43]. ECN is by definition equal to ECL for saturated FA. The main difference between the two is that ECN is calculated directly from the molecular structure and it can only have integer values, while ECL describes the actual retention. ECL is typically a measured value or a prediction that aims to describe the observed retention. According to the "ECN rule", ECL in RP-LC should fall with approximately 2 units each time a double bond is introduced in a molecule. When discussing retention patterns and the chromatographic overlaps it is important to consider whether the ECN rule fit to the observed ECL data, and also whether the ECL values can be altered by varying the chromatographic conditions. If there are factors that significantly influence the ECL values, these can be used to "tune" retention patterns to resolve overlap of important peaks. Several conditions can be changed in LC to modify the chromatographic retention. The elution patterns of FAMEs in GC are affected by the polarity of the stationary phase and sometimes by the applied temperatures [44]. In LC, in addition to the stationary phase, the retention can also be affected by the mobile phase composition, which is an advantage since it offers more possibilities for optimization. It has been shown that selectivity can vary with column temperature and gradient steepness [45].

1.8. Multivariate methods

1.8.1. Response surface methodology

It has been found that response surface methodology can be applied for accurate predictions of ECL values as functions of the applied chromatographic conditions in GC [39]. In this methodology, response functions are obtained from experiments which are carried out by varying a number of predictor variables (for instance the chromatographic conditions) systematically according to a predetermined plan: the experimental design. Response surface methodology can be divided into three major areas: the design of experiments, model fitting, and prediction. The response functions are typically polynomial models obtained by regression, that link the response to the experimental parameters [46]. Equation 21 shows a typical quadratic equation for two independent variables where x_1 and x_2 represent the main effects, x_1x_2 represent their interaction and x_1^2 and x_2^2 are the squared terms of variables 1 and 2 respectively:

$$\hat{y} = b_0 + b_1x_1 + b_2x_2 + b_{12}x_1x_2 + b_{11}x_1^2 + b_{22}x_2^2 \qquad \textit{Equation 21}$$

Finding the response surface means solving an equation explaining how the response, *y*, varies as function of the *x*-variables, the interactions between the variables and usually also higher order (squared) terms of the main variables [24, 47]. The complexity of the model will increase with the number of variables and if higher order terms are included. To optimize chromatographic separations, experimental design may be the best way to set up the experiments, and through response surface methodology it can be seen how the response varies with the different conditions.

1.8.2. Experimental design

One variable at a time approach dates back to the beginnings of systematic scientific research. In this approach, to simplify control and interpretation of the results, only one of the factors is varied by keeping the rest of them at constant values. This has some disadvantages like unnecessarily large number of experiments required and the possibility of missing the optimum in optimization studies [48]. Design of experiments refers to the process of planning the experiments, collecting appropriate data to be

analysed by statistical methods resulting in valid and objective conclusions. In this way the number of experiments is reduced and also the experimental costs.

The most commonly used multivariate designs in chromatography are the full and fractional factorial designs, central composite design, Box-Behnken design, Doehlert design and mixture designs. The factorial designs are often applied to investigate which are the most important factors and which factors that do not significantly affect the experimental results. Central composite or Doehlert designs are more frequently applied to optimize a process or to obtain response functions [49].

- ***Factorial design***

In a full factorial design, **Figure 6 (A**), the influence of all experimental variables are investigated. If the combinations of k factors at two levels are investigated, the factorial design will consist of 2^k experiments. The levels of the factors are given by – (minus) for low level and + (plus) for high level. The number of experiments significantly increases with the number of levels. The number of experiments can be reduced by applying fractional designs, but this may imply loss of information and reduction of the reliability of the results.

Factorial design is a classic tool for estimating the significance of main and interaction effects. Two-level full factorial design is applicable only for linear polynomial models. Polynomial models of second order (or higher) can be obtained by extending the approach to three-level designs. Three-level full factorial design is a composite design constructed by augmenting a two-level design with additional points, thereby saving the time and expense of replacing the measurements already performed [48].

- ***Central composite design***

Central composite design is the most popular class of design used to fitting second order models. Axial points are added to the factorial design to incorporate quadratic terms into the model and to get a better fit (**Figure 6 (B)**). Generally central composite design consists of a 2^k factorial with factorial points, $2k$ axial or star points and centre points [50]. The factorial points are important to determine the interaction terms, whereas the star points are important to determine the quadratic terms. Three different types of CCD

exist depending on the distance of the star points to the center; the star points and factorial points can be equidistant from the center (circumscribed), the star points may lie within the space of the factorial design (inscribed) or they can be on the faces of the factorial design points (faced). CCD needs L^k + Lk + nc experiments, where L is the number of levels, k is the number of factors and nc are the number of replicated centre points [51].

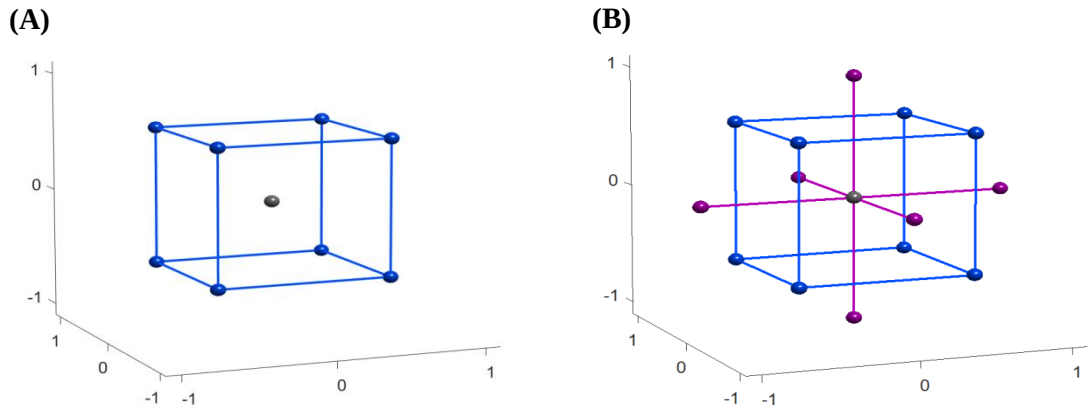

Figure 6 - (A) Full factorial design for three factors two levels. (B) Central composite design for three factors and two levels.

1.8.3. Principal component analysis

Principal Component Analysis (PCA) is probably the most widespread multivariate statistical technique in which a set of correlated variables are transformed into a set of uncorrelated variables called principal components. Usually, the first few components explain most of the variation in the data [41]. If M is a data matrix with m rows and n columns, with each variable being a column and each sample a row, PCA decomposes M as the sum of *r* t_i and p_i, where *r* is the rank of the matrix M [42]:

$$M = t_1 p_1^T + t_2 p_2^T + \ldots + t_k p_k^T + \ldots + t_r p_r^T \qquad \textit{Equation 22}$$

t is called score vector and contain information of the samples (objects) and p^T is called loading vector and contain information of the variables. $t_1 p^T{}_1$ represent the first principal component, PC1, which best represents the variation in the original data matrix. PC1 will never show a perfect representation of M using real data; the remaining variance factor is incorporated into a residual matrix E.

$$E_1 = M - PC1 = M - t_1 p_1^T \qquad \textit{Equation 23}$$

The second principal component PC2 is extracted from E_1 and the residual matrix E_2 is calculated according Equation 24:

$$E_2 = E_1 - PC2 \qquad \textit{Equation 24}$$

The procedure may continue until the number of principal components equals the least of the numbers of variables or objects.

Once scores (relating to the samples) and loadings (relating to the variables) have been calculated they can be graphically represented by plots of score vectors against score vectors (score plots) and loading vectors against loading vectors (loading plots). It is possible to plot any PC against any other PC, the most common is PC_1 vs PC_2. If the correlation between the variables is large, the first principal components will explain a large proportion of the total variance in M.

1.8.4. Multivariate regression techniques

Multivariate regression techniques are applied when a response variable, *y*, can be modeled from a number of *x*-variables (independent variables or predictors). The regressions can be performed directly with the values of the variables like in ordinary multiple linear regressions (MLR) or the *x*-variables can be first transformed into a set of a few intermediate linear latent variables (LV), and these LV are used for regression with the dependent variable *y*, as in partial least squares regression (PLSR). In PLSR the latent variables are extracted considering the maximum covariance (common variance) between the X matrix and the *y* vector [3].

To evaluate the performance of the model and in order to obtain a large number of predictions, cross validation is the most common strategy. The optimum number of latent variables for prediction it is also usually estimated by cross validation [52]. In cross validation, the dataset with n objects is split into segments (S) of approximately equal size where one segment (test set) is left out for validation. The other segments (S-1) called the training set, is used as calibration set to create the model. The model created is then applied for prediction for the objects in the test set and evaluated by

comparing predicted (y_p) and measured (y_m) values of the response variable y. The procedure is repeated until all segments have been used as test set. Finally, the model is evaluated from the residuals ($y_p - y_m$) of all objects combined. If S is equal to the number of samples the method is called leave one out or full cross validation.
A common way to evaluate the model performance is the root mean square error of cross validation (RMSECV):

$$\mathrm{RMSECV} = \sqrt{\frac{1}{n}\sum_{1}^{n}(y_p - y_m)^2} \qquad \textit{Equation 25}$$

Root mean square error of calibration (RMSEC) can also be calculated, but on the calibration residuals, where the calibration and validation set are identical [3].

Usually, a pretreatment or weighting of the variables is done in order to all variables have the same influence. A common solution is to apply standardization, where each variable is divided by its own standard deviation. Mean centering, where the mean is subtracted from each variable is another common procedure before multivariate analysis [3].

1.9. Aim of the thesis

As explained in the previous sections, GC is typically the preferred method for analyses of fatty acids. The aims of this work are to find out to which extent HPLC-MS can be a complementary technique to GC and GC-MS, or if it can be a replacement for these techniques. The work has the following sub-goals:

- To study which qualitative information that can be gained from electrospray mass spectra, and how this can complement or replace information from electron ionization GC-MS.
- To study whether the signals from DI-ESI-MS and HPLC-ESI-MS are suitable for quantitative studies, with particular focus on linearity and differences in response (detection limits are rarely an issue in fatty acid analysis because there is usually plenty of sample material).
- To study how chromatographic parameters (solvent strength, temperature, solvent gradient) affect the retention pattern, chromatographic efficiency and ionization efficiency (detector sensitivity).
- To study the feasibility of using retention indexes (ECL values) in RP-LC of FFA and whether the retention patterns can be predicted from molecular structure and ECL values acquired on GC.

2. Experimental

2.1. Chemicals

Methanol (HPLC grade, 99,9%) was purchased from Honeywell. Acetonitrile (GC grade, 99,8%), Acetone (GC grade, 99,5%) and Chloroform (GC grade, 99,0%) were purchased from Sigma-Aldrich. Isopropanol (LC grade), Ammonium acetate (MS Grade, 99,0%) and Formic acid (LC-MS grade) were purchased from Fluka. Tetrahydrofuran (LC grade) was purchased from Merck. Iso-octane and Hydrochloric acid were purchased from Nofima, Bergen Norway. Deionized water was of milli-Q grade and purified in a Milli-Q system from Millipore, USA.

2.2. Instrument

DI-MS and HPLC-MS analysis were performed on a 6420 A triple quadrupole mass spectrometer equipped with a binary pump and auto-sampler. Electrospray ionization was used in negative mode for the analysis of FFA and in positive mode for the analysis of FAME. The instrument was operated in full scan and selected ion monitoring (SIM) modes and for fragmentation studies in product ion scan. The volume of injection was 1 µl. The cell accelerator voltage and gas flow rate were maintained in 7 V and 6 l/min respectively and the gas temperature was 280 ºC in all experiments. Other conditions are described in more detail in each particular section. Nitrogen was used as source gas, curtain gas and collision gas. A fragmenter of 135 V, needle voltage of 4500 V and nebulizer of 35 psi were applied unless other condition are specified. Different columns, column temperatures and mobile phases are specified in the following sections. The system was controlled by Agilent Mass Hunter (B.06.00, Agilent Technologies).

2.3. HPLC Columns

The following columns were used:

- SB C18, 1,8 µm, 2,1x50 mm Agilent, (Method development)
- Zorbax Eclipse XDB-C18, 1,8 µm, 4,6x50 mm Agilent, (Method development)
- Poroshell 120 EC18, 2,7 µm, 3.0x50 mm Agilent, (Method development)

- Zorbax Eclipse Plus C18 Rapid resolution HD, 1,8 µm, 2.1x50 mm Agilent, (Sensitivity and linearity)
- Zorbax SB C8 Rapid resolution HD, 1,8 µm, 2.1x50 mm Agilent, (Effect of chromatographic parameters and retention pattern studies)

2.4. Solvent systems

Different solvent systems including solvents like acetonitrile (ACN), methanol (MeOH), acetone (ACO) and tetrahydrofluran (THF) were used.

- For investigation of fragmentation patterns, MeOH was used as solvent for the analysis of FFA and MeOH+0.5% of formic acid for FAME.
- For sensitivity and linearity studies, the mobile phase used for LC-MS was H_2O:(ACN:MeOH 50:50) 20:80, gradient: 0 min. 80% B, 3 min. 100% B, 10 min. 100% B and the flow rate 0.4 ml/min. For DI-MS the mobile phases used were ACN:MeOH 50:50 (DI-MS) and H_2O:(ACN:MeOH 50:50) 20:80 (DI-MS (H_2O)) with a flow rate of 0.2 ml/min.
- For effects of chromatographic parameters and retention pattern studies, 4 solvent systems of similar polarity with linear gradients and a flow rate of 0.35 ml/min were applied:

    - H_2O:ACN 44:56 increasing to 100% ACN
    - H_2O:MeOH 25:75 increasing to 100% MeOH
    - H_2O:ACO 38:62 increasing to 85% ACO
    - H_2O:THF 55:45 increasing to 60% THF

For the evaluation of chromatographic parameters, different temperatures from 30 to 60 ºC and different gradient times (time required to increase to the maximum percentage of the organic solvent) from 10 to 20 minutes were tested. For retention patterns study, the four solvents system mentioned above where used with a column temperature of 30 ºC and a gradient time of 20 minutes.

2.5. Samples

All samples prepared were dissolved in MeOH and stored at -20 ºC.

- ***Method development***

FFA and FAME single standards were obtained from Nu-Chek Prep, MN, USA. Each sample and mixture including FFA and FAME from 8 to 24 carbons and from saturated to polyunsaturated was prepared from stock solutions of 5 mg/ml in chloroform to a final concentration of approximately 50 µg/ml in methanol.

- ***Sensitivity and linearity***

Reference mixtures of saturated FFA: 8:0, 10:0, 12:0, 14:0, 16:0, 18:0, 20:0, 22:0 and 24:0; were accurately prepared from stock solutions of 5 mg/ml in chloroform and diluted with methanol at 7 levels of concentrations (from 6 to 100 µg/ml approximately) for calibration curves. FFA18:0 was used as internal standard (IS) in a concentration of approximately 30 µg/ml in each calibration sample. Exact concentrations are given in **Appendix a**. In direct infusion analysis, extracted ion chromatogram (EIC) was applied to extract the area of each compound from the TIC (total ion current chromatogram).

- ***Effects of chromatographic parameters***

The reference mixture GLC-793 (Nu-Chek Prep, MN,USA) containing the following 28 FAMEs: 12:0, 14:0, 14:1 *n*-5, 15:0, 16:0, 16:1 *n*-7, 17:0, 17:1 *n*-7, 18:0, 18:1 *n*-9, 18:2 *n*-6, 18:3 *n*-3, 18:3 *n*-6, 20:0, 20:1 *n*-9, 20:2 *n*-6, 20:3 *n*-3, 20:3 *n*-6, 20:4 *n*-6, 20:5 *n*-3, 22:0, 22:1 *n*-9, 22:4 *n*-6, 22:5 *n*-3, 22:6 *n*-3, 23:0, 24:0, and 24:1 *n*-9 was converted to FFA as explained in Section 2.6 and analysed by LC-MS using different chromatographic systems.

- ***Studies of retention patterns***

Reference mixtures, single standards and algae samples were used to investigate the retention of fatty acids. Three reference mixtures were used: GLC-793 and GLC-461 from Nu-Chek Prep, MN, USA and Bacterial Acid Methyl Ester (BAME) Mix from Sigma-Aldrich. Three mixtures (MIX 1, MIX 2 and MIX 3) where prepared with single standards in order to analyze separately the isomers of some compounds like 18:3 and 20:3. Eight algae samples, coming from previous studies made elsewhere and containing different FAMEs were also included in the study. All FAME samples were

converted to FFA and spiked with a reference mixture of saturated fatty acids: 12:0, 14:0, 16:0, 17:0, 18:0, 20:0, 22:0 and 24:0 for calibration of ECL values, after that they were analysed by LC-MS with different solvent systems. The fatty acids analysed (with exception of saturated ones) with the samples where they were contained are detailed in **Table 1**.

Table 1 - Samples and fatty acids analyzed

Fatty acid short name	Sample
13:1 *n*-1	MIX 2
14:0 2-OH	BAME
14:1 *n*-5	GLC 739, GLC 461
14:0 2-OH	BAME
14:0 2-CH_3	BAME
14:0 3-CH_3	BAME
15:0 2-CH_3	BAME
16:0 2-CH_3	BAME
16:0 2-OH	BAME
16:4 *n*-1	ALGAE (PSL006, PSL041, PSL027, PSM020, GV23)
16:3 *n*-6	ALGAE (GV23)
16:3 n-4	ALGAE (PSL006, PSL041, PSL027, PSM020, GV19, GV23, GV25, GV27)
16:2 *n*-4	ALGAE (PSL006, PSL041, PSL027, PSM020, GV19, GV25, GV27)
16:1 *n*-7	GLC 739, GLC 461 BAME, ALGAE (PSL006, PSL041, PSL027, PSM020, GV19, GV25, GV27)
17:1 *n*-7	GLC 739, GLC 461
18:0 12-OH	MIX 3
18:4 *n*-3	ALGAE (PSL006, PSL041, GV19, GV23, GV25, GV27)
18:3 *n*-3	MIX 1, GLC 739, GLC 461, ALGAE (GV23, GV27)
18:3 *n*-6	MIX 2, GLC 739, GLC 461, ALGAE (PSL006, PSL041, PSL027, PSM020, GV19)
18:2 *n*-6	MIX3, GLC 739, GLC 461, BAME, ALGAE (PSL006, PSL041, PSL027, PSM020, GV23)
18:1 *n*-9 7-OH	MIX 2
18:1 *n*-7	MIX 2
18:1 *n*-9	MIX 3, GLC 739, GLC 461, BAME, ALGAE (PSL006, PSL041, PSL027, PSM020, GV23)
18:1 *n*-12	MIX 1
19:1 *n*-9	MIX 1
20:5 *n*-3	GLC 739, GLC 461, ALGAE (PSL006, PSL041, PSL027, PSM020, GV23)

20:4 *n*-6	GLC 739, GLC 461, ALGAE (PSL027, PSM020, GV19, GV23, GV25, GV27)
20:3 *n*-3	MIX1, GLC 739, GLC 461
20:3 *n*-6	MIX 2, GLC 739, GLC 461
20:2 *n*-6	GLC 739, GLC 461
20:1 *n*-9	GLC 739, GLC 461 ALGAE GV19, GV23, GV25
22:6 *n*-3	MIX 2, GLC 739, GLC 461, ALGAE (PSL006, PSL041, PSL027, PSM020, GV19, GV25,GV27)
22:5 *n*-3	GLC 739, GLC 461
22:4 *n*-6	GLC 739, GLC 461
22:3 *n*-3	MIX 1
22:2 *n*-6	GLC 461
22:1 *n*-9	GLC 739, GLC 461
24:1 *n*-9	GLC 739, GLC 461, ALGAE (PSL006, PSL041, PSL027, PSM020)

In the cases of the same fatty acid appeared in several samples, the average of the calculated ECL values was used in the dataset for the study (after exclusion of outliers by Grubbs test). All samples were prepared to a final concentration of approximately 800 µg/ml (sum of all compounds) and dissolved in MeOH.

2.6. Making FFA from FAME

Approximately 5 mg of FAMEs sample were heated at 90 ºC with 1 ml of KOH (1 M, dissolved in 90:10 EtOH Abs:H_2O) for 1 hour. After cooling to room temperature, 2.5 ml of H_2O and 1 ml of HCl (2 M) were added and the sample was extracted twice with 1 ml of iso-octane. The extracts were combined and the iso-octane evaporated at 60 ºC under inert atmosphere (N_2). The remaining was dissolved in MeOH and the concentration was adjusted to the study.

The conversion process was controlled by GC with an Agilent 7890A gas chromatograph with FID detector. The injection mode was split (split ratio 100:1) at 280 ºC with an injection volume of 1 µl. The oven program was the following: 60 ºC for 2 minutes, then 60 ºC/min to 150 ºC and then 1 ºC/min to 250 ºC. The column used was DB-FFAP Agilent Technologies, 30 m x 250 µm x 0.25 µm and helium was the carrier gas.

2.7. Software and Data handling

- Agilent Mass Hunter (B.06.00, Agilent Technologies) was used for data acquisition on the mass spectrometer and to obtain the peak area values for linearity and sensitivity studies.

- Sirius (version 11.0, Pattern Recognition Systems A.S) was used for the analysis of the experimental design applied for the evaluation of instrumental settings in section 3.1.1 (Screening of ionization settings for FFA).

- MS convert (Stanford University, http://proteowizard.sourceforge.net/index.shtml) was used to convert the data format from the Agilent software to a MZ5 format, for import into Chrombox D (www.chrombox.org).

- Chrombox D and C were used to resolve, integrate and identify the studied compounds from the spectra and chromatograms.

- Chrombox O was applied for response surface models of chromatographic selectivity (ECL) and efficiency.

- MATLAB (version R2017b, Mathworks)/PLS toolbox (Eigenvector Research, Manson, WA, USA) was used to perform principal component analysis (PCA) and partial least squared regressions (PLSR). Chrombox was also run under the same Matlab version.

Once the samples were analysed and spectra were obtained, a theoretical spectral library was generated and the compounds were resolved and identified by fitting the real data with the theoretical spectra by least square spectral resolution (LSSR) approach [53, 54] applied by Chrombox D. The program also applies deconvolution methods for resolution of overlapping peaks. The basic idea of these methods is to decompose the raw data matrix X into matrices containing pure spectra, S^T, in row vectors and pure chromatographic profiles, C, in column vectors [55]. The result is a list of resolved lipid species with their identities and corresponding abundances.

ECL values were obtained from Chrombox C. This program converts the entire retention time scale to retention indices by second order local regressions [38], and also calculates peak widths in retention index units. The peak apex was used to determine the retention time and the unbranched saturated fatty acids were used as references. The independent variable (*x*-variable), is the t_R of the reference compounds, and the dependent variable (*y*-variable) is the RI, defined for the corresponding compounds. These regressions will give a smooth curve passing through all the regression points of the standard series. After integration and calibration, a list off all the compounds with its retention indices was obtained.

For the study of the effect of chromatographic parameters, Chrombox O (Optimizer) was used for setting up the experimental designs, creating the response surface models and to calculate model fits and errors. All models were calculated by MLR and the quality of the predictions was evaluated by the root mean squared error (RMSE) calculated according Equation 26, where *n* is the number of experiments in the design and *p* is the number of regression coefficients in the models. The squared correlation coefficient (R^2) from the linear regression between y_p and y_m was also used as an indication of the precision of the model.

$$RMSE = \sqrt{\frac{1}{n-p}\sum_{1}^{n}(y_m - y_p)^2} \qquad \textit{Equation 26}$$

For the retention study, the models for ECL values were obtained by PLSR. Leave one out was selected as method for cross validation. RMSEC (calibration) and RMSECV (cross validation) were obtained according Equation 25. The number of latent variables was selected when the RMSECV got its lower stable value.

Mean centring (the mean values were subtracted) and standardization (each variable was divided by the standard deviation) were applied to the *x*-variables for the multivariate regression methods.

3. Results and discussion

3.1. Initial tests and method development

A direct infusion method and a liquid chromatography method using ESI were developed to analyze fatty acids, either as free fatty acids (FFA) or fatty acids methyl esters (FAME). It was expected that these two groups of compounds (acids and esters) showed different ionization properties and therefore required different conditions to be analyzed. In preliminary studies, both groups of compounds were analyzed in ESI positive and negative mode. Different mobile phases were tested including the most typical organic solvents like acetonitrile, methanol, isopropanol, etc. Instrumental settings were optimized applying experimental design. To study if qualitative information can be obtained from ESI spectra, the fragmentation pattern using collision induced dissociation was also investigated.

3.1.1. Direct Infusion-Mass Spectrometry

- ***Selection of the ionization mode***

In a preliminary study, single samples of four compounds of short and long carbon chain (FFA 12:0 and 22:6 and FAME 12:0 and 22:6) were analyzed by DI-MS applying ESI in positive and negative mode. Best results were expected for FFA in negative mode due to the tendency of carboxyl acids to be deprotonated. On the contrary, the ester group of FAME has tendency to be ionized and in this way be analyzed in positive mode.

Considering FFA, the spectra showed a very pure signal of the $[M-H]^-$ ion and minimal fragmentation or adducts when they were analyzed in ESI- (**Figure 7 (A, B)**). The highest intensity was seen when MeOH was used as solvent. FFA were also analyzed in ESI+ but the intensity was lower, less clean spectra was obtained and the main ions seen in the spectra for FFA 12:0 were $[M+45]^+$ and $[M+23]^+$ which corresponds to Na and COOH adducts and for FFA 22:6 $[M+18]^+$ and $[M+23]^+$, corresponding to H_2O and Na adducts.

Regarding FAME, less noisy spectra were obtained in positive mode **(Figure 7 (C, D)**). Formic acid was added in the mobile phase in order to promote the protonation of FAMEs obtaining the highest signal using 0.5% formic acid in MeOH. It was noticed that the use of an acidified solvent increased the signal of $[M+H]^+$. However, the signal of the sodium adduct $[M+Na]^+$ was in most of the cases higher. Many low signals were obtained in the spectra when FAMEs were analysed in negative mode.

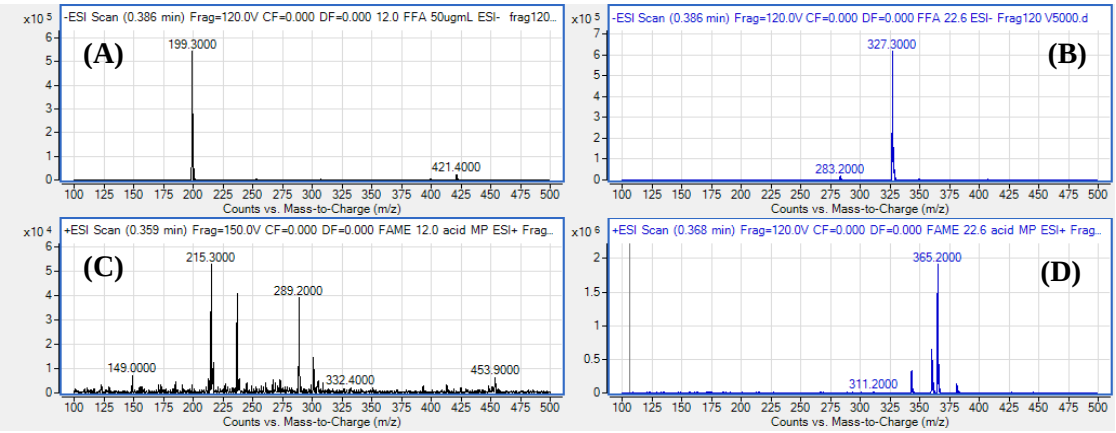

Figure 7 - Full scan spectra (A) FFA 12:0 ESI- MeOH. (B) FFA 22:6 ESI- MeOH. (C) FAME 12:0 ESI+ 0.5% HCOOH MeOH. (D) FAME 22:6 ESI+ 0.5% HCOOH MeOH. Monoisotopic mass of FFA 12:0 200.2 Da; FAME 12:0 214.2 Da; FFA 22:6 328.2 Da and FAME 22:6 342.3 Da.

After selecting the ionization mode for each class of compounds: ESI- for FFA and ESI+ for FAME, new compounds were analysed using MeOH as solvent. FFA12:0, 14:0, 16:0, 18:0, 20:0, 22:0, 24:0, 18:0 12-OH, 18:1 *n*-9, 18:1 *n*-12, 18:2 *n*-6, 18:3 *n*-3 and 18:3 *n*-6 showed a very clean signal of the deprotonated molecular anion $[M-H]^-$. Regarding FAME, the higher signal corresponded to $[M+Na]^+$ ion. The molecular ion $[M+H]^+$ was present with lower intensity.

- ***Investigation of the fragmentation pattern with MS-MS***

With the aim of getting more information about the structure of the FAs, the fragmentation pattern was investigated applying different collision energy (ce) by MS/MS. The compounds were analysed in product ion scan mode, where a molecular ion selected in Q1 is collisionally activated in the collision cell, q2, and the fragment ions formed are analysed in Q3. The ce were tested from 8 to 50 eV since the optimal ce may vary with the length of the acyl chain [56].

Very low fragmentation was seen for FFAs, particularly for the saturated ones, despite of increasing the ce. More fragment ions were obtained when the unsaturated compounds were analyzed. Fragments around *m/z* 59 and sometimes *m/z* 73 were present, denoting the presence of CH_3CO_2 and $CH_3CH_2CO_2$ respectively. D. Perret et al. [57] makes reference in her work to a fragment ion at *m/z* 183 due to charge remote fragmentations, this was seen in some of the FFA, like 20:0 and 22:0, but with very low intensity. According to J. Kerwin et al. [58], fragment ions at *m/z* 181 and 207 can be seen in 18:3 *n*-3. On the other hand, an ion at *m/z* 165 and a more intense ion at 205 can be present in FFA 18:3 *n*-6. In the compounds analysed, the fragment ion at *m/z* 181 was observed for FFA 18:3 *n*-3 (**Figure 8 (A)**); 18:3 *n*-6 (**Figure 8 (B)**) showed the fragment at *m/z* 205, however their intensities were low. Even though the fragmentation was lower for monounsaturated FFA, fragment ions at *m/z* 59 and 83 where seen for 18:1 *n*-12 **Figure 8 (D)**), and no for *n*-9 (**Figure 8 (C)**) with a ce of 25 eV**,** which can mean that these fragments are favored in compounds with a double bound in the position Δ-6. Fragmentation increased with the number of double bonds.

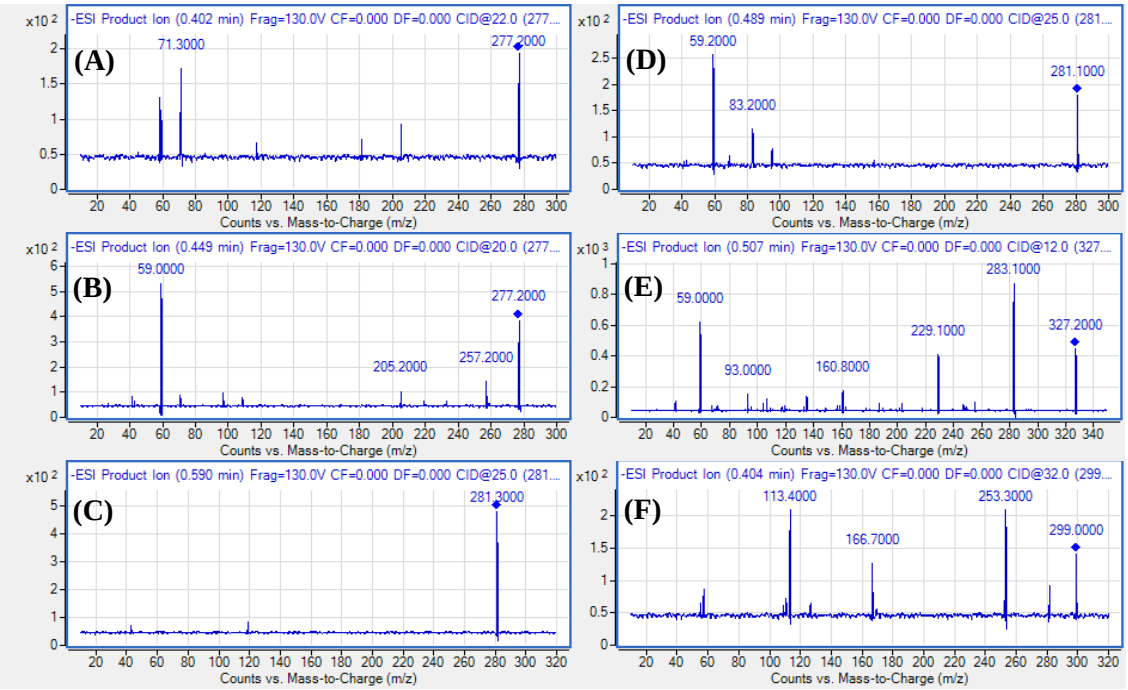

Figure 8 - Product ion scan spectra obtained with different collision energies (ce). (A) FFA 18:3 n-3 ce 22 eV. (B) FFA 18:3 n-6 ce 20 eV. (C) FFA 18:1 n-9 ce 25 eV. (D) FFA 18:1 n-12 ce 25 eV. (E) FFA 22:6 n-3 ce 12 eV. (F) FFA 18:0 12-OH ce 32 eV.

The highest number of fragments was seen for the FFA 22:6 *n*-3 (**Figure 8 (E)**). It was also noticed that lower ce is needed to get fragments of polyunsaturated fatty acids. **Figure 8 (F)** shows particular fragment ions at *m/z* 113, 167 and 253 for the hydroxy fatty acid 18:0 12-OH.

Regarding FAME, in a first step, MeOH was used as mobile phase, selecting $[M+Na]^+$ as precursor ion because it was the major ion in the entire spectra. The fragmentation so obtained was extremely low. Therefore, in a second step, 0.5% formic acid in MeOH was used as mobile phase and $[M+H]^+$ was selected as precursor ion, which increased the fragmentation considerably, even when the signal of the $[M+H]^+$ was smaller than that of sodium. It was necessary to increase the collision energy as the chain length increases in the saturated compounds to get more fragmentation. In most of the cases 15 eV was suitable to get a total fragmentation, only FAME 22:0 and 24:0 needed higher energies of 20 and 30 eV, respectively. Fragment ions at *m/z* 43, 57, 71, 85, 103 and 117 were in general present for all compounds, an inter-peak spacing of *m/z*= 14 representing cleavages of consecutive C-C single bonds in the fatty acid chain [57]. **Figure 9** shows the differences in the spectra of isomers compounds of 18:1 and 18:3.

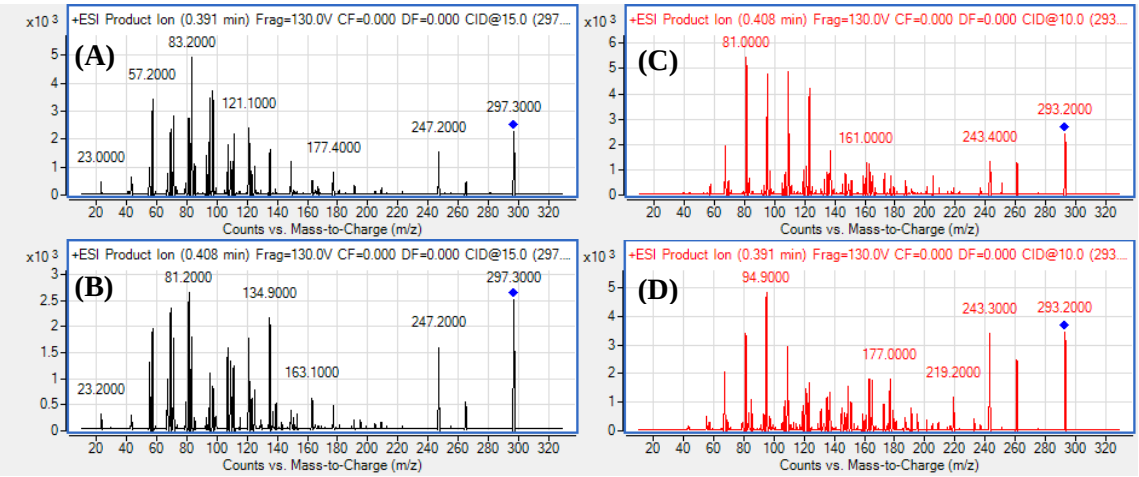

Figure 9 - Product ion scan spectra. (A) FAME 18:1 n-9 ce 15 eV. (B) FAME 18:1 n-12 ce 15 eV. (C) FAME 18:3 n-3 ce 10 eV. (D) FAME 18:3 n-6 ce 10 eV.

- ***Screening of ionization settings for FFA***

Multiple settings could be adjusted on the triple quadrupole. Fragmentor voltage, needle voltage and nebulizer pressure were suspected to cause variation in the ion abundance,

hence a central composite design including these parameters was applied. Low and high levels were defined according to results from preliminary studies and instrumental limitations. The total abundance was the sum of $[M-H]^-$ signal (area) of each compound present in a mixture sample containing 9 saturated FFA (8:0, 10:0, 12:0, 14:0, 16:0, 18:0, 20:0, 22:0, 24:0). The experiments were performed in DI-ESI-MS with SIM mode using ACN:MeOH 50:50 as solvent. The 18 experiments performed, with the levels selected and the total abundances are shown in **Table 2**.

Table 2 - Central Composite Design experiments, 4 replicates for central point. Fragmentor 80-180 V, nebulizer 20-40 psi and needle voltage 3500-5500 V were the low and high levels respectively. The abundance is the sum of $[M-H]^-$ of each compound in the TIC.

Experiment		**Fragmentor (V)**		**Nebulizer (psi)**		**Needle Voltage (V)**		**Signal detector Abundance·10^6**
1	Center	0	130	0	30	0	4500	14.4
2	Center	0	130	0	30	0	4500	13.5
3	Center	0	130	0	30	0	4500	12.2
4	Center	0	130	0	30	0	4500	11.7
5	Factor	-0.577	101	-0.577	24	-0.577	3900	14.1
6	Factor	+0.577	159	-0.577	24	-0.577	3900	13.5
7	Factor	-0.577	101	+0.577	36	-0.577	3900	12.5
8	Factor	+0.577	159	+0.577	35	-0.577	3900	12.1
9	Factor	-0.577	101	-0.577	24	+0.577	5100	15.7
10	Factor	+0.577	159	-0.577	24	+0.577	5100	15.1
11	Factor	-0.577	101	+0.577	36	+0.577	5100	13.9
12	Factor	+0.577	159	+0.577	36	+0.577	5100	13.3
13	Start	-1	80	0	30	0	4500	12.0
14	Start	+1	180	0	30	0	4500	11.6
15	Start	0	130	-1	20	0	4500	13.7
16	Start	0	130	+1	40	0	4500	11.5
17	Start	0	130	0	30	-1	3500	10.3
18	Start	0	130	0	30	+1	5500	13.6

According the regression coefficients plot in **Figure 10**, needle voltage and nebulizer are significant factors. The needle voltage shows the highest and positive value therefore should be kept at the highest level to get the best response. On the opposite, nebulizer, which shows a high and negative regression coefficient, should be set at the lowest level. No significant interaction factors were observed, p-values obtained from ANOVA are showed in **Appendix b**.

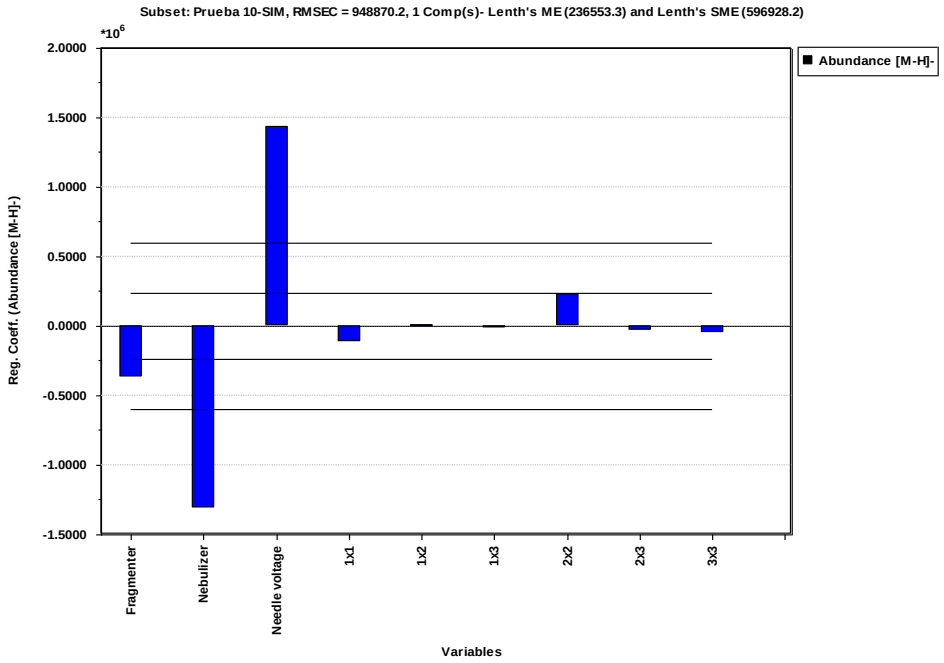

Figure 10 - Regression coefficients plot from the CCD performed in Sirius. Coded values of the variables were used for regression.

A problem found with these results was that the model obtained from the experimental design only captured the 56% of the total variance in the response. This can have different explanations. Looking at the abundances in **Table 2**, the highest needle voltage applied (exp. 18) did not show the highest response. It is possible that undesirable fragmentations could occur when the needle voltage is at the highest level and therefore the abundance of the expected ions decreases. A similar situation occurs with nebulizer pressure, where the lowest value (exp. 15) did not show the highest abundance. Another important fact is the lack of reproducibility in the analyses. Considering the centre point, the analyses showed a coefficient of variation of 9.4% for 4 replicates. The high

variation on the data makes difficult to attribute the differences to instrumental parameters.

To conclude this part of the work, initial experiments showed that a very clean signal of the molecular ion is obtained when FFA were analysed in ESI-. The main signal obtained for FAME was the sodium adduct for almost all the compounds analysed in ESI+. Highly fragmented spectra of FAME were obtained (compared to FFA) when collision induced dissociation was applied. Although the spectra of FAME positional isomers were different, it was not possible to identify diagnostic ions that indicate double bound positions which is possible with chemical ionization in GC-MS [59]. Although the results showed a lack of reproducibility, a needle voltage around 5000 V combined with nebulizer pressure of 24 psi maintaining the fragmentor around 130 V gave the highest abundance of $[M-H]^-$ precursor ions of FFAs.

3.1.2. Liquid Chromatography-Mass Spectrometry

The HPLC method was developed considering three goals: highest signal and highest resolution in the shortest possible anaysis time. In a first attempt both groups of compounds were analyzed applying reverse phase (RP) liquid chromatography using a C18 column, but due to difficulties to elute FAMEs from the column, the development continued only for FFAs. Different mobile phases, columns and instrumental parameters were tested. The process started by injecting single samples of FFAs using mixtures of H_2O, ACN and MeOH as solvents. The solvent composition was adjusted to have adequate retention times and good peaks shape, considering mainly long chain FA that had the highest retention times and widest peaks. Peak symmetry was improved when MeOH was present in the mobile phase, and the t_R was also lower with this solvent (**Figure 11**). On the contrary, the presence of H_2O increased analysis time and peak width.

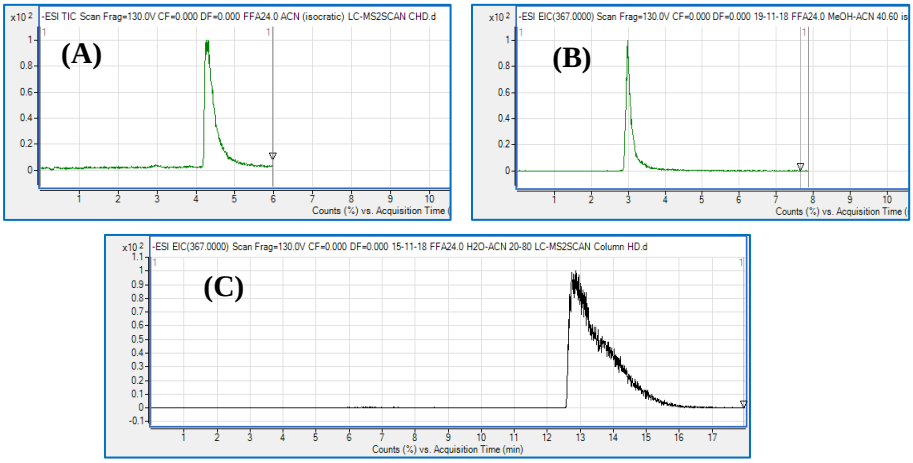

Figure 11 - TIC full scan chromatograms of FFA 24:0. (A) ACN. (B) MeOH:ACN 40:60. (C) H_2O:ACN 20:80. Flow rate 0.5 ml/min.

- ***Selecting the column***

Four different available C18 columns with different particle size and internal diameter were tested to analyse a mixture sample of FFA 20:0, 22:0 and 24:0:

- SB C18, 1,8 µm, 2,1x50 mm Agilent (**Figure 12 (A)**)
- Zorbax Eclipse XDB-C18, 1,8 µm, 4,6x50 mm Agilent (**Figure 12 (B)**)
- Poroshell 120 EC18, 2,7 µm, 3.0x50 mm Agilent (**Figure 12 (C)**)
- Zorbax Eclipse Plus C18 Rapid resolution HD, 1,8 µm, 2.1x50 mm Agilent (**Figure 12 (D)**)

For the columns with higher internal diameter (Zorbax Eclipse XDB) or higher particle size (Poroshell) the flow rate was increased from 0.5 ml/min to 0.7 ml/min to avoid too long retention times due to low back pressure. It can be seen from **Figure 12** that Eclipse XDB, Poroshell and Eclipse Plus columns showed symmetrical peak shapes and good resolution. The last column mentioned showed the lowest retention time with the lowest flow rate and was therefore selected for further studies. Besides, it had lower particle size than Porshell, which contribute to increased resolution by decreasing the A and C terms in the van Deemter equation.

After performing some analyses with the Eclipse plus column, it was observed a constant increase in back pressure, near the limit set for the instrument (400 bar),

probably caused by the retention and accumulation of FAs on the column and/or from the previous uses of the column, which were unknown. This resulted in changing the column to a C8 (Zorbax SB C8 Rapid resolution HD, 1,8 µm, 2.1x50 mm Agilent), expecting that the shorter carbon chains of the column material avoided the strong interaction and accumulation of the fatty acids. C8 and C18 are the most common hydrophobic phases used in reverse phase liquid chromatography. However due to the longer carbon chains of C18, it has greater retention capacity [60], (www.chromacademy.com). It was therefore expected a decrease in retention times with the C8 column.

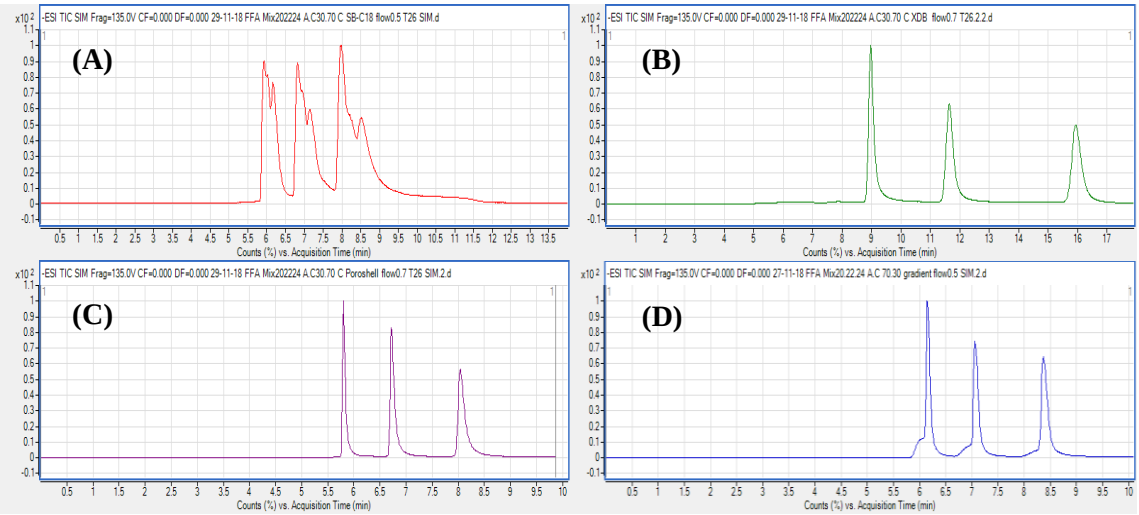

Figure 12 - SIM chromatograms obtained for a mixture of FFA20:0, 22:0 and 24:0 with different Columns. (A) SB C18 1,8µm, 2,1x50 mm Agilent. (B) Zorbax Eclipse XDB-C18 1,8µm, 4,6x50. (C) Poroshell 120 EC18 Agilent 2,7µm, 3.0x50 mm Agilent. (D). Zorbax Eclipse Plus C18 Agilent Rapid Resolution HD 1,8µm, 2.1x50 mm. Mobile phase: 0 min H_2O:MeOH:ACN 20:30:50; 4 min MeOH:ACN 30:70. Column temperature 26 ºC.

- ***Selecting the mobile phase***

Methanol, acetonitrile, isopropanol and water are commonly used mobile phase constituents in reverse phase LC-MS separation of FFA. As mentioned, because of the wide polarity range of fatty acids, analysis with isocratic elution is not feasible. To increase resolution and avoid peak overlapping, gradient elution with a percentage of water at the start point of the run was used. **Figure 13** shows that the use of water in the mobile phase decreases the ionization efficiency of the compounds, evidencing lower responses. It also indicates the improvement in intensity when the proportion of MeOH

is increased in the mobile phase, as well as a decrease in retention time. The proportion of water in the gradient was a compromise between intensity and resolution, therefore it was adjusted to have some resolution between the first two peaks (FFA 8:0 and 10:0) but without losing too much intensity. When the organic solvent was increased to 100% in few minutes it was possible to elute all the studied saturated fatty acids in less than 9 minutes on C18 column and less than 6 minutes on C8 column.

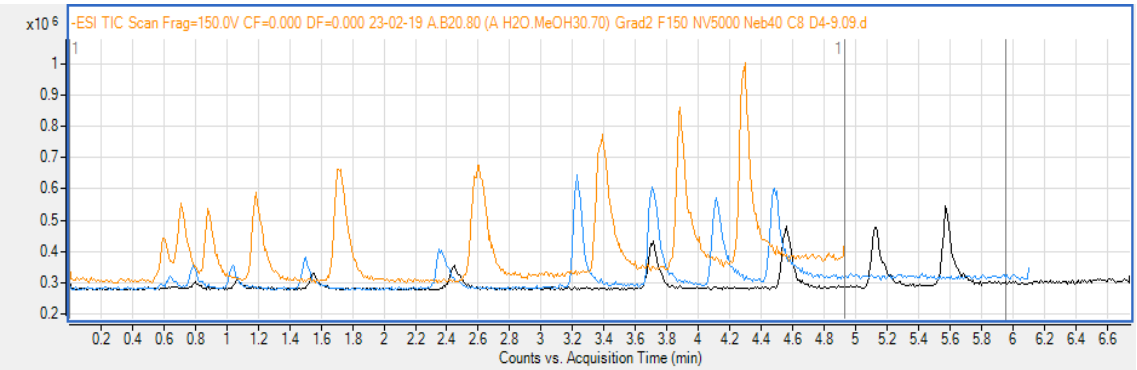

Figure 13 - TIC full scan chromatograms obtained for a mixture of 9 saturated FFA (from 8:0 to 24:0) on C8 column with three MP gradient programs containing different proportions of ***H_2O:ACN:MeOH***. ***Black*:20:40:40** *increasing to ACN:MeOH 50:50 in 3 minutes (notice that the FFA8:0 is almost not visible).* ***Blue*: 12:48:40**. ***Orange*:12:40:48** *(the highest intensity and lowest retention time). The last to gradients were increased to ACN:MeOH 50:50 in 1 minute and maintained until the last compound eluted. Flow rate 0.4 ml/min and column temperature 26 ºC.*

The use of IPA decreased retention times but caused a high increment in the backpressure due to its high viscosity. It was seen that ammonium ions may stabilize long chain fatty acid negative ions [2], therefore, ammonium acetate was tested in the mobile phase, however no improvement in the response was seen.

- ***Effect of column temperature***

Figure 14 shows that increasing temperature from 26 to 40 ºC caused a decrease of more than 1 minute in the total analysis time, while the signal intensity remained almost the same.

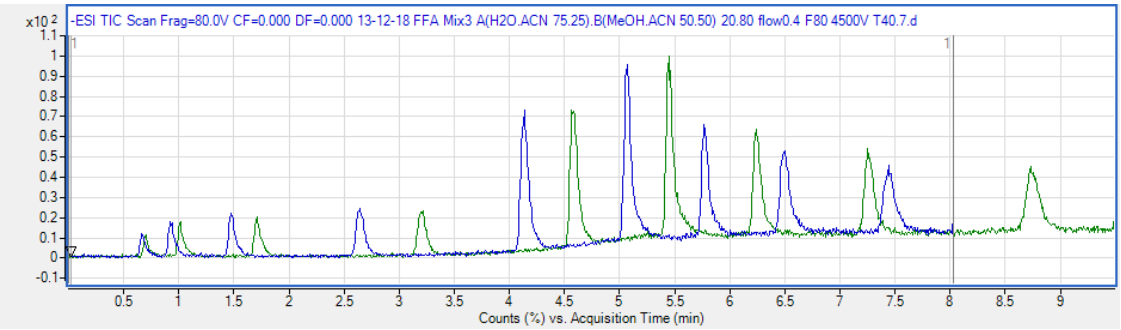

Figure 14 - TIC full scan chromatograms obtained for a mixture of 9 saturated FFA (from 8:0 to 24:0) on C18 column, H_2O:(ACN:MeOH) 15:85 to 100% B in 3 minutes. Flow 0.4 ml/min. Green: 26 ºC, blue: 40 ºC.

- ***Differences in response***

All the chromatograms showed so far where obtained from mixtures containing approximately the same mass concentration (µg/ml) of all FAs, and it was therefore expected higher signal (peak area) for FAs with lower molecular mass, since they contained higher number of molecules. It can be easily noticed from the chromatograms showed in previous sections that there were large differences in response between the different FAs, where the shortest chain FAs showed the lower responses. This effect is investigated in the following section.

- ***Study of unsaturated FFA***

Additionally, a mixture of 6 unsaturated FFA: 18:3 *n*-3, 18:3 *n*-6, 22:6 *n*-3, 18:2 *n*-6, 18:1 *n*-9 and 18:1 *n*-12 was prepared, and the proportions of the solvents were again adjusted to get separation of the compounds. It was possible to partially resolve the isomers of 18:3 and 18:1 FFA (**Figure 15**).

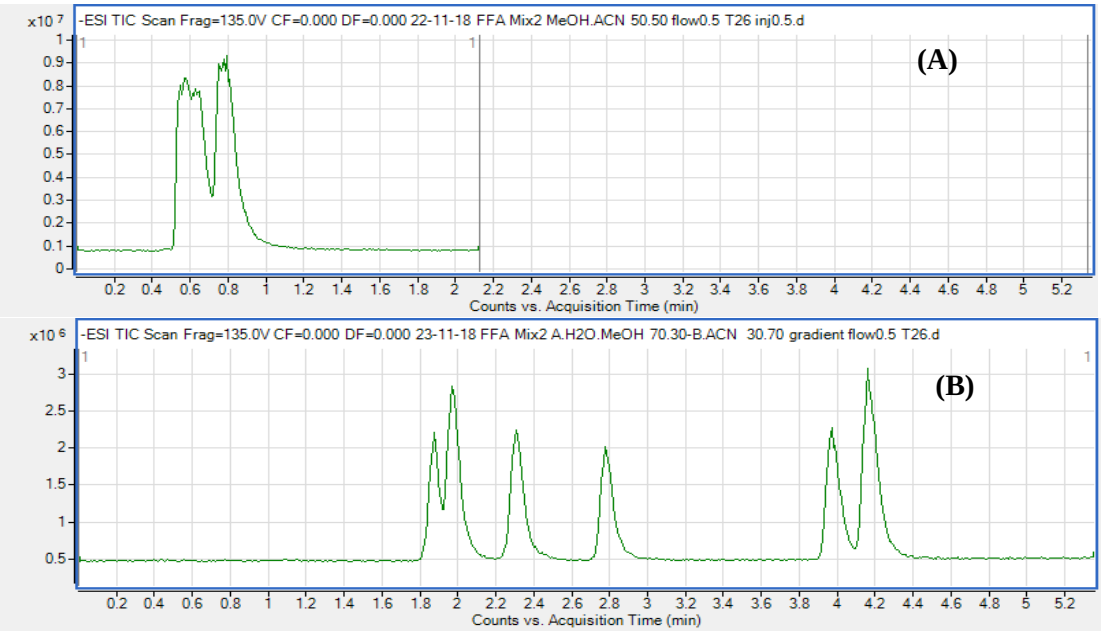

Figure 15 – TIC full scan chromatograms obtained for a mixture of 6 unsaturated FFA on C18 column. (A) MeOH:ACN 50:50 isocratic. (B) (H_2O:MeOH (70:30)):ACN 30:70, Gradient: 0 min 70% B, 5 min 100% B. Flow rate 0.5 ml/min.

The order of elution was confirmed with the injection of single compounds: FFA 18:3 *n*-3, FFA 18:3 *n*-6, FFA 22:6 *n*-3, FFA 18:2 *n*-6, FFA 18:1 *n*-9 and FFA 18:1 *n*-12. The higher the number of the double bonds, the earlier elutes the compound relative to the analogue saturated fatty acid. This confirms that the retention increase as the ECN increases.

In this section it was demonstrated that FFA can be analyzed by RP-LC using C18 and C8 columns with relatively good resolution and in short running times. Less than 9 minutes and less than 6 minutes were the running times necessary to separate a mixture of saturated FFA in C18 and C8, respectively. MeOH in the mobile phase increases the signal but decrease resolution due to a less favorable selectivity. On the contrary, water decreases the signal while increasing the separation between the compounds. The ionization efficiency seemed to be lower for short chain FFA. Column temperature affects the retention times, but seems not to affect signal response. Finally, isomers of 18:1 and 18:3 can be partially resolved using a mobile phase containing water, ACN and MeOH.

3.1.3. Making FFA from FAME

As the developed chromatographic method implies the analysis of FFA it was necessary to find a procedure to convert FAME to FFA since many samples are available as FAME or triglycerides. A modified version of the method suggested by W. W. Christie [61] was applied. In this procedure potassium hydroxide and heat were applied to hydrolyze the esters and convert them into potassium salts of the carboxylic acid. After that, a washing step with iso-octane was made and hydrochloric acid was added to form the FFA. Finally, FFAs were extracted with iso-octane. A scheme of the process is show in **Figure 16.**

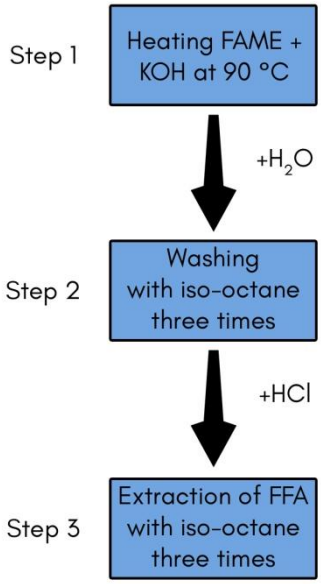

Figure 16 - Conversion process: FAME to FFA.

Step 2 was the most complicated due to the formation of foam, which makes it very difficult to separate the phases. The three final extracts were analyzed separately, and also the washing phases to investigate the necessity of this step. The method was evaluated by GC, where references mixtures were injected to indicate where FFAs 12:0 and 18:0 elute and where FAME should be expected in the chromatogram. **Figure 17** shows the chromatograms obtained from the GC analysis.

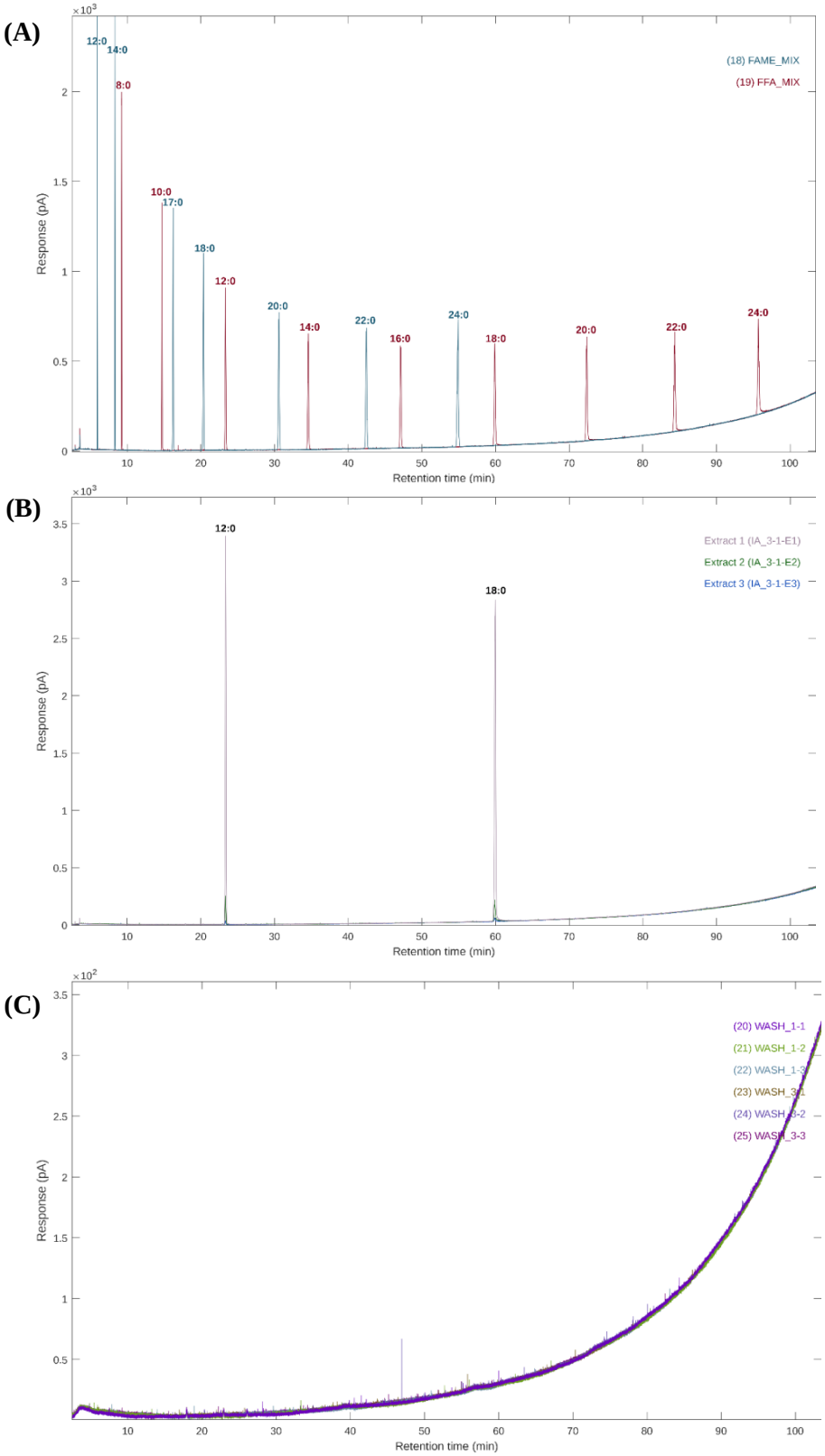

Figure 17 - Chromatograms obtained from GC-FID analysis. (A) Reference mixture of FAME and FFA. (B) Injection of the three final extracts. (C) Three washes for a duplicate sample.

As can be seen, the washing contained no FFA (**Figure 17 (C)**), showing that there is no loss in this step. The washing also did not contain FAME, showing that conversion of FFA to FAME was complete; hence there was no point in using the washing step when working with pure FAME. Regarding the analysis of the final extracts, **Figure 17 (B)** shows low quantity of FFA in extract 2 and almost nothing in extract 3. **Table 3** shows the total areas and the percentages of the FFA obtained in each extract on triplicate analyses. It can be notice that almost everything is extracted in the two first extractions, resulting in a minimal loss.

Table 3 - Total areas after repeated extraction of FFA 12:0 and 18:0.

12:0						
	Exp. 3-1	**Percent**	**Exp. 3-2**	**Percent**	**Exp. 3-3**	**Percent**
E1	24350	93.1%	21098	85.1%	15707	87.6%
E2	1604	6.1%	3226	13.0%	2073	11.6%
E3	196	0.8%	477	1.9%	155	0.8%
Total extr.	26150		24801		17936	
18:0						
	Exp. 3-1	**Percent**	**Exp. 3-2**	**Percent**	**Exp. 3-3**	**Percent**
E1	32758	94.1%	28266	86.5%	20649	88.8%
E2	1775	5.1%	4006	12.3%	2404	10.3%
E3	287	0.8%	401	1.2%	213	0.9%
Total extr.	34820		32673		23267	

To analyze the samples by HPLC, iso-octane was evaporated at 50 °C under N_2 from the extracts and the remaining was reconstituted in MeOH. Then, appropriate dilutions were made to inject into the LC-MS spectrometer.

3.2. Sensitivity and linearity

It was noticed in the previous sections the differences in peak area between the different FAs when they were analyzed by LC-MS. In order to evaluate if it was possible to obtain useful information for quantitative analysis, the differences in response signal were investigated, and whether or not this differences where dependent on the concentration. For this, calibration curves were built, using mixtures of saturated fatty acids of different chain length analyzed by DI-MS and LC-MS.

3.2.1. Calibration experiments

Mixtures of FFA 8:0, 10:0. 12:0, 14:0, 16:0, 18:0, 20:0, 22:0 and 24:0, at seven levels of concentrations were accurately prepared (from 6 to 100 µg/ml) and analyzed by LC-MS and DI-MS. In gradient LC elution the solvent composition is varied from low to high mobile phase strength. To evaluate if the differences in solvent composition affect the ionization efficiency, two conditions were applied in DI: one with a solvent composition corresponding to the start point of the LC program (H_2O:(ACN:MeOH 50:50) 20:80= DI-MS (H_2O)) and the other corresponding to the gradient end (ACN:MeOH 50:50= DI-MS). Since many studies have reported difficulties in reproducibility when these techniques are used [21], and due to the variability on the responses previously seen, FFA 18:0 was used as internal standard (IS). Besides, the quantification of lipids is usually done through direct comparison with an IS, since all analytes and internal standard are subjected to the same ion suppression and matrix effect and also to the same instrumental variability [62]. To get more sensitivity, SIM mode was chosen for the analysis. The sequences were run three times with different randomization. Each calibration mixture contained approximately 30 µg/ml of IS. Plots of A_x/A_{IS} vs C_x/C_{IS} are showed in **Figure 18**, where A denotes area (signal strength), C denotes concentration, IS denotes internal standard, and x is any fatty acid. Ideally, the slopes of these curves should be 1 if all fatty acids have equal response. Chromatograms and peak areas are given in **Appendix c** and **d**. Due to a backpressure increase (nearly 400 bar) on the C18 column, the first sequence analyzed by LC-MS was run at 26 ºC, while the second and the third at 40 ºC. Since previous results seemed to indicate that

temperature had no significant influence in the peak area of the compounds, the calibration curves were constructed with the results of all three sequences.

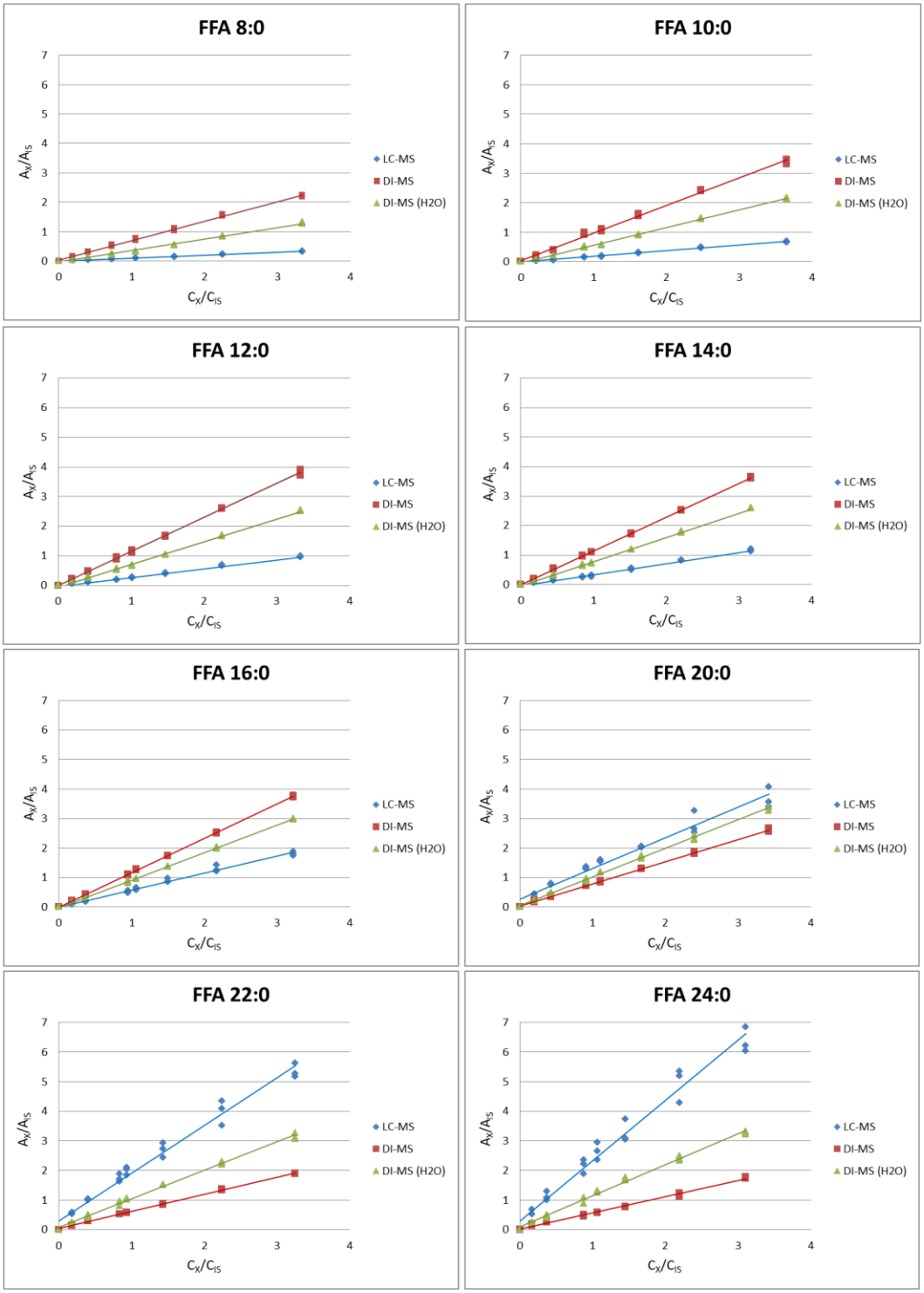

Figure 18 - Calibration curves obtained from: Blue: LC-MS with H_2O:(ACN:MeOH 50:50) 20:80, 0 min. 80% B, 3 min. 100% B, flow rate 0.4 ml/min; Red: DI-MS with ACN:MeOH 50:50, flow rate 0.2 ml/min and Green: DI-MS with H_2O:(ACN:MeOH 50:50) 20:80, flow rate 0.2 ml/min (DI-MS (H_2O)).

A summary with the coefficients of determination (R^2) and slope values for the three methods for all the compounds is given in **Table 4**.

Table 4 - Slope and R^2 obtained from the calibrations curves. Slope values correspond with the response factor: $C_{IS} \cdot A_X / C_X \cdot A_{IS}$

FFA	DI-MS		DI-MS (H_2O)		LC-MS	
	Slope	**R^2**	**Slope**	**R^2**	**Slope**	**R^2**
8:0	0.664	0.9972	0.3912	0.9952	0.0982	0.9953
10:0	0.9394	0.9957	0.5979	0.9974	0.1897	0.9940
12:0	1.1506	0.9990	0.7640	0.9978	0.2962	0.9944
14:0	1.1449	0.9995	0.8191	0.9985	0.3741	0.9930
16:0	1.161	0.9997	0.9308	0.9996	0.5746	0.9911
20:0	0.7534	0.9988	0.9677	0.9975	1.0425	0.9703
22:0	0.5737	0.9987	0.9600	0.9956	1.6062	0.9818
24:0	0.5481	0.9940	1.0498	0.9944	2.0344	0.9765

According to these results, there seemed to be no systematic deviation from the regression lines that could indicate a non-linear response. Direct infusion using an organic solvent without water (DI-MS) shows better fit to the regression line, which gave the highest R^2 values. According with the slope, this method also showed the highest sensitivity for compounds FFA 8:0 to FFA16:0 and, LC-MS for FFA 20:0, 22:0 and 24:0 (compounds which elute with 100% of organic phase). It has been reported that poor sensitivity using ESI has been observed for some lipids. The pre-formation of ions is very important in the ESI detection mode. The sensitivity of detection is dependent on the solution environment as well as the properties of the analyte [63].

Much less time and less consumption of solvents is required to perform a direct infusion analysis compared with chromatographic separation. Considering these advantages, direct infusion can be preferred to analyze simple mixtures of fatty acids. When chromatography is used, an additional separation step exist, which makes this method more reliable since phenomena like ion suppression and ion enhancement are minimized, and isomers may be chromatographically separated.

3.2.2. Differences in response

As mentioned before, since all the samples were prepared in approximately the same concentration of each compound in mass/volume units, it was expected that FAs having lower molecular weight (and therefore higher number of moles) showed higher responses if the ionization efficiency was equal for all the compounds. However, this was not the case. **Table 5** shows the mass, mol and area percentage for one of the calibration mixtures that contains a concentration of approximately 30 µg/ml of each compound (including the IS, FFA18:0). Short chain fatty acids, which had the highest mol percentage, did not show the highest area percentage in any of the three methods applied.

Table 5 - Mass, mol and area percentage for one calibration mixture analyzed by LC-MS, DI-MS and DI-MS (H_2O) methods.

FFA	Conentration (µg/ml)	Mass%	Mol%	Area%		
				LC-MS	DI-MS	DI-MS (H_2O)
8:0	30.8	11.3	18.3	1.1	8.7	4.3
10:0	32.5	11.9	16.2	2.2	12.8	7.4
12:0	29.6	10.8	12.6	2.8	13.7	8.5
14:0	28.5	10.4	10.7	3.3	13.3	9.2
16:0	31.2	11.4	10.4	7.1	15.3	12.4
18:0	29.4	10.8	8.8	11.6	11.9	12.9
20:0	32.5	11.9	8.9	18.1	10.4	15.2
22:0	27.3	10.9	6.9	23.0	6.9	13.4
24:0	31.2	11.4	7.2	30.7	6.9	16.6

The behavior of the analysed fatty acids was very different. Comparing LC with the two DI methods, DI-MS (H_2O) seemed to present the same behavior, where the response percentage is increasing with the chain length; however the difference is more pronounced in LC, where the last compounds, which elute without water in the MP, presented the highest responses. Completely different is what DI-MS with only organic solvent showed, where a higher response was obtained for short FAs. It is also more equal in response between different fatty acids than the solvent mixture with water, and it has responses that are at maximum around medium chain lengths (FFA12, 14 and 16).

According to this it seems clear that the presence of water decrease the ionization efficiency of the FAs and comparing both DI methods it seems that the short FAs are more affected by high proportion of water.

It is also known that ion suppression can occur when ESI is applied, where the presence of other analytes (or matrix) will influence the ionization efficiency of a compound. It has been demonstrated that molecules with higher mass can suppress the signal of smaller molecules, and also that more polar analytes are more susceptible to suppression [20]. This could happen in DI where all the analytes enter together in the ion source and can explain why the response is lower in DI-MS for short chain fatty acids even though the mobile phase did not contain water. In LC-MS the analytes enter one by one on the ionization source, thus ion suppression due to competition with other analytes should not be present, but there may still be other molecules in the solvent that affect ionization efficiency. If it is considered that ion suppression has no influence in LC and that the efficiency is affected by the presence of water, the first eluting compounds will be more affected, and in this way the signal obtained will be smaller. Since LC-MS and DI-MS (H_2O) showed the same tendency and the differences in response are higher in LC, it seems that the main cause for the difference in the response is that the FAs have different ionization efficiencies in different solvents and that some of these compounds have an intrinsic poor ability to be ionized [64], such as short fatty acids.

Regarding the raw areas, these are much higher when MeOH:ACN (50:50) is used as solvent instead of H_2O:(MeOH:ACN 50:50) in DI. In LC-MS the solvent that gives poorest response (water) is used when the short FA elute, while longer chain FA elute with a solvent that give better signal. This will therefore amplify the differences in response caused by the different solvent compositions, which leads to the very large differences in response seen for the LC method. According to Xie et al, using MS to accurately quantify complex lipid mixtures may be difficult, because lipids have different responses to mass analyzer due to the different total carbon numbers and double bonds [62].

It can be concluded that there are large differences in response and it is always lower for the shortest FAs. The fact that the response differences are higher in LC-MS than in DI-MS rules out ion suppression as the main cause. Ionization efficiency in different solvents seems to be an important cause for the differences in response. The same differences in response were seen for all levels of concentrations and none of the responses showed strong deviations from linearity in the relationship between signal and concentration (relative to the IS), which indicates that response factors based on a single reference sample may work well for quantification.

3.3. Effect of chromatographic parameters

The purpose of the work described in this section was to evaluate how different chromatographic parameters: the choice of apolar solvent, temperature and gradient time affected the retention patterns, chromatographic efficiency and detector sensitivity. Three level factorial design and response surface methodology was applied for the purpose. The following apolar solvents were evaluated: methanol (MeOH), acetonitrile (ACN), acetone (ACO) and tetrahydrofuran (THF). Because these solvents have different polarity it was necessary to standardize the solvent composition (fraction of water/apolar solvent) so that the mobile phase had approximately equal strength at the start and at the end of the gradient. The flow rate applied was 0.35 ml/min for all the solvents systems.

3.3.1. Description of HPLC-programs

It is known that selectivity can vary significantly as a function of gradient steepness or temperature [45]. These two factors: column temperature and gradient time (time in which the organic solvent is increased) were selected as variables using 4 different solvents systems including water and ACN, MeOH, ACO and THF. The mobile phase used so far consisted of water in combination with an organic solvent, where water is the weakest solvent. The organic modifier is less polar and therefore has higher elution strength in reverse phase chromatography as it speeds up elution and reduces the retention times. Although the polarity of a certain solvent can be known the properties of mixture solutions are difficult to comprehend [65]. The proportion of each organic solvent (solvent B) combined with water (solvent A) was adjusted to give approximately the same t_R for the first and last eluting compound (FFA 12:0 and FFA 24:0 respectively). The initial conditions of the chromatographic run which gave the best linear relationship between ECL and t_R were selected in order to obtain accurate estimates of ECL values from Chrombox C. The saturated FFAs contained in the GLC-793 mixture were used for calibration of the ECL values.

3.3.2. Standardization of solvents

ACN and MeOH were first evaluated, ACO was evaluated in a second stage and finally THF was incorporated to the experimental design. A reference mixture containing 9 FFAs (8:0, 10:0, 12:0, 14:0, 16:0, 18:0, 20:0, 22:0, 24:0) was analyzed using different mixtures of A(water):B(organic solvent), from 0 to 25% of A, with the aim to find the composition of ACN:H_2O and MeOH:H_2O that gave the same t_R for FFA 24:0. **Table 6** shows the polarity and viscosity of the different solvent used in the study. As it was expected, the retention times follow the polarity properties when they are used as pure solvents, FFA 24:0 showed lower t_R for MeOH than ACN. However, when they were mixed with water it was the opposite. The ACN mixture had higher elution strength than MeOH. The increase in the proportion of water when it is mixed with MeOH causes an exponential increase in the retention time (**Figure 19**). Chromatograms are shown in **Figure 20**.

Table 6 - Synder polarity indices and viscosity.

Solvent	Polarity index	Viscosity (cP) at 20 ºC
Acetonitrile	5.8	0.38
Methanol	5.1	0.55
Acetone	5.1	0.36
Tetrahydrofuran	4.0	0.55

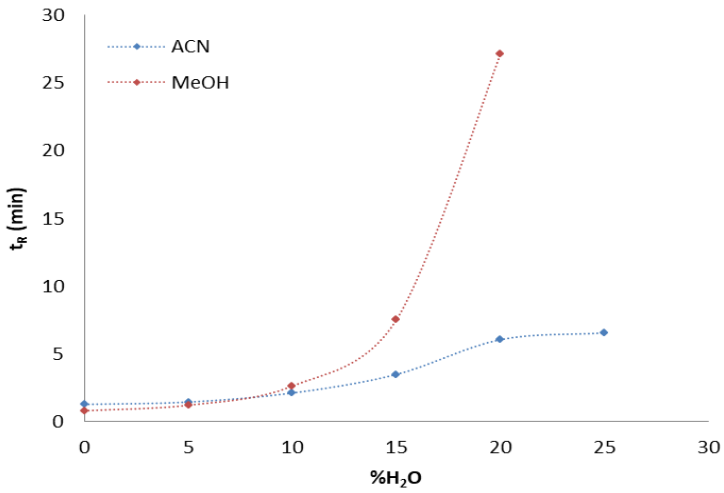

Figure 19 - Retention time of FFA 24:0 vs H_2O percentage on the mixtures with ACN and MeOH. (FFA 24:0 did not elute in less than 45 minutes using MeOH with 25% of H_2O).

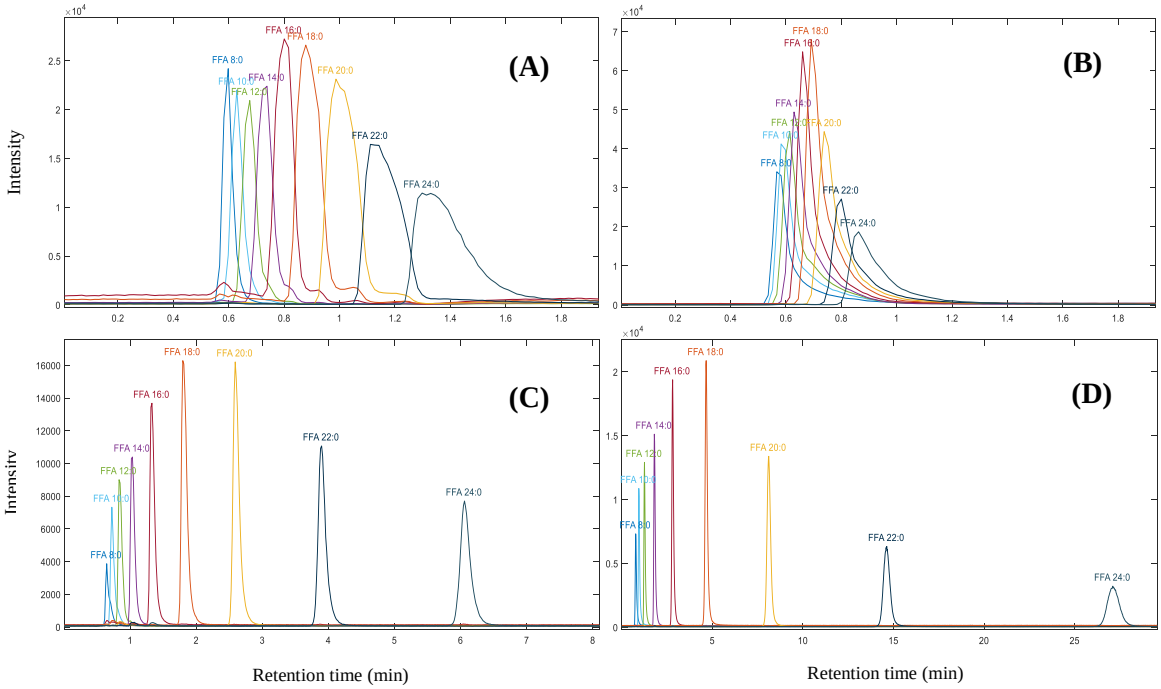

Figure 20 - SIM chromatograms obtained with different mobile phases: (A) 100% ACN. (B) 100% MeOH. (C) H_2O:ACN 20:80. (D) H_2O:MeOH 20:80. Isocratic elution, flow rate 0.35 ml/min, column temperature 26 ºC.

Both solvents gave similar t_R when mixed with a percentage of water between 0 and 10%, thus it was expected to have similar retention when the solvents were increased to 100% in the gradient. However, these differences in the behavior when they were combined with water required standardization of the start point of the chromatographic run for each solvent separately.

- ***Standardize start point***

The criterion used for standardization of the start point was that there should be a linear relationship between chain length (ECL) and retention times for normal saturated FA from C12 to C24. With an intermediate temperature of 45 ºC, the reference mixture GLC-793 was analysed trying different mixtures of water and organic solvent at the start point of the run, and increasing to 100% B in 15 minutes.

ACN

Proportions of water from 20 to 50% in increments of 10% where tested as solvent A. According to ECL vs t_R plots **(Figure 21**), the best linear relationship would be obtained

with a proportion of water between 40%, where the relationship between ECL and t_R was slightly concave, and 50%, where it is slightly convex. Based on these two conditions linear regression models where built to predict the t_R of each fatty acid in the range 40-50% water. 44% of H_2O gave the best linear fit for ECL vs t_R predicted. Thus H_2O:ACN 44:56 was selected as the start point for the design.

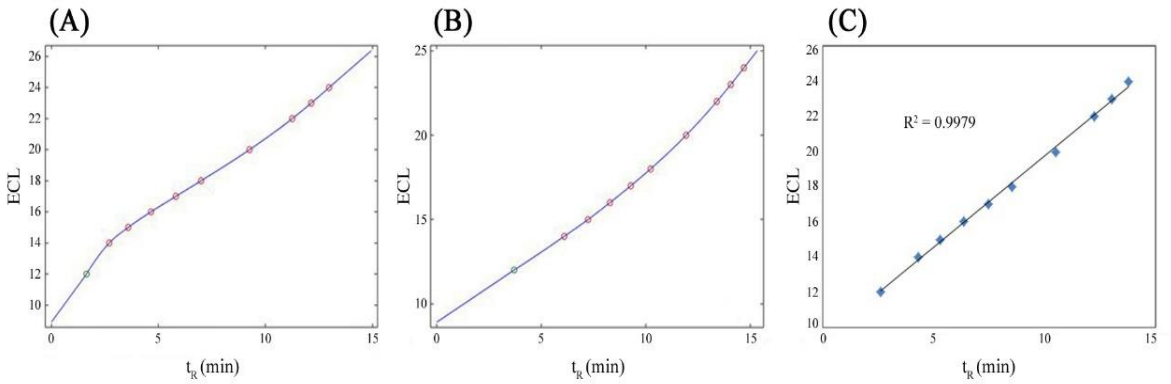

Figure 21 - Regressions curves for ACN. (A) ECL vs t_R with 40% H_2O. (B) ECL vs t_R with 50% H_2O. (C) ECL vs t_R predicted with 44% of water.

MeOH

Regarding MeOH the procedure applied was the same but with a lower percentage of water in the tested mobile phases. The water percentage was tested in the range of 20-30% to build the models. In this case 25% was found to give the best linear relationship between ECL and t_R (**Figure 22**). Therefore H_2O:MeOH 25:75 was selected as the start point for the design.

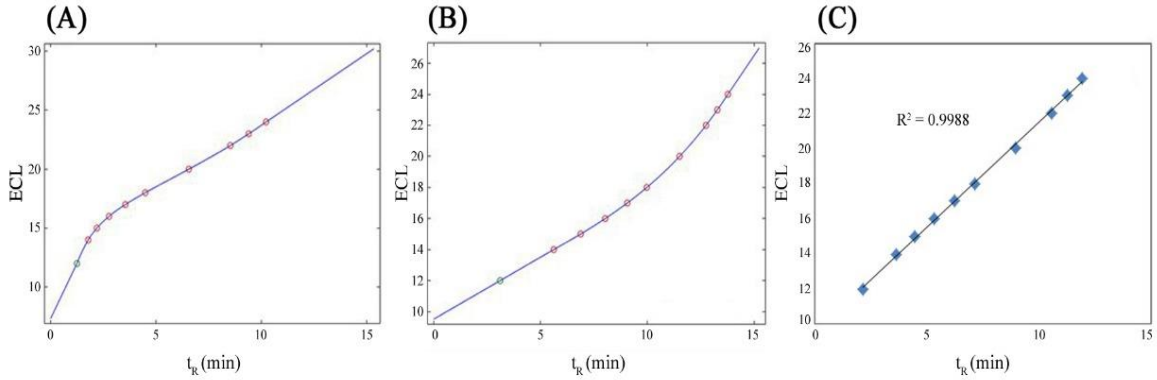

Figure 22 - Regressions curves for MeOH. (A) ECL vs t_R with 20% H_2O. (B) ECL vs t_R with 30% H_2O. (C) ECL vs t_R pred with 25% of water.

ACO

ACO was added later to the design and the proportion of the mixture ACO-water at the start point of the chromatographic run was adjusted. In this case the procedure was different. The aim when standardizing the mobile phase composition was that the retention times for 12:0 and 24:0, contained in the GLC-793 mixture, should be close to the corresponding values for MeOH and ACN, and ideally between the values for these solvents.

After testing different mobile phase compositions at the start and end point of the chromatographic run, the best conditions found were: H_2O:ACO 38:62 increasing to 85% of ACO in 15 min, which gave a t_R of 2.52 and 14.07 min for FFA12:0 and 24:0 respectively at 30 ºC. This program was chosen to set the experimental design for ACO.

THF

THF was standardized the same way as ACO. However, THF has very different properties than the other three solvents, which put constraints on the possible conditions to use. Its less polar behavior leads to use higher proportion of H_2O, this in combination with its medium-high viscosity (**Table 6**) resulted in the highest column back pressures around 450 bar. The proportion of water was increased to higher percentages than the other solvents to get separation of the compounds. **Figure 23** shows the differences in resolution with small increments in water proportion. The final proportion of the THF in the gradient could not be increased to more than 60%, otherwise, the retention time for FFA 24:0 was too low. H_2O:THF 55:45 was selected as start point and H_2O:THF 40:60 as the end point of the chromatographic run. **Table 7** shows the retention times obtained for FFA 12:0 and FFA 24:0 for the different solvents at 30 and 45 ºC. To perform the experiments at 30 ºC with THF, it was necessary to increase the pressure limit of the instrument from 400 to 500 bar. The pressure obtained for these experiments were 457 bar which is far below the pressure limit for the column (1200 bar).

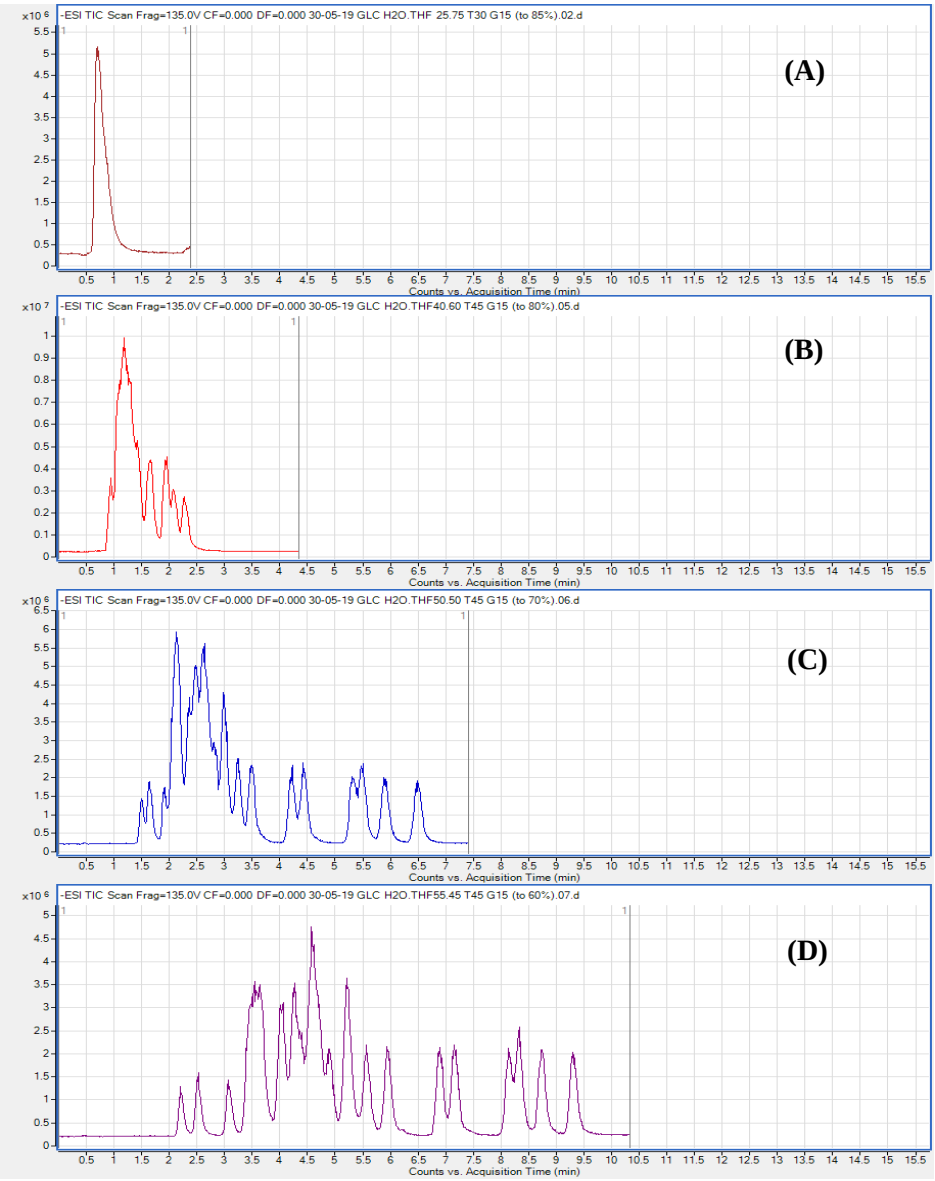

Figure 23 - TIC chromatograms obtained for GLC-793 with different proportions of H_2O:THF at 40ºC. (A) 25% of H_2O (There is no separation due to co-elution). (B) 40% of H_2O. (C) 50% of H_2O. (D) 55% of H_2O.

Table 7 - Retention times in minutes of FFA 12:0 and 24:0 with 15 minutes gradient time with the different programs: H_2O:ACN 44:56 to 100% of ACN, H_2O:MeOH 25:75 to 100% of MeOH, H_2O:ACO 38:62 to 85% of ACO and H_2O:THF 55:45 to 60% of THF.

	ACN		**MeOH**		**ACO**		**THF**	
	30 ºC	**45 ºC**	**30 ºC**	**45 ºC**	**30 ºC**	**40 ºC**	**30 ºC**	**45 ºC**
FFA 12:0	2.66	2.36	2.26	1.81	2.52	2.02	2.85	2.22
FFA 24:0	14.82	13.79	13.21	12.26	14.07	13.01	13.97	10.73

3.3.3. Experimental design

After the solvent compositions had been properly standardized it was possible to study the effects of temperature and gradient time for the four different apolar modifiers. A full factorial design with two factors and three levels (30, 45 and 60 ºC for temperature and 10, 15 and 20 min for gradient time) was performed. The temperature levels were selected according to the limitations of the column, and gradient time was selected in order to have appropriate retention times**.** A total of 36 experiments were done: 9 with ACN, 9 with MeOH, 9 with ACO and 9 with THF system (**Table 8**). Because ACO has some limitations regarding its low boiling point (56 ºC), the highest level for column temperature in the design was decreased to 50 ºC for this modifier. As was mentioned before the compounds were resolved and identified using Chrombox D, then in Chrombox C the retention times were converted to ECL by second order local regressions [38] using the saturated unbranched fatty acids for calibration. These values were then analysed using Chrombox O. In general, quite linear relationships for regressions between t_R and ECL were obtained.

Table 8 - 3^2 experimental design for the study of chromatographic parameters.

Experiment	Temp. (ºC)		Gradient time (min)
	ACN/MeOH/THF	ACO	
1	30	30	20
2	30	30	15
3	30	30	10
4	45	40	20
5	45	40	15
6	45	40	10
7	60	50	20
8	60	50	15
9	60	50	10

3.3.4. Effects on retention patterns

One of the purposes of the experiment was to evaluate to which degree the retention pattern is affected by the chromatographic parameters. As **Table 9** shows, the biggest

variation in ECL is given for compounds with at least 3 double bounds; denoting that there is a connection between the fatty acid structure and the variation in ECL values. ECL values were very similar among the different solvents except for THF which showed more unique values. In general, the highest shifts between the different experiments were observed for THF and ACN systems and the lowest for ACO and MeOH. Higher variation was seen for the highly unsaturated FFA of 22 and 20 carbons. The shift was also very high for FFA 18:3 for the experiments performed with THF. Regarding ACO, FFA18:3 and 20:3 were the compounds that showed the biggest variation in ECL. However the isomers of these compounds are not resolved in the majority of the experiments with this solvent.

Table 9 - ECL average and range (max-min) for the 9 experiments with the different solvents (ACN, MeOH, ACO and THF), and calculated range between the averages for the solvents. The highest shifts for each solvent are shown in red.

FA	ACN		MeOH		ACO		THF		Max-min (between the solvents)
	Average	Max-Min	Average	Max-Min	Average	Max-Min	Average	Max-Min	
14:1	12.614	0.034	12.616	0.072	12.619	0.083	12.769	0.110	0.155
16:1	14.618	0.055	14.612	0.056	14.595	0.046	14.741	0.097	0.146
17:1	15.571	0.057	15.574	0.047	15.571	0.038	15.723	0.112	0.152
18:3 n-3	14.326	0.108	14.378	0.072	14.261	0.176	14.700	0.345	0.438
18:3 n-6	14.326	0.108	14.378	0.072	14.331	0.058	14.842	0.110	0.516
18:2	15.314	0.100	15.371	0.066	15.292	0.045	15.630	0.143	0.338
18:1	16.547	0.072	16.549	0.057	16.557	0.039	16.743	0.154	0.196
20:5	14.218	0.116	14.320	0.067	14.198	0.069	15.067	0.198	0.869
20:4	15.233	0.138	15.308	0.084	15.253	0.075	16.036	0.182	0.803
20:3 n-6	16.051	0.162	16.165	0.102	16.057	0.137	16.499	0.147	0.448
20:3 n-3	16.051	0.162	16.290	0.067	16.057	0.137	16.499	0.147	0.448
20:2	17.160	0.122	17.283	0.084	17.142	0.040	17.439	0.095	0.297
20:1	18.431	0.091	18.457	0.084	18.382	0.079	18.565	0.159	0.183
22:6	15.136	0.185	15.295	0.097	15.165	0.082	16.240	**0.226**	**1.103**
22:5	15.759	**0.201**	15.982	0.106	15.792	0.072	16.554	0.216	0.795
22:4	16.764	0.200	16.954	**0.134**	16.801	0.084	17.503	0.200	0.739
22:1	20.347	0.071	20.383	0.082	20.267	0.081	20.409	0.145	0.142
24:1	22.286	0.079	22.333	0.111	22.186	**0.122**	22.306	0.141	0.147

It also can be observed that the range between the averages of ECL values for each solvent is much larger than the within solvent ranges, evidencing the importance of the solvent used, more than the other chromatographic conditions. All the experiments performed with MeOH were capable of partially resolving the two isomers of FFA20:3. Experiments at 30 and 45 ºC with THF partially resolve FFA18:3 isomers. All the experiments with ACO at 30 ºC partially resolved 18:3 isomers.

- ***Principal component analysis of ECL***

With the ECL values obtained for the three first solvents investigated (ACN, MeOH and ACO) a principal component analysis (PCA) was performed considering the 27 experiments as objects and the unsaturated fatty acids as variables. The scores plot and the loadings plot are showed in **Figure 24**. The PC1 and PC2 together explain the 94.13% of the variation in the original data matrix. It can be seen that there are three clear clusters in the plot corresponding to the difference in the solvent, where PC2 is quite important for the separation. It also can be seen that the experiments performed at the same temperature form sub-groups within each solvent (with exception of ACO, probably because of the range of temperature tested for this solvent was smaller). No clear effect of the gradient time can be seen in the score plot. According to the loading plot, the main effect seems to be the degree of unsaturation, which is explained by a combination of PC1 and PC2, but mainly by PC1. There also seem to be an effect of the chain length in monoenes, basically along PC2, with greater differences for the longest chain. However, the differences between ECL of monoenes are low, so this effect may have limited practical significance. 18:3 A (18:3 *n*-3) deviates from this pattern, probably because it was an overlapping peak in most chromatograms, and therefore can be more affected by noise.

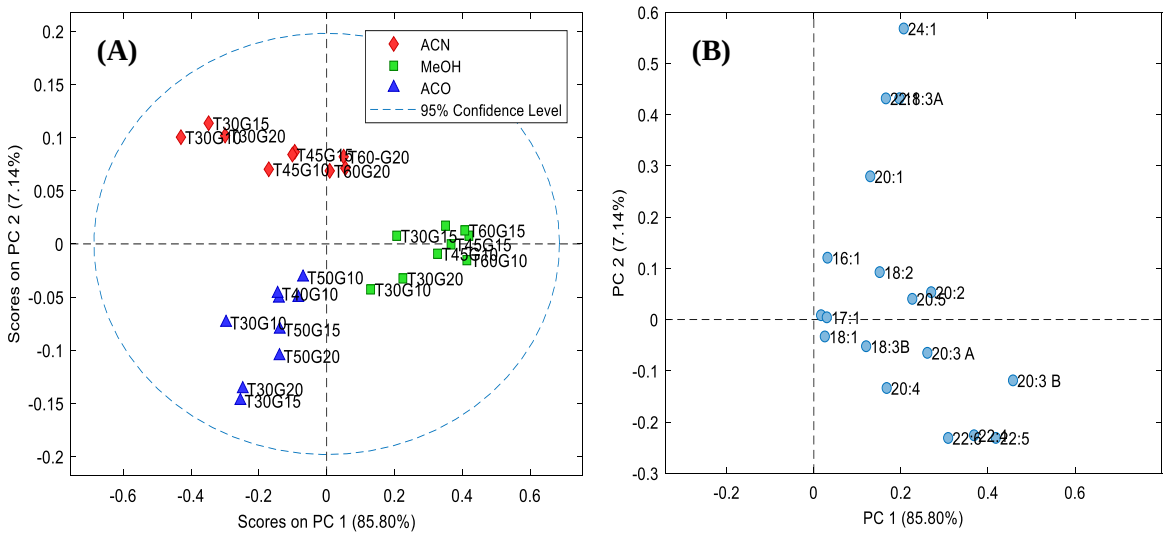

Figure 24 - PCA of ECL for ACN, MeOH and ACO. (A) PCA score plot showing similarities between the 27 programs from the design. (B) PCA loading plot.

Figure 25 shows when THF was included in the PCA. As THF showed more unique ECL values, a cluster containing THF experiments can be observed far from the other experiments. Another cluster can also be seen for the experiments corresponded to MeOH, while the experiments performed with ACN and ACO are mixed, which seem reasonable due to the similarity in ECL values, mainly between the ACN experiments at 30 and 45 ºC with the ACO experiments at 40 and 50 ºC. According the loading plot, the main effect explained by PC1 (97%) is the degree of unsaturation. One can draw almost vertical lines in the loading plot that will fit to the fatty acids with the same number of double bonds. There is also a tendency that the shortest FAs have the lowest values along PC2. This component explains the effect of temperature that is seen within each main group, but it also separates the three solvents that are not THF.

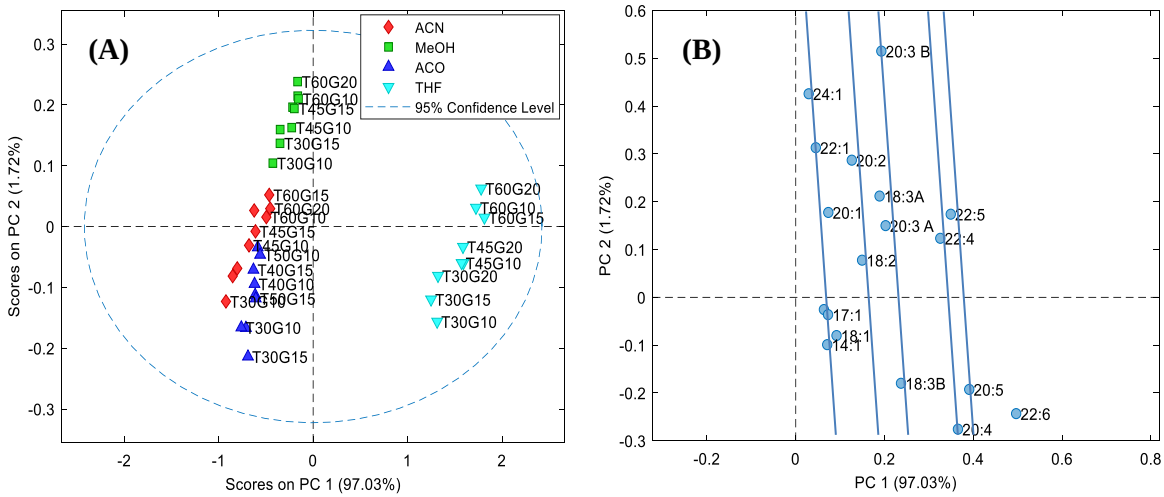

Figure 25 - PCA of ECL for ACN, MeOH, ACO and THF. (A) PCA score plot. (B) PCA loading plot.

As was mentioned before, some experiments were able to partially resolve 18:3 and 20:3 isomers. To check if these compounds could generate noise in the data, a new PCA was built but removing the values for FFA18:3 and 20:3 from the data set. The only difference observed is that the clusters of MeOH and ACN are closer to each other so these compounds contributes to the differentiation between the experiments with these two solvents. According to the ECL values, 20:3 has more influence, probably because its isomers are resolved with MeOH. PCA plots are showed in the **Appendix e**.

- ***Response surface models of ECL***

Models to predict ECL values for each compound were created in Chrombox O considering temperature and gradient time as variables. The models were evaluated by the coefficient of determination (R^2) and RMSE between predicted and measured. The plots of predicted vs. measured for FFA 22:6 are shown in **Figure 26**.

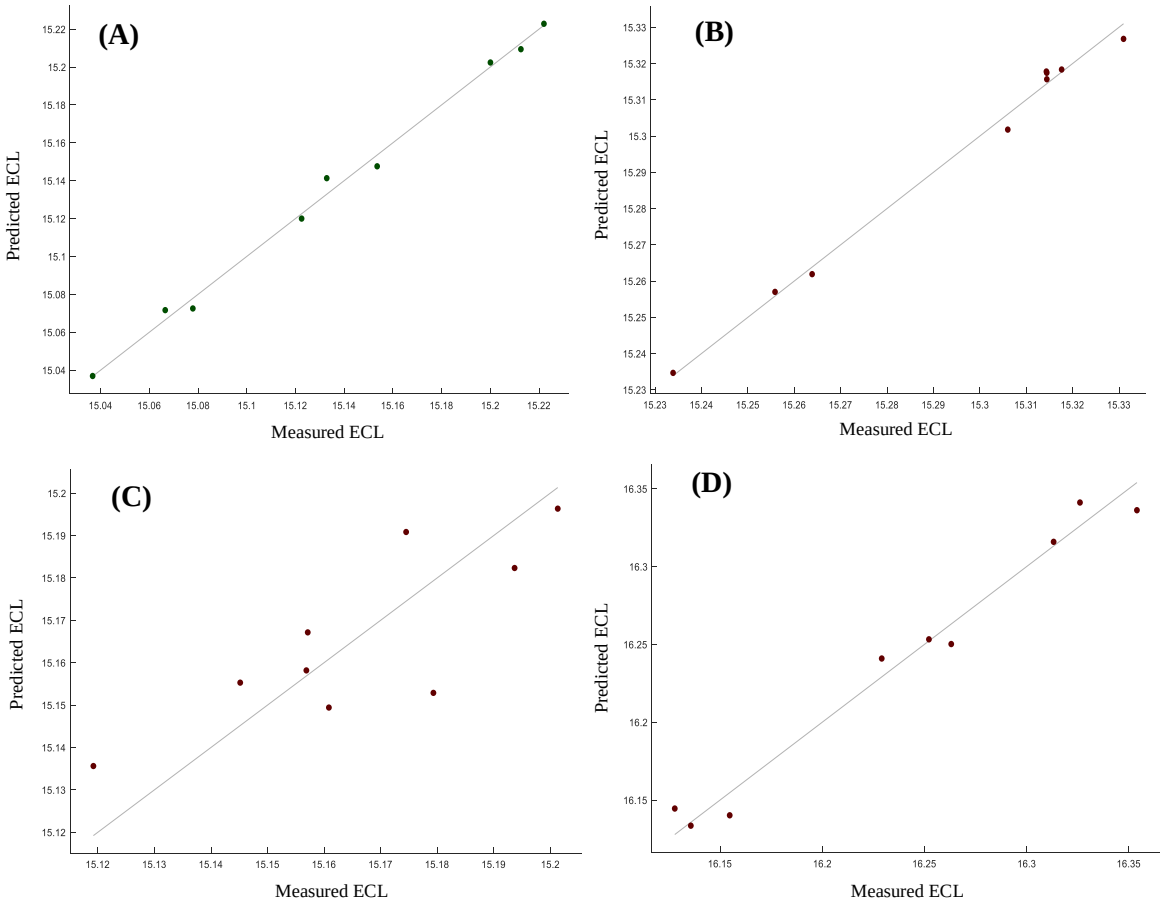

Figure 26 - ECL values predicted vs measured for FFA 22:6. (A) ACN. (B) MeOH. (C) ACO. (D) THF.

A summary with the R^2 and RMSE values is given in **Table 10** and **Figure 27** respectively. ACN showed higher R^2 and lower RMSEs for predictions than the other solvents. ACO had the lowest R^2 values. This can be partially explained by the range in the response, which was low for ACO. Besides, ACO and THF also tend to have higher RMSE than the other solvents. In general, better models were obtained for polyunsaturated fatty acids which showed the highest ECL shifts. FFA 22:6 showed the highest R^2 with ACN and MeOH systems and 18:3 *n*-3, which is partially resolved from its isomer in some of the systems, show the most accurate models with THF. For some monounsaturated compounds like 18:1, 20:1, 22:1 and 24:1, the MeOH system presented the highest values of R^2 and the lowest RMSE.

Table 10 - R^2 values for ECL predicted vs measured of the response surface models for ACN, MeOH, ACO and THF.

FA	R^2 (Predicted vs Measured)			
	ACN	**MeOH**	**ACO**	**THF**
14:1	0.6919	0.8233	0.8641	0.8903
16:1	0.9744	0.4408	0.4849	0.7994
17:1	0.9747	0.8832	0.8274	0.9621
18:1	0.9002	0.9534	0.6084	0.9010
18:2	0.9917	0.9741	0.9449	0.9656
18:3 *n*-3	0.9543	0.8980	0.9042	0.9947
18:3 *n*-6	-	-	-	0.9458
20:1	0.9774	0.9910	0.9343	0.9576
20:2	0.9836	0.9753	0.9862	0.8880
20:3 *n*-6	0.9822	0.9339	0.8932	0.9817
20:3 *n*-3	-	0.9345	-	-
20:4	0.9986	0.9890	0.7271	0.9879
20:5	0.9307	0.8914	0.8491	0.9461
22:1	0.8847	0.9882	0.8341	0.8868
22:4	0.9948	0.9900	0.9517	0.9099
22:5	0.9950	0.9683	0.8737	0.8913
22:6	0.9949	0.9931	0.6600	0.9764
24:1	0.9369	0.9696	0.9106	0.9791

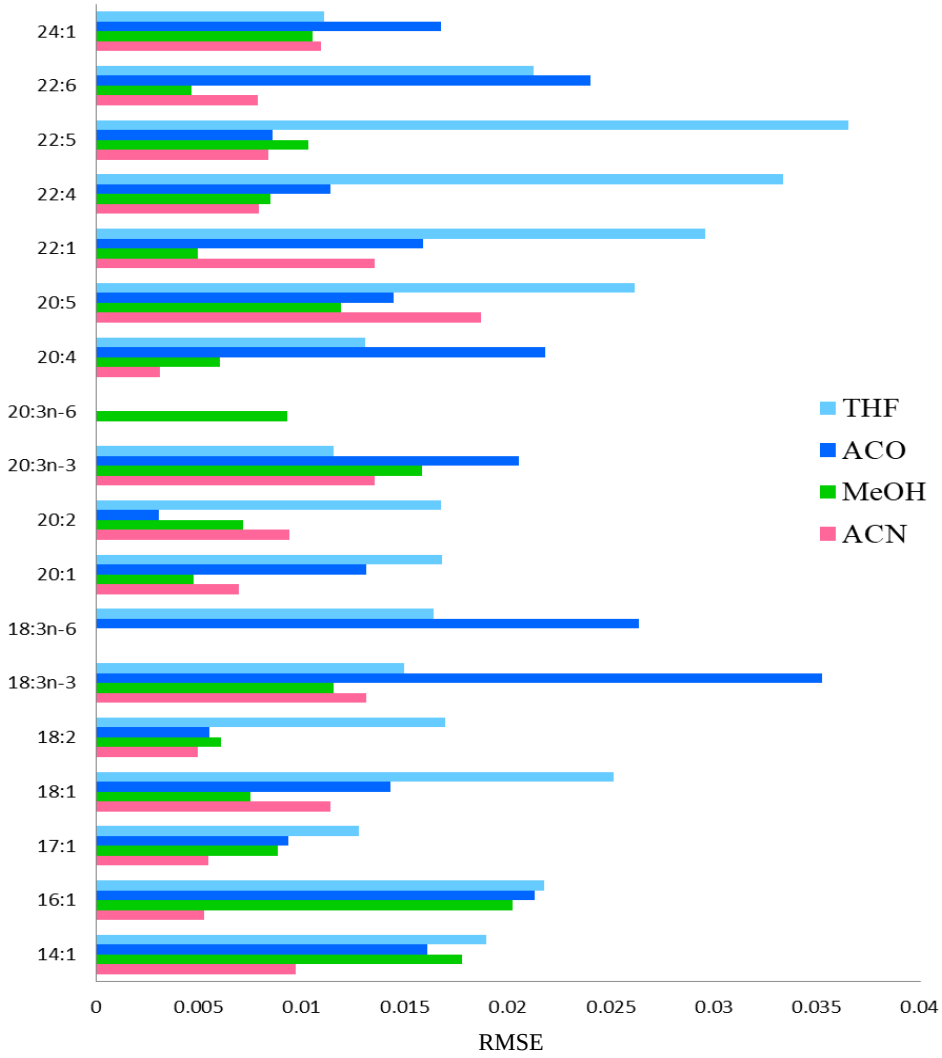

Figure 27 - RMSE values obtained from the models for all the unsaturated compounds analysed with ACN, MeOH, ACO and THF.

The fatty acid 16:1 showed the worst R^2 and RMSE with the MeOH system. There was a general tendency for all fatty acids to increase ECL values with temperature. This was not the case for 16:1, which seem to have a more random variation of ECL than the other FA. It is possible that the estimation of ECL for 16:1 was inaccurate since the plot of retention time vs ECL has a strong a curvature around ECL=14, which is more noticed in the case of MeOH (**Figure 28**). Moreover, considering compounds showing little variation in ECL, for example 18:1 in ACO systems, the model is just marginally more accurate than using the mean value.

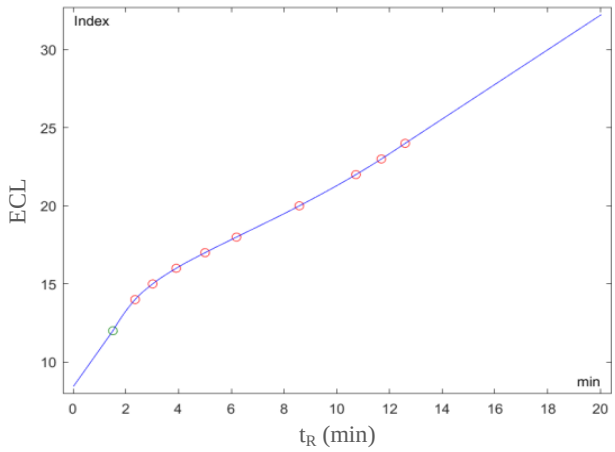

Figure 28 - ECL vs t_R, H_2O:MeOH 25:75. Temperature: 30 ºC, Gradient: 20 min.

The peak width values, in retention index units in the different experiments were between 0.2 and 0.6, where the lower value corresponded mainly to MeOH and ACN experiments and the highest peak width values corresponded to THF and ACO. Comparing the obtained RMSEs with the peak width, the majority of the RMSEs are below 0.02 and none of them are above 0.04 ECL units, which means that the errors are fractions (typically below 10%) of a peak width at baseline.

A weak point of the LC-MS methodology is the ability to distinguish between isomers such as 18:3 *n*-6/18:3 *n*-3 and 20:3 *n*-6/20:3 *n*-3. The largest observed difference in ECL within these pairs was 0.14 on the C8 column, while it is between 0.3 and 0.5 in typical GC columns like BPX70, BP20 and IL100 (www.chrombox.org/data).

- ***Response surface plots***

The response surface plots were also evaluated (**Figure 29**) to check the influence of the variables. In general, the plots show the same trends as PCA, the main effect on the ECL values is the temperature, while the gradient time has almost zero effect. This is particularly clear for ACN and THF, where it looks like an almost linear dependence of ELC on temperature. For the two other solvents (MeOH and ACO) the models are slightly more complex. For MeOH there is a larger effect of increasing from low to medium temperature, than from medium to high temperature (also visible in the PCA plot). ACO shows more complex models with maxima and saddle points, and less clear

effect of temperature. But in these experiments there were also lower temperature range (30 to 50 ºC vs. 30 to 60 ºC for the other).

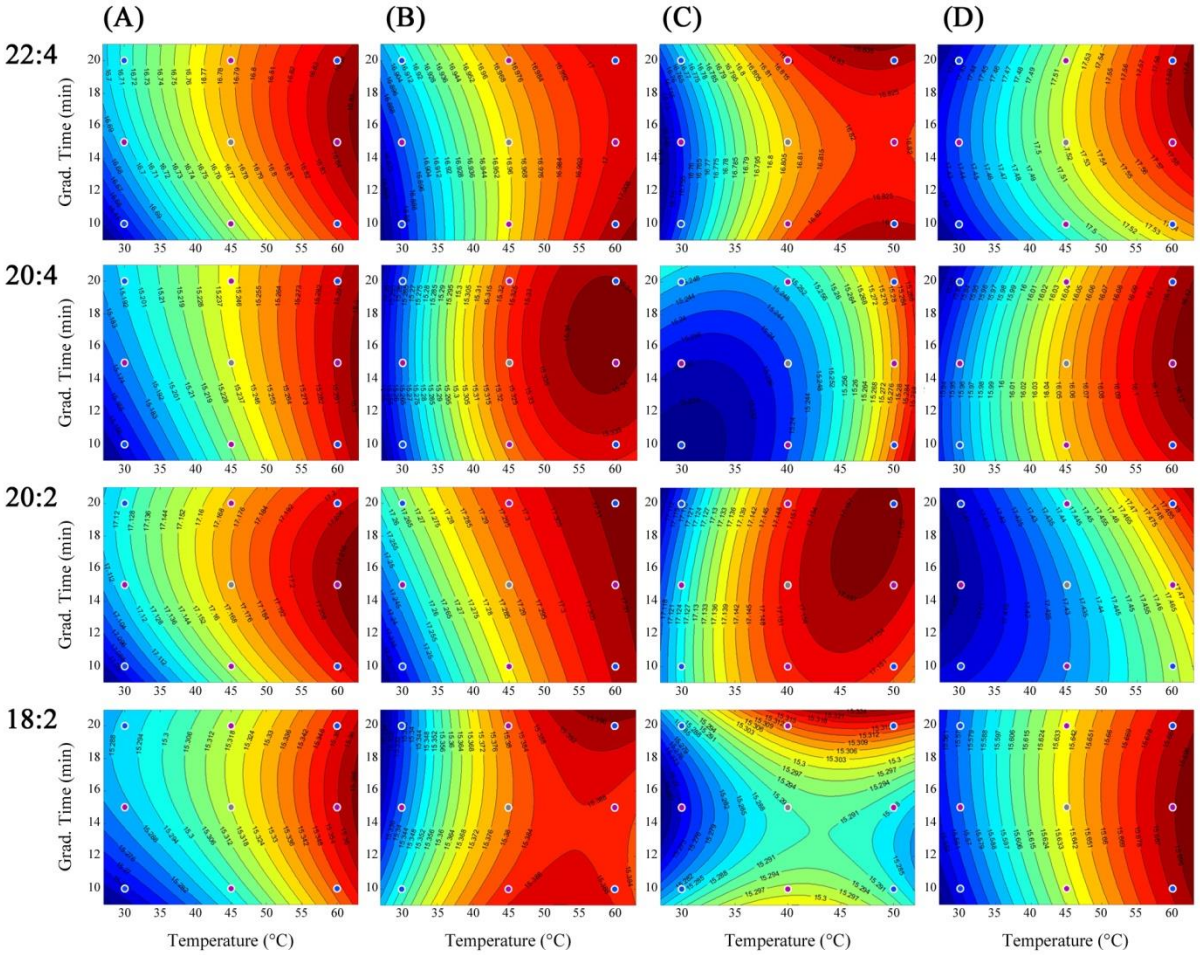

Figure 29 - Response surface plots of FFA 22:4, 20:4, 20:2 and 18:2. (A) ACN. (B) MeOH. (C) ACO. (D) THF.

From this section it may be concluded that although temperature has some effect on retention the largest effect is the choice of the apolar modifier, and THF is the one that stands out from the three other. The effect is that THF has higher ECL values than the three other organic modifiers. This may mean weaker interactions between the solvent and the double bounds in the analytes with THF than with the other solvents. However, it is emphasized that THF is not the only solvent in the system. The mobile phases with THF also had more water than the other mobile phases, and water stabilize the THF-THF interactions [66], which may, reduce the interactions of this solvent with the double bonds. The differences between the organic modifiers means that in order to

fine-tune the retention pattern, e.g. for the purpose of resolving chromatographic overlaps, the best way of doing so may be to use ternary mixtures of THF, water, and one of the three other solvents used, because they showed similar retention patterns. Good predictions of ECL were obtained for highly unsaturated FAs that showed the highest shifts in ECL values. The RMSE were quite low for all the solvents, representing only a small fraction of the peak width.

3.3.5. Effects on efficiency

Other purpose of the work was to investigate how the different chromatographic conditions affect the efficiency. The efficiency was evaluated by the PPC, which were calculated according Equation 17. It should be emphasized that PPC is not a pure estimate of efficiency, the way efficiency is defined in isocratic chromatography by Purnell and van Deemter equations. By the Purnell equation, resolution in isocratic chromatography is a function of efficiency (plate number, N), selectivity (relative retention, α) and retention (retention factor, *k*). In programmed chromatography, *k* is not constant and these two functions are not valid. However, the resolution is still a result of the same factors that gives the A, B and C terms in the van Deemter equation, and the retention. Resolution can be calculated from PPC and ECL (Equation 18), and ECL is a pure selectivity estimate (α can be calculated from ECL). PPC is therefore a function of the effects leading to the A, B and C terms in the van Deemter equation, and the retention. The retention factor, *k*, is gradually decreasing when the elution strength is increased in solvent programmed LC. Shorter time from low to high solvent strength will always lead to lower average *k*, and longer gradient times should in theory give higher PPC if all other factors are the same, but the magnitude of the effects can be difficult to predict.

The effect of temperature is much more challenging to predict than the effect of gradient time. Higher temperatures increase the B and decrease the C terms in the van Deemter equation. Whether there is a positive or negative effect of increased temperature depend on whether the B or C terms are dominating, or whether the mobile phase velocity is lower or higher than the optimal velocity (*u*) given by Equation 10. Furthermore, it is

complicated by the fact that the temperature may have large effect on retention. So a positive effect on the efficiency may be compensated by lower retention factors.

The peak width in retention index units increases in the following way: MeOH < ACN < ACO < THF, which implies that the best and the poorest efficiency were for MeOH and THF respectively. This can be easily noticed from the bar graphs in **Figure 30** where the efficiency for THF is almost half of MeOH. According to the chromatograms seen in **Figure 31**, peaks corresponding to THF and ACO were broader and with more tailing.

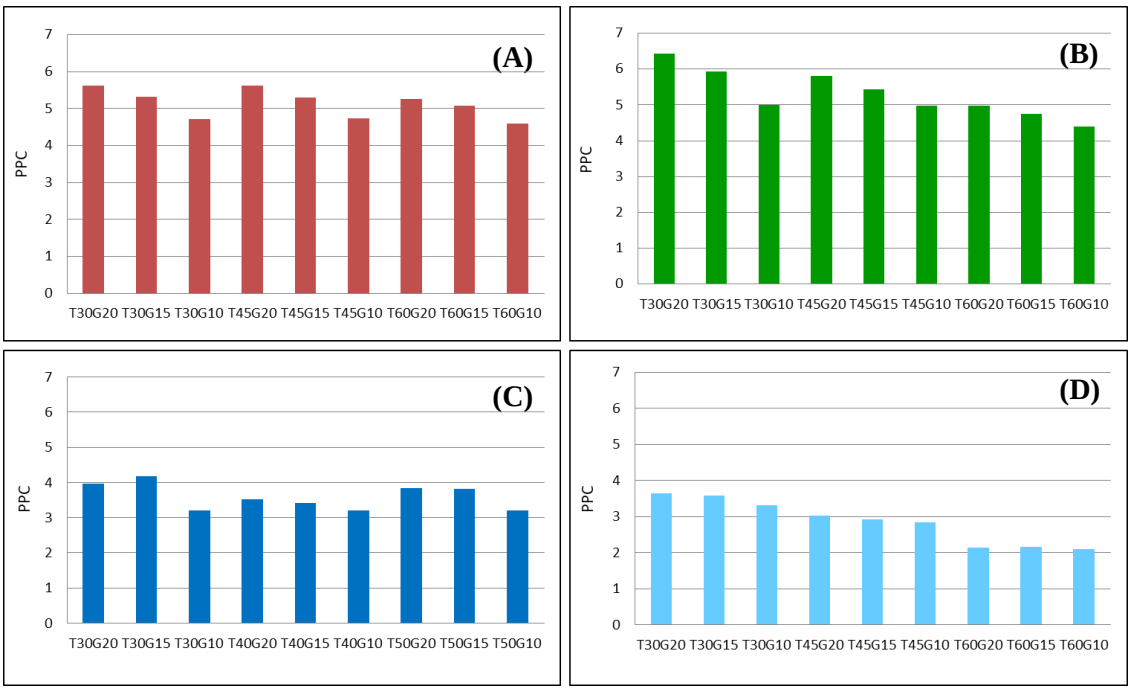

Figure 30 - Average PPC of all FAs present in GLC-793 showing the different experimental conditions for the four solvents. (A) ACN. (B) MeOH. (C) ACO. (D) THF.

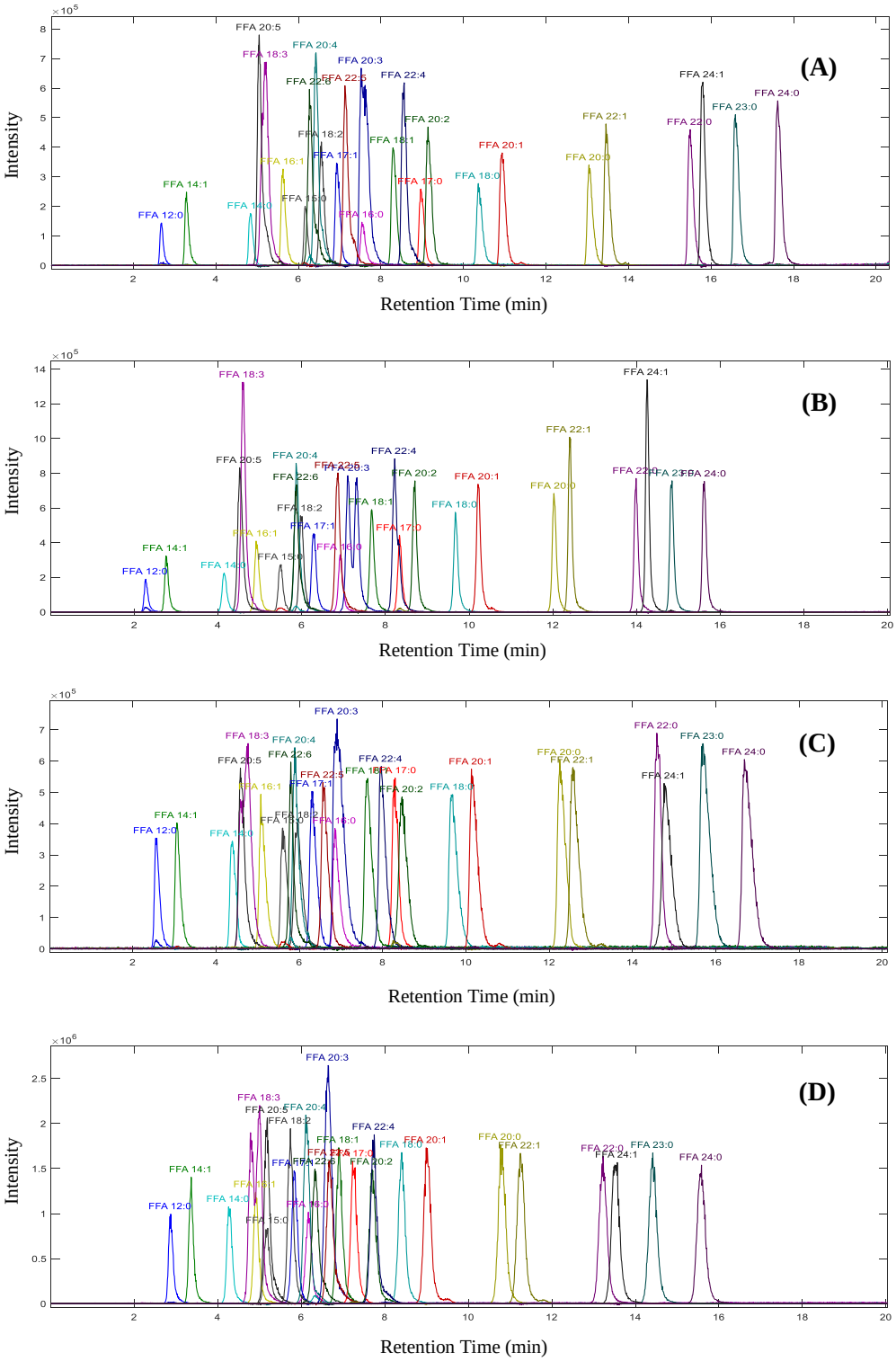

Figure 31 - TIC full scan chromatograms obtained at 30 ºC. (A) H_2O:ACN 44:56 Grad: 0 min 56% B, 20 min 100% B. (B) H_2O:MeOH 25:75 Grad: 0 min 75% B, 20 min 100% B. (C) H_2O:ACO 38:62. Grad: 0 min 62% B, 20 min to 85% B. (D) H_2O:THF 55:45 Grad: 0 min 45% B, 20 min to 60% B.

All solvents systems showed differences in efficiency with gradient time, with exception of THF. As expected the efficiency decreases as the gradient time decreases. Besides, considering the same temperature, the peak shape is better (narrower and more symmetric peaks) with higher gradient times. Efficiency also decreases as temperature decrease; this effect is more visible with MeOH and THF systems.

The PCA score plot in **Figure 32** shows an increase in PPC along PC1 with decreasing temperature and also with increasing gradient time for MeOH. This is the only solvent where PPC have a clear dependence on both factors. Regarding ACN there was a positive effect when increasing gradient time and less difference was observed with temperature. On the contrary, THF shows a clear effect of temperature, where low temperature has a positive effect and no differences are seen regarding gradient time. The case of ACO is less clear, there is a minimal effect of temperature (also less variation in the design) and in general it seems to be that the gradient time is positive but the largest PPC was found with intermediate gradient time (15 minutes).

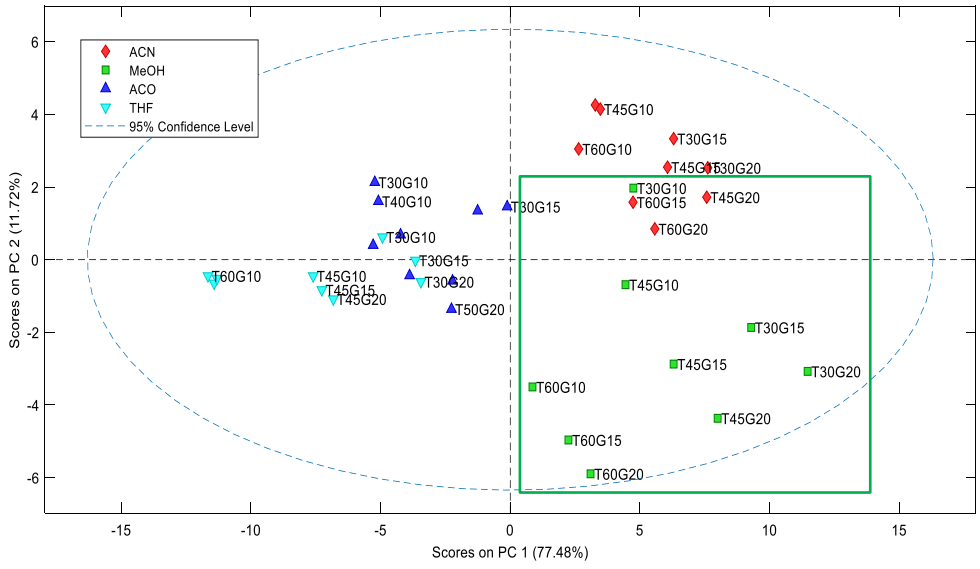

Figure 32 - PCA score plot of PPC for all the solvents (Experiments with MeOH are framed).

As explained above, the PPC depends on the factors leading to the A, B and C terms in van Deemter equation, as well as the mobile phase velocity *u* and the retention. In this study the velocity was not investigated and it is unknown how close to the optimum

velocity the experiments were performed. Probably the u_{op} is different for each solvent system and this can explain some differences found between the solvents. The effects on PPC with the different gradient times were expected because of a decrease on retention with faster increase of solvent strength. Regarding temperature, B and C terms are affected by diffusion factors where $B=2 \cdot D_M$ (diffusion coefficient) and C is inversely proportional to D_M. Therefore, an increase in temperature will cause an increase in the B term and a decrease in the C term. It is in this case not known which terms in the van Deemter equation that have the largest effect. If it is considered that the B term has larger effect than the C term, this will result in a loss of efficiency when temperature is increased. This is clearly the case when MeOH and THF systems are used.

From this section it can be concluded that MeOH system presented the lowest values of peak width and the best efficiency with a maximum PPC value near 7. This is lower efficiency than with GC methods, where PPC for FAME are typically around 20-30 [67] and can be above 10 even for fast separations in less than 10 minutes [68]. The efficiency in LC may be optimized mainly by using longer gradient times and reducing the column temperature. An interesting fact is that the gradient time showed minimal effect on selectivity, but it affects efficiency, which is important when it comes to optimization. In GC, the temperature rate (corresponding to gradient in LC) typically has strong influence on efficiency and selectivity. This makes it challenging to optimize both factors, where one easily end up with compromises that are not ideal for any of the two. If the gradient has limited influence on selectivity in LC, it may be easier to optimize both efficiency and selectivity.

- ***Time-efficiency trade-off***

The time of analysis is also effected by temperature and gradient time. There are combinations of gradient time and column temperature that maximize the efficiency in a certain amount of time. If the time of the analysis is considered as the retention time of the last eluting compound (FFA 24:0), increasing column temperature or decreasing the gradient time lead to decrease retention time, **Figure 33**. Again, decreasing the gradient time has a larger effect than the increase in temperature, with exception of THF where the temperature has the larger effect. It is known from the previous section that higher

temperatures reduce *k*, for all the solvents, and to a large degree for THF. That fits with the results showing reduction in PPC with increasing temperature, and this was the most visible effect for THF. However, we are not able to separate the effect from reduced k and any changes in the B and C terms, so it is not known how important the reduction in *k* is relative to the other factors. To do this, it must be done a large number of experiments at different temperatures so that the factors in the van Deemter and Purnell equation could be found.

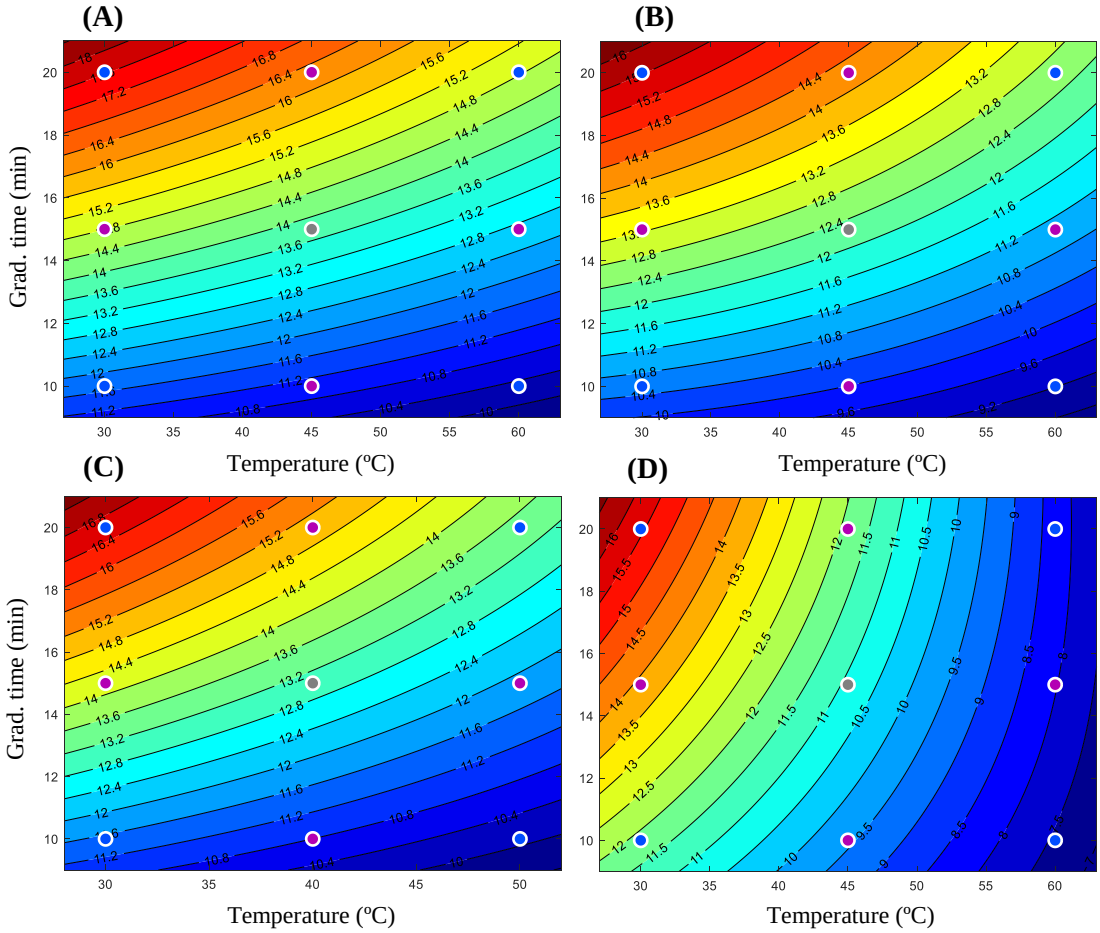

Figure 33 - Retention time response surface plots of the last eluting FFA: 24:0. (A) ACN. (B) MeOH. (C) ACO. (D) THF.

The last section shows that the decrease in the gradient time or increase in temperature causes a drop in efficiency, whereas shorter analysis times require higher temperatures and shorter gradient times. Although shorter retention times are always desired, it may not be obtained without losing efficiency; therefore there is a trade-off between time and efficiency. The shortest time of analysis (7.4 min) was obtained for THF at 60 ºC

with a gradient of 10 minutes; however, worst efficiency was obtained. MeOH has still a low time of analysis (9,2 min at 60 ºC and 10 min gradient) but much better efficiency than THF.

3.3.6. Effects on response

For the analyzed GLC-793 mixture where all the FAs were present in equal mass concentration, the peak areas obtained follow the tendency previously seen, to increase as chain length increase for all the solvents. The peak areas obtained with THF were more than the double of the areas obtained with the other solvent systems for all the compounds, and the lowest peak areas were in general obtained with ACN (**Figure 34**). Regarding ACO and MeOH, MeOH showed higher responses for polyunsaturated FAs and ACO for saturated and monounsaturated FAs. This is useful information for choosing solvent for purposes of quantification. For instance, despite short fatty acids showed lower responses for all solvents, it may be a good idea analyze them using THF in order to obtain higher sensitivity. In the same way, to quantify a mixture of saturated FAs it is probably better use ACO instead of MeOH.

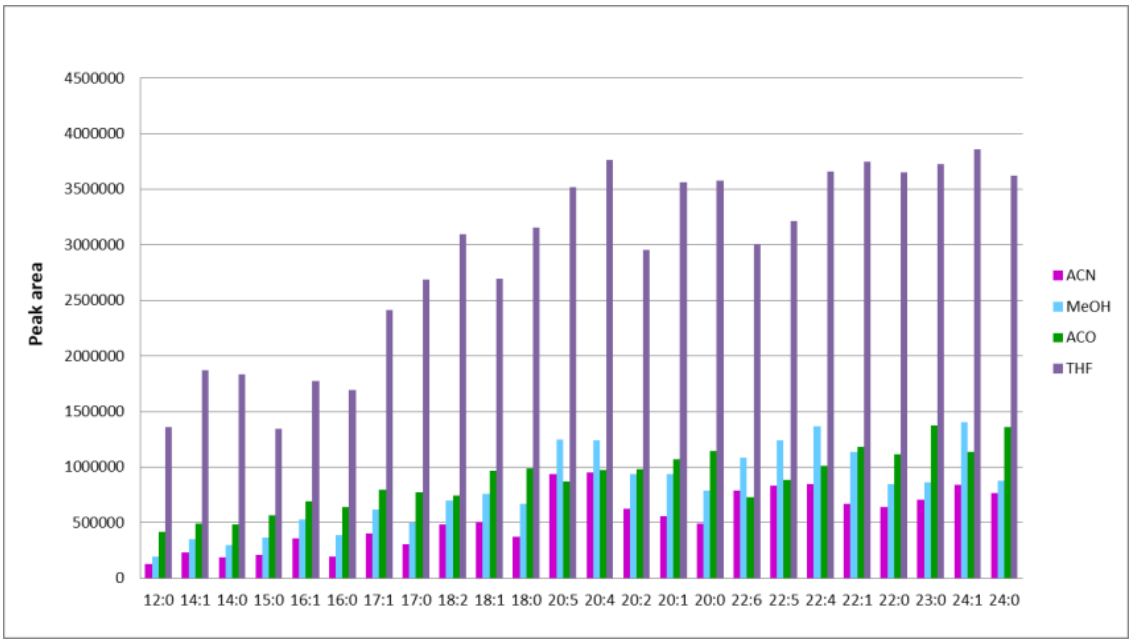

Figure 34 - Peak area average obtained for all the solvents. (Averages for all the programs).

The PCA score plot showed in **Figure 35** matches with the previous figure where THF present the highest values, and is separated from the other solvent systems (which have

similar values) along PC1 (98% of the variance). The other chromatographic parameters (temperature and gradient time) have limited effect compared with the choice of the solvent. They have almost no effect with ACN, and based on PC2, temperature seems to affect in some degree the response obtained with THF, being higher at higher temperatures.

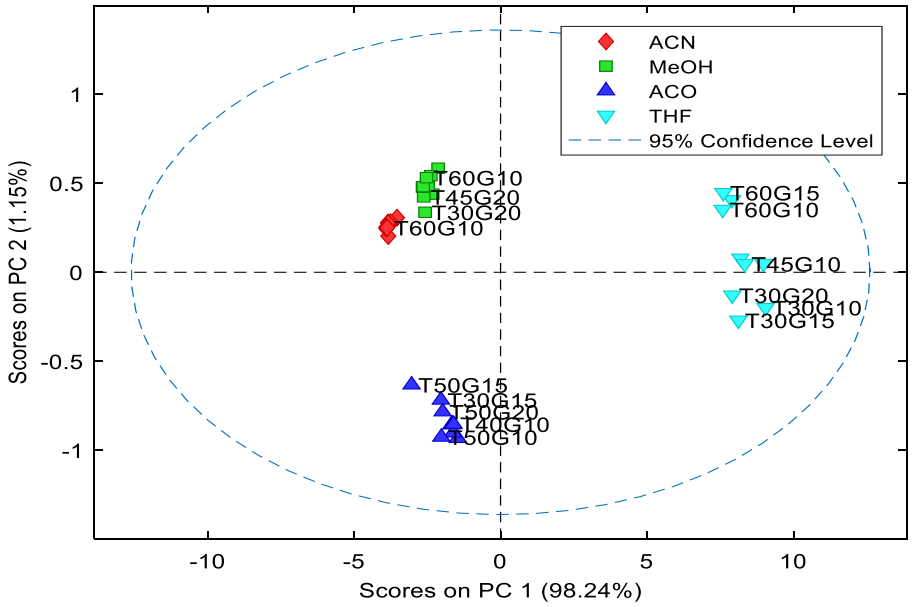

Figure 35 - PCA Score plot of peak area.

3.4. Studies of retention patterns

In this last part of the work a more detailed study of retention patterns were conducted, with particular focus on models that could predict ECL values, This required more FAs than present in the GLC-793 mixture and FFAs were therefore prepared from additional samples. 14 samples from different origin containing a total of 50 FAME where first converted to FFA and then analysed by LC-MS using the 4 chromatographic systems (one of each solvent) which gave the best linear dependence between the ECL values and retention time (**Figure 36**):

1- H_2O:ACN 44:56 0 min 56% ACN, 20 min 100% ACN
2- H_2O:MeOH 25:75 0 min 75% MeOH, 20 min 100% MeOH
3- H_2O:ACO 38:62 0 min 62% ACO, 20 min 85% ACO
4- H_2O:THF 55:45 0 min 45% THF, 20 min to 60% THF

A mixture of saturated fatty acids was spiked to the samples for calibration of ECL values.

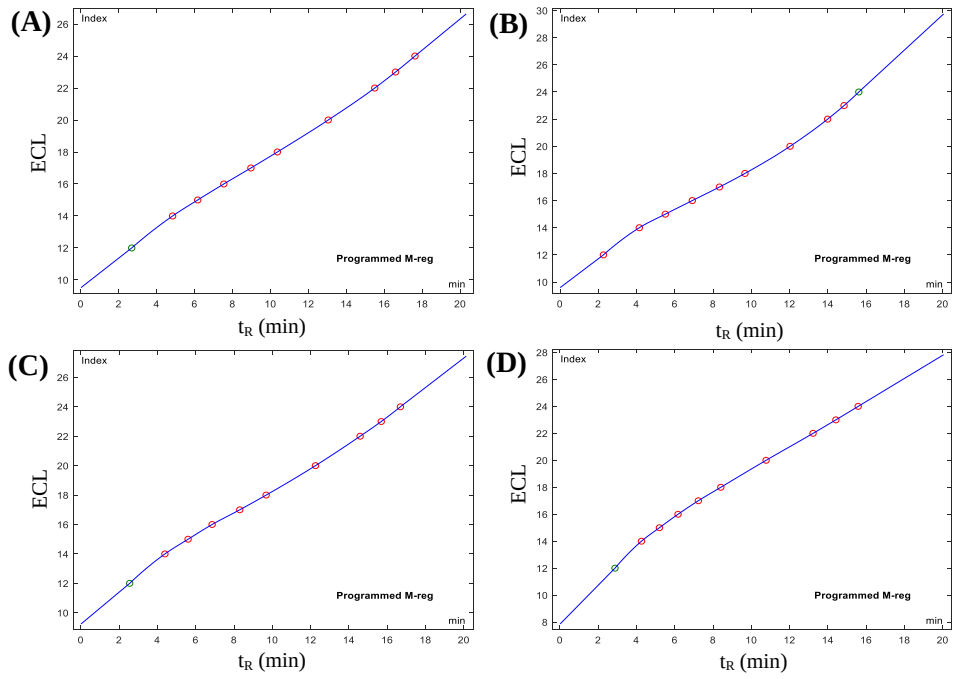

Figure 36 - ECL values vs retention time at 30 ºC with 20 minutes gradient, 0.35 ml/min for GLC-793. (A) ACN. (B) MeOH. (C) ACO. (D) THF.

The mean of the estimated ECL values was calculated for fatty acids present in more than one sample. To check outliers, a Grubbs test with 95% confidence was performed. Only a few values were removed where some interference on the chromatogram was seen. It is important to mention that for the MeOH system, more chromatographic runs were carried out and therefore the quality of the data may be better. In the same way fewer analyses were performed with THF system. Thus, no values were removed.

It is known from previous sections that the elution patterns are affected by the polarity of the mobile phase and it also have some dependence on other parameters, like column temperature. It has been studied that FCL values obtained from GC in some phases like polyethylene glycol are similar for members of homologous series (e.g. *a*:3 *n*-3, where *a* is a varying number of carbons) and this has been used for identification purposes in GC analysis [69]. In all the systems studied, there was a tendency for the FCL of compounds coming from same series to increase with increasing chain length as it is shown in **Table 11**. Although there was similarity between the FCL, large variations within some of the groups studied like *a*:3 *n*-6, *a*:5 *n*-3 and *a*:4 *n*-6 were seen. The largest difference within these groups is between FAs with the first double bound in Δ-4 and Δ-5 position (16:3 *n*-6, 20:5 *n*-3 and 20:4 *n*-6) and the following FAs in the group with first double bound in Δ-6 and Δ-7 position (18:3 n-6, 22:5 *n*-3 and 22:4 *n*-6) . This indicates that the Δ-DB in these particular positions has a relevant effect on retention of FAs. The lowest differences between the FCL values were seen for MeOH and the highest for THF.

Table 11 - FCL values of the homologous series analysed with the different solvent systems (ACN, MeOH, ACO and THF) and ECL average for all solvents.

Serie	FA	FCL				ECL average for all the solvents
		ACN	ACO	MeOH	THF	
***a*:1 *n*-9**	18:1 *n*-9 (Δ9)	-1.47	-1.47	-1.47	-1.32	16.57
	19:1 *n*-9 (Δ10)	-1.54	-1.55	-1.52	-1.39	17.59
	20:1 *n*-9 (Δ11)	-1.57	-1.62	-1.57	-1.47	18.44
	22:1 *n*-9 (Δ13)	-1.68	-1.75	-1.64	-1.62	20.33
	24:1 *n*-9 (Δ15)	-1.70	-1.85	-1.71	-1.75	22.26
	Range	**0.23**	**0.37**	**0.24**	**0.43**	
***a*:2 *n*-6**	18:2 *n*-6 (Δ9)	-2.70	-2.70	-2.64	-2.41	15.39
	20:2 *n*-6 (Δ11)	-2.88	-2.88	-2.74	-2.59	17.23
	22:2 *n*-6 (Δ13)	-2.99	-3.02	-2.84	-2.74	19.10
	Range	**0.29**	**0.33**	**0.21**	**0.33**	
***a*:3 *n*-6**	16:3 *n*-6 (Δ4)	-3.27	-3.18	-3.34	-2.65	12.89
	18:3 *n*-6 (Δ6)	-3.71	-3.64	-3.61	-3.17	14.47
	20:3 *n*-6 (Δ8)	-4.02	-3.99	-3.87	-3.47	16.16
	Range	**0.75**	**0.80**	**0.53**	**0.83**	
***a*:3 *n*-3**	18:3 *n*-3 (Δ9)	-3.76	-3.78	-3.62	-3.39	14.36
	20:3 *n*-3 (Δ11)	-3.99	-3.96	-3.73	-3.54	16.20
	22:3 *n*-3 (Δ13)	-4.09	-4.09	-3.81	-3.68	18.08
	Range	**0.32**	**0.32**	**0.19**	**0.29**	
***a*:1 *n*-7**	16:1 *n*-7 (Δ9)	-1.38	-1.39	-1.37	-1.26	14.65
	17:1 *n*-7 (Δ10)	-1.44	-1.45	-1.44	-1.33	15.58
	18:1 *n*-7 (Δ11)	-1.50	-1.52	-1.51	-1.39	16.52
	Range	**0.12**	**0.13**	**0.14**	**0.14**	
***a*:5 *n*-3**	20:5 *n*-3 (Δ5)	-5.82	-5.75	-5.67	-4.97	14.45
	22:5 *n*-3 (Δ7)	-6.30	-6.22	-6.04	-5.50	16.00
	Range	**0.47**	**0.48**	**0.37**	**0.53**	
***a*:4 *n*-6**	20:4 *n*-6 (Δ5)	-4.79	-4.69	-4.70	-4.03	15.45
	22:4 *n*-6 (Δ7)	-5.30	-5.22	-5.09	-4.55	17.00
	Range	**0.51**	**0.53**	**0.39**	**0.51**	

- ***Effect of introducing double bonds and functional groups***

As in GC, ECL values obtained from RP-LC are affected by the introduction of double bonds, the more C atoms and the less DB, the more retention. As the number of double bonds increase, the molecule is becoming more polar and the interaction with the nonpolar mobile phase is weakened, accelerating the elution process and therefore decreasing the ECL values [43, 70]. How much the ECL value decreases, would depend on the *n* or Δ position of the double bound. It can be seen from **Figure 37 (A)** that the effect of introducing the first double bond in monounsaturated fatty acids in n-9 position is higher with the chain length and increases as the double bound is moved further away from the carboxyl group. However, there is almost no difference between fatty acids of different chain lengths when an additional n-6 or n-3 double bound is introduced (**Figure 37 (B, C)**). Even more, unlike GC, where the methylene group between two double bounds has an important role in the retention [29], the addition of the second double bound has less effect than the addition of the first double bound (**Figure 37 (C)**). All of these, together with the information of FCL from the previous section seem to indicate that the Δ-position has the highest influence regarding retention in RP-LC. **Figure 37 (D)** shows that the effect of introducing a double bound in polyunsaturated fatty acids is higher in Δ-6 than in Δ-5, and it has the lowest effect when it is introduced in Δ-4, closer to the carboxyl group. This means less retention as the double bound is moving away from the acid group, at least, until Δ-6. This same behavior was observed for all the solvents systems.

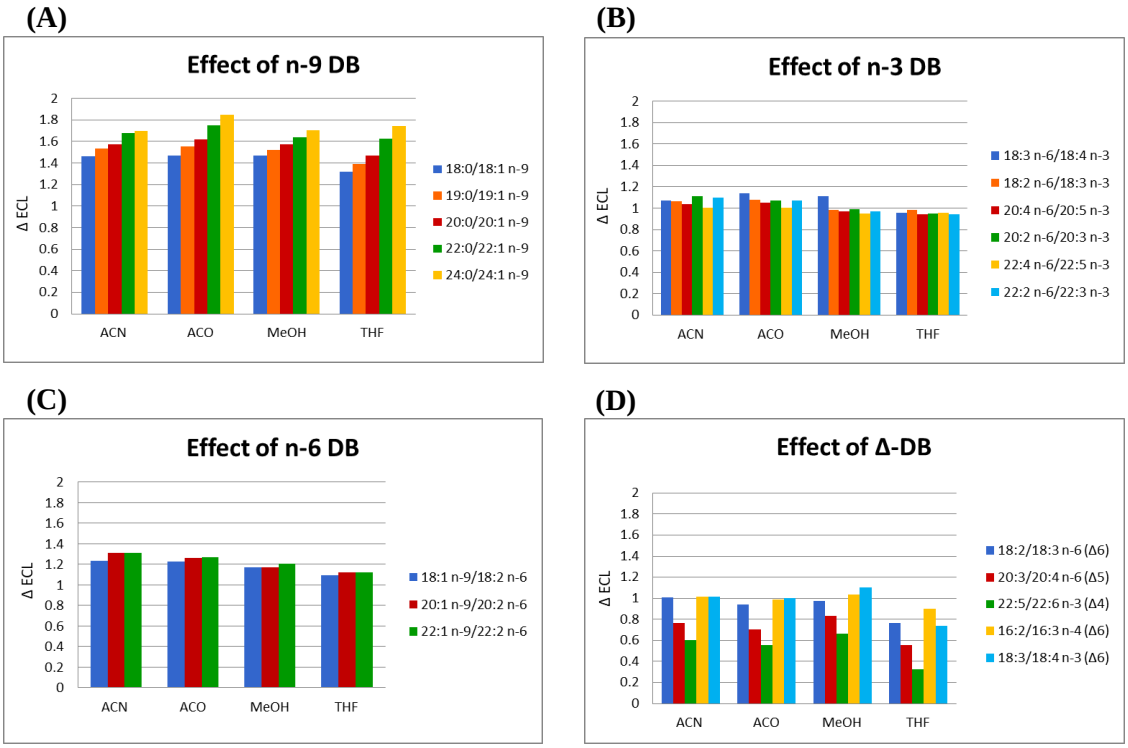

Figure 37 - Effects on ECL values of introducing double bonds in different positions. (A) n-9. (B) n-3. (C) n-6 (notice the effect of introducing a second double bond). (D) Δ-double bond. ΔECL was calculated by subtracting the ECL value of the most unsaturated to the less unsaturated fatty acid.

From the results given above it is clear that ECN rule is not a good predictor for the retention of unsaturated FFA, this is clarified in **Figure 38** where the changes in ECL values can be seen for FFAs of 20 carbons. A change in ECL close to 2 was only observed for saturated FA versus monoenes with the double bound in n-9 and the introduction of a new double bound in some cases led to changes of much less than 1. It is emphasized that ECN was originally used as a rough estimate in isocratic conditions, where the mobile phase strength is constant. In gradient experiments used in this work, the mobile phase strength was higher when the last of the compared compounds eluted than when the first eluted. Still, it is clear that there are large differences depending on the position where the double bound is introduced. The low effect of introducing a double bound close to the carboxyl group is similar to what is observed for several GC phases [44]. The ECN, where all double bonds are equal, cannot account for these observations.

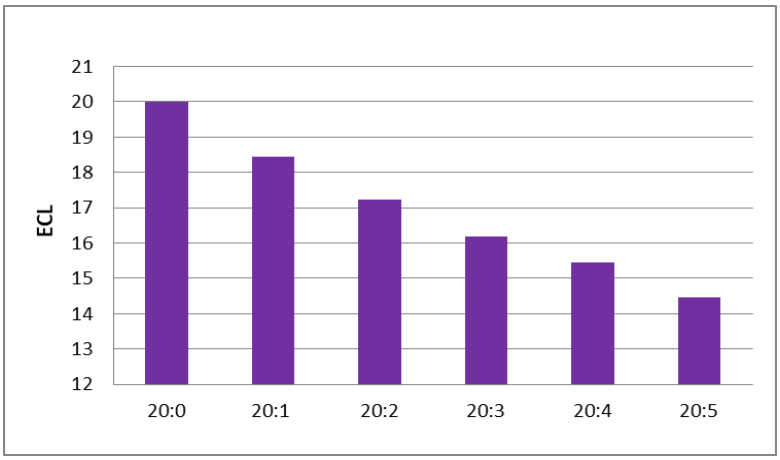

Figure 38 - Changes in ECL values as the number of double bonds increases for FA of 20 carbons.

- ***Introduction of hydroxyl, methyl and ethyl groups***

The introduction of a OH group has a very high effect on the ECL value, making them elute early from the column. As can be seen from **Figure 39 (A)** the effect is higher when the OH group is further away from the carboxyl group. This sounds reasonable since the molecule is more polar. Predicted log P values for 2-hydroxy octadecanoic acid is 7.3, while it is lower than 7 for the hydroxyl octadecanoic acids with the hydroxyl groups further from the carboxyl group (www.chemspider.com). For all the hydroxyl compounds studied the effect is higher for THF, probably due to the high content of water that has stronger interaction with hydroxy groups. The effect is lowest for the MeOH system that has the lowest content of water at the beginning of the run.

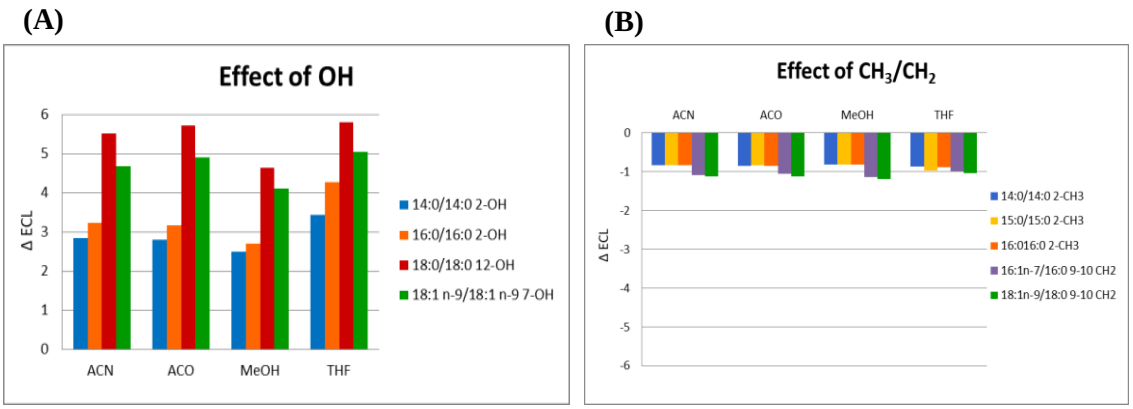

Figure 39 - Effect on ECL of introducing functional groups. (A) Hydroxyl group. (B) CH_3 and CH_2 groups.

Figure 39 (B) shows that the introduction of a methyl or a cyclopropane group makes the fatty acid more apolar eluting after their analogues (negative values). The effect of introducing the methyl group in Δ-2 is less then adding an extra carbon to the chain, since ΔECL is lower than 1. The effect of introducing a cyclopropane is calculated by comparing with a compound with the same number of double bound equivalents. In this case the effect is slightly higher than 1. Almost no difference was observed between the solvent systems.

3.4.1. Models of ECL based on chemical structure

As it have already been done in GC, and since there is a general increase in the ECL values with the chain length within a homologous series, it may be possible to establish mathematical models that can predict ECL values as a function of chemical structure. Multivariate calibration was applied to predict ECL values from molecular descriptors related to the chemical structure of the molecule. **Table 12** summarized the molecular descriptors which were selected according to the work of Mjøs and Grahl-Nielsen [29]. Methylene-interrupted unsaturated fatty acids can be described by the number of carbons, the number of double bonds and their position in the molecule, either by the n or Δ position. Hence, these were selected as variables. However, it is known from GC that the relationship between the position of the double bonds and the ECL is not linear [29]. To deal with this and improve the accuracy of the models, higher order terms were included.

Table 12 - Molecular descriptors for PLSR models.

Variable	Description
A	Number of carbons
B	Number of double bonds
C	Δ-position
D	Δ-position^2
E	Δ-position^3
F	Δ-position^4
G	n-position
H	n-position^2
I	n-position^3

PLSR models were built using different combination of these variables and were evaluated according RMSE. Models containing no information of n-position or Δ-position gave the highest values of RMSE, indicating that models should include both double bond positions. Better models were obtained including higher order terms of both positions, finding that the best model was obtained when variables from A to H were used. Including the cubic term of n-position had no positive effect. It was also seen in the previous section that the Δ-position has the major effect on retention of FAs. The results obtained for these models are shown in **Appendix f**. Variables A to H were used to build models for all solvent systems. Results are given in **Table 13**.

The different solvent systems gave small differences in the calculated errors. The MeOH system showed the lowest errors of calibration and cross validation as well as the highest R^2 values for predicted versus measured. On the opposite, the THF system showed the worst prediction models. The quality of the models improved considerably when 18:1 *n*-12 was removed from the data set (**Table 13**). This fatty acid shows particular characteristics, probably because it contains one double bond very close to the carboxyl group, which implies a different behavior from the other monoenes that have the double bond near the center of the molecule. Because it is the only one compound with this characteristic, the model is not able to accurately predict its ECL value. Predicted vs measured plots for MeOH are showed in **Figure 40**. The average peak width in RI units was around 0.2 and 0.3, indicating that, again the RMSEC is only a small fraction of a peak. However they are higher than the errors obtained from the response surface models that predicted ECL as a function of chromatographic parameters, where none of them was above 0.04. Additionally, is important to mention that in similar works made on GC, the monoenes had a different behavior than the other saturated FAs and therefore they were not included in the models. In this study, monounsaturated FAs seemed to fit well into the models.

Table 13 - Merits of PLSR models with ABCDEFGH variables, 4 LV with and without 18:1 n-12.

Solvent system	With 18:1 *n*-12				Without 18:1 *n*-12			
	RMSEC	RMSECV	R^2 C	R^2 CV	RMSEC	RMSECV	R^2 C	R^2 CV
ACN	0.0807	0.1338	0.9989	0.9970	0.0620	0.0778	0.9993	0.9990
ACO	0.0797	0.1264	0.9989	0.9973	0.0673	0.0860	0.9992	0.9988
MeOH	0.0675	0.1041	0.9992	0.9982	0.0599	0.0750	0.9994	0.9991
THF	0.0821	0.1293	0.9988	0.9970	0.0703	0.0936	0.9991	0.9985

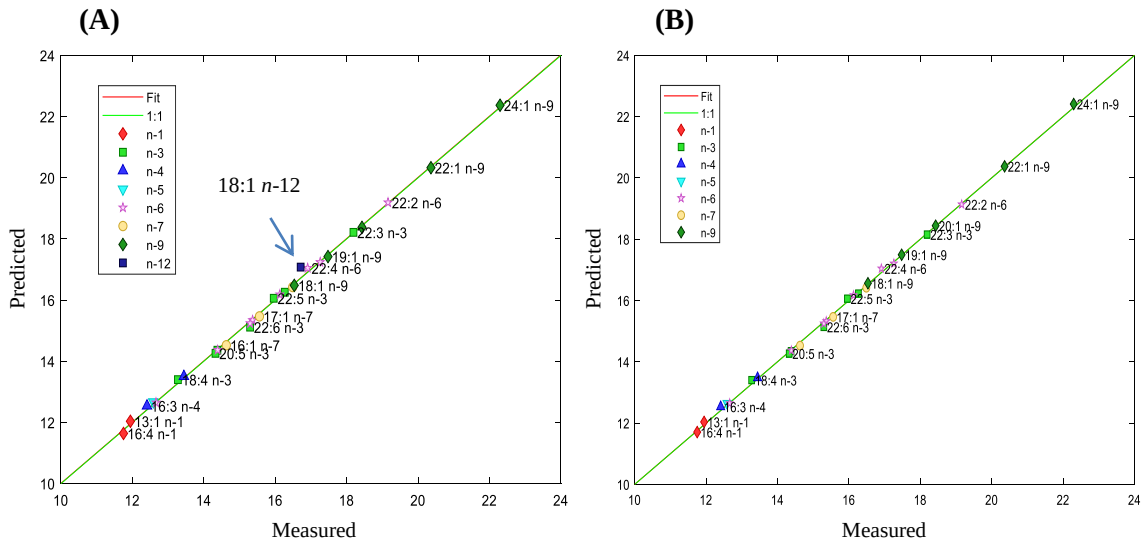

Figure 40 - Plots of Predicted vs Measured ECL for the calculated models with MeOH system. (A) With 18:1 n-12. (B) Without 18:1 n-12.

With the models created, the effect of moving the first double bound closer to the carboxyl group can be illustrated by predicting ECLs for triunsaturated FA, as shown in **Figure 41** and **Figure 42**, where ECL values vs. n-position is showed for 18:3 and 20:3 respectively. Regarding 18:3 and considering the MeOH system, ECL decreases to n-5 (Δ-7) that shows the lowest retention and then starts to increase again as the double bond becomes closer to the carboxyl group. Therefore there is a particular intermediate position where the retention is the lowest (e.g. between Δ-6 and Δ-8 for MeOH) and begins to rise when moving to both sides. The effect is more pronounced from n-5 on, where the ECL increases to higher values as Δ decreases. Regarding the other solvents, from *n*-4 the increase in ECL is more remarkable. In ACO and THF systems, which

have a similar behavior, the ECL decreases to *n*-4 and starts to increase steeply. In these two systems the isomers *n*-3 and *n*-6 are partially resolved where 18:3 *n*-3 elutes before 18:3 *n*-6.

The case of 20:3 fatty acids is a bit different (**Figure 42**). The MeOH system shows approximately the same retention for n-1 and n-9. With two more carbons between the carboxyl group and the first double bond the molecule is more retained. This is also why, unlike the 18:3, the ECL of n-6 is slightly lower than n-3. The lowest ECL values are for *n*-4 and *n*-5 for all the solvents where the saturated parts of the carbon chain are relatively short for both sides.

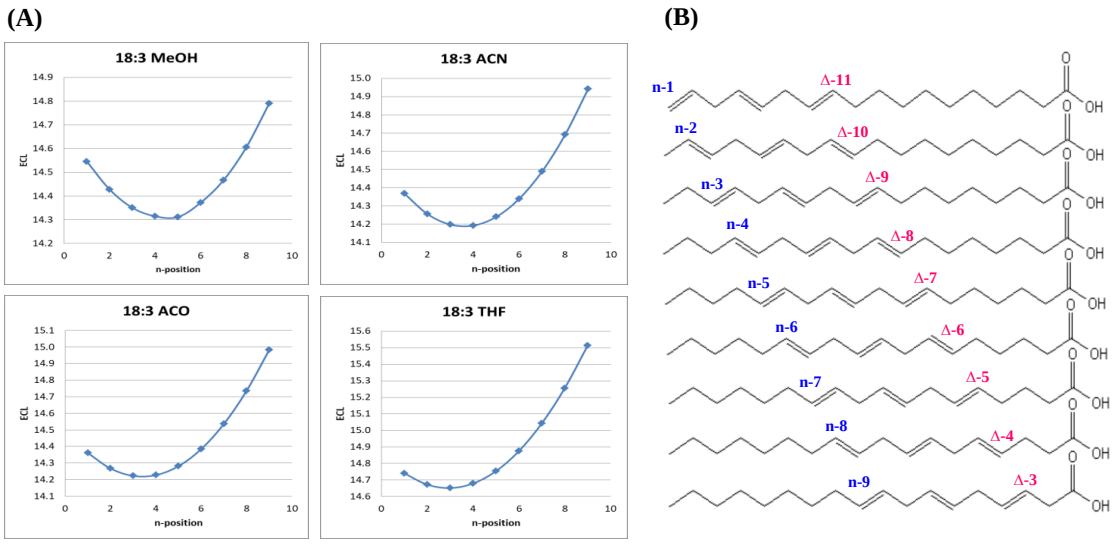

Figure 41 - (A) Effect of ECL values depending on the position of the double bond for fatty acid 18:3. (B) Chemical structure representation of the different isomers of fatty acid 18:3.

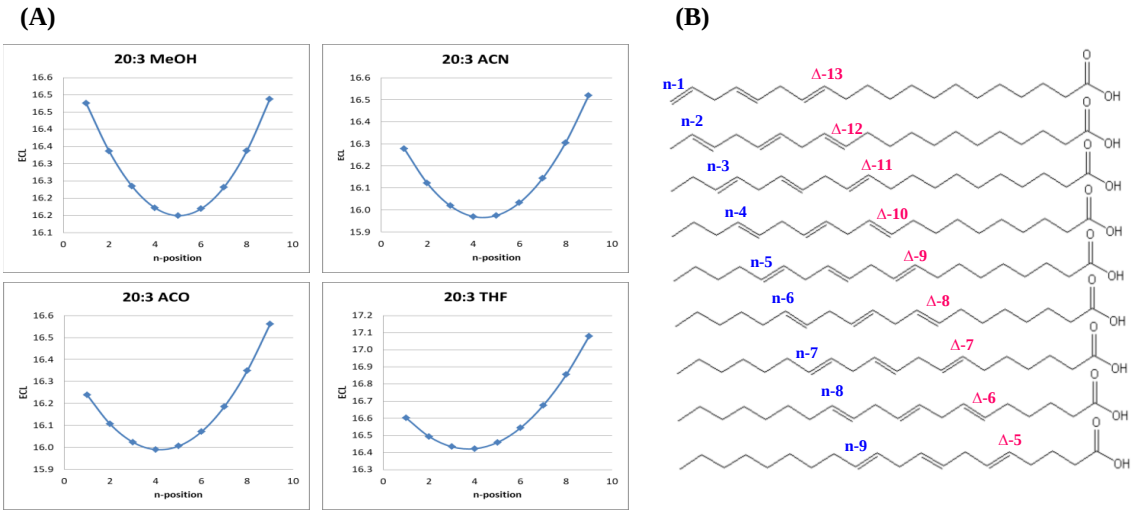

Figure 42 - (A) Effect of ECL values depending on the position of the double bond for fatty acid 20:3. (B) Chemical structure representation of the different isomers of fatty acid 20:3.

All of this indicates that these polyunsaturated compounds are more retained when the first double bound is far away (more than 10 carbons) or close enough (approximately less than 6 carbons) to the carboxyl group.

3.4.2. Models of ECL based on GC data

It is well known that much more has been done regarding retention indices in gas chromatography than in liquid chromatography. This is probably due to the retention variability observed in LC. It is an even higher problem with gradient elution systems, where the composition of the mobile phase is continuously changing [71]. To investigate whether retention data acquired by LC can be predicted from GC-retention data, PLSR models were built using the GC-FCL data on different columns obtained from www.chrombox.org/data. FCL from seven GC-columns of different polarity (BPX 70, BP20, BD225, SLB-IL61, SLB-IL82, SLB-IL100 and HP5) were used as variables in the models. FCL values can be used as indication of the polarity of the compound. As it was seen, on reverse phase liquid chromatography the unsaturated FA have negative FCL values, which mean they elute before the saturated FA with the same number of carbons. This is also the case with apolar columns in GC. When the HP5 column was included into the models, better results were obtained because this non polar GC column gave more similar results (negative values) to the LC-data obtained using the

C8 column. On the other hand, it was necessary to remove the BPX70 column from the dataset to have good prediction for 16:4 *n*-1 (**Figure 43**). It is possible that the ECL for this compound is not accurately determined on this column since it was hidden under 18:1 *n*-9, which was present in much higher percentage [69]**.** The merits of the models with and without BPX70 are shown in **Table 14**.

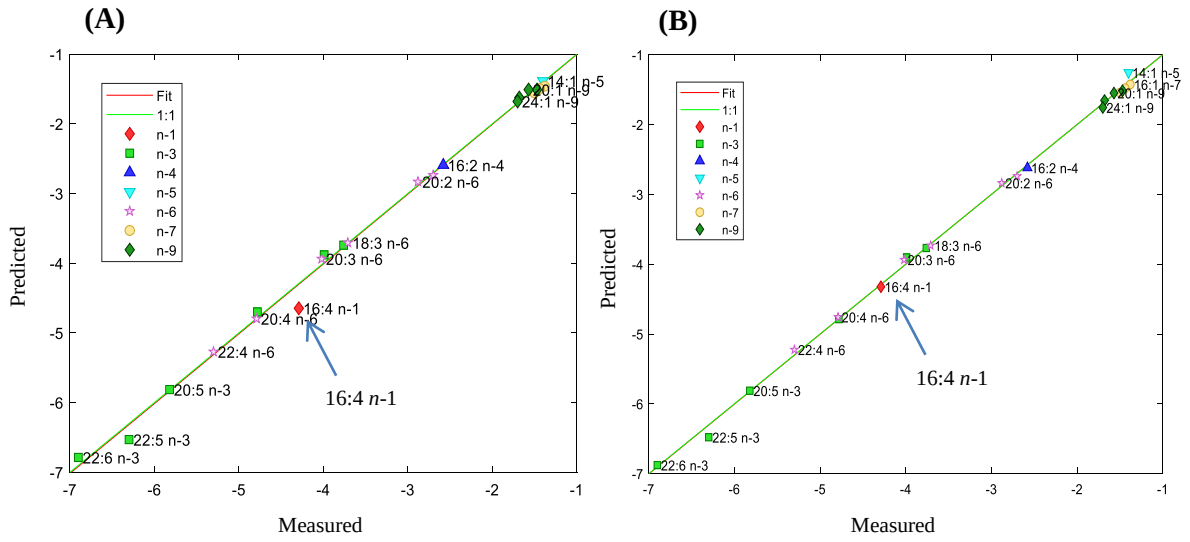

Figure 43 - Plots of Predicted vs Measured ECL for the calculated models with ACN system. (A) With BPX70. (B) Without BPX70.

Table 14 - Merits of the PLSR models with and without BPX70.

Solvent	All columns				Without BPX70			
system	RMSEC	RMSECV	R^2 C	R^2 CV	RMSEC	RMSECV	R^2 C	R^2 CV
ACN	0.0485	0.1056	0.9992	0.9963	0.0459	0.0651	0.9992	0.9986
ACO	0.0644	0.1243	0.9985	0.9947	0.0441	0.0617	0.9993	0.9987
MeOH	0.0486	0.0748	0.9991	0.9979	0.0434	0.0599	0.9993	0.9987
THF	0.0589	0.1183	0.9983	0.9934	0.0347	0.0533	0.9994	0.9986

Good models were obtained for all the solvent systems, demonstrating that although the elution behavior is very different between GC and LC, ECL values can be accurately predicted from one methodology to the other. The lowest errors were obtained with the THF system when BPX70 column was removed from the dataset.

Information of ECL of hydroxyl compounds was available for BPX70, BP20, DB225 and HP5 columns, but, probably due to the lack of information and the different behavior of these fatty acids it was not possible to obtain good models. The worst predictions were obtained for THF which gave ECL values more different from the other solvents. This could be due to the high proportion of water used at the beginning of the run and therefore the interaction with hydroxyl compounds is different. The merits of these models are shown in **Table 15**.

Table 15 - Merits of the PLSR models including 16:0 2-OH and 18:0 12-OH

Solvent system	Variables included	RMSEC	RMSECV	R^2 C	R^2 CV
ACN	BPX70/BP20/DB225/HP5	0.1137	0.1281	0.9953	0.9941
ACO	BPX70/BP20/DB225/HP5	0.1421	0.1772	0.9924	0.9882
MeOH	BPX70/BP20/DB225/HP5	0.0819	0.0959	0.9972	0.9962
THF	BPX70/BP20/DB225/HP5	0.1893	0.3051	0.9834	0.9608

To summarize this section, the distance of the first double bond to the carbonyl group seemed to be the most important factor related to the retention of FAs in RP-LC systems. There is a particular intermediate Δ-position were the retention is the lowest, and this position increases with chain length. ECL values of fatty acids containing 13 to 24 carbons with double bounds in *n*-1, *n*-3, *n*-4, *n*-5, *n*-6, *n*-7 and *n*-9 positions, can be predicted based on its molecular structure. Good models were obtained with the different solvent systems, and these were more accurate accurate for MeOH. Good predictions of ECL values can also be made from the GC data, being the best for THF, which is valuable because of the large amount of information available for different GC columns. Besides, ECL of monoenes can be predicted from LC, which is a problem on GC. Based on the errors obtained for the models created, better predictions of ECL can be made with the data from different chromatographic conditions on LC or different columns in GC than from the chemical structure of the molecules. However the errors for predictions for all the models were within fractions of a peak width.

3.5. Quality control of the C8 column

Due to the back pressure problems with C18 column, which resulted in the interruption of the analyses, a quality control of the new C8 to check its stability was carried out. A control chart was prepared to check the stability of the pressure during the runs under the same conditions (**Figure 44**). The chart was constructed with the first 20 measurements obtained with MeOH system. The central line, lower/upper warning limits and lower/upper action limits were calculated after checking for outliers. After that, the following measurements were introduced in the graph after performing the Snedecor's F test and confirm that there was statistical difference between the variances of both series. All the measurements were inside the control conditions indicating a good performance of the column.

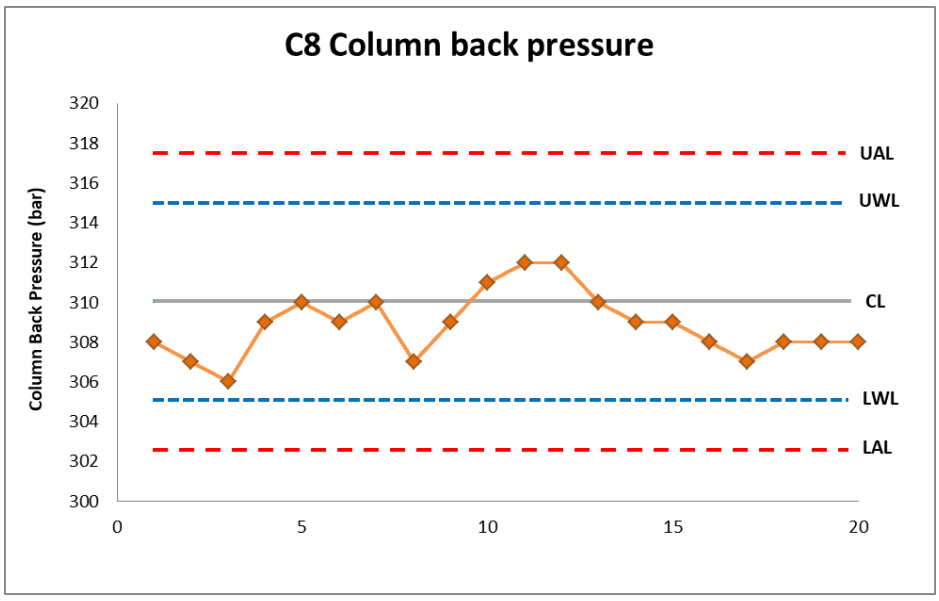

Figure 44 - Pressure control chart of C8 column.

A record of the column use was created (**Figure 47, appendix g**) and filled with C8 column data from its first use in order to have available all historical information of the column. This is additional information to check the performance of the column and can be also useful for futures uses.

4. Conclusions

It was possible to develop a DI-MS to analyse FFA and FAME, however, acceptable results from the initial experiments by HPLC-MS were only achieved with FFA. The tests with collision activated dissociation gave highly fragmented spectra of FAME positional isomers that were different. However, it was not possible to find diagnostic ions that clearly indicated double bond positions, which is possible using electron ionization in GC-MS. For FFA it was not possible to get good signals for fragments. Thus, HPLC-ESI-MS seems not to be more feasible than GC-MS for structure elucidation of FAs.

When it comes to the possible replacement of GC by HPLC-MS for the quantitative analyses of fatty acids, the weakest point of the LC-MS methodology is the ability to distinguish between isomers such as 18:3 *n*-6/18:3 *n*-3 and 20:3 *n*-6/20:3 *n*-3. Since there is no fragmentation, the negative ion mass spectra of FFA provide no information that distinguish these compounds. In addition, the chromatographic separation between these positional isomers was low or absent with every solvent system tested. The cause of the poor chromatographic separation can be partially explained by the lower efficiency of LC. The maximum PPC was around 7 in this study, while it is typically above 10 in GC, even for fast separations (<10 min). However, efficiency is not the only explanation for the poor separation in LC. The selectivity is also much poorer than with GC. If there is no need to separate the isomers HPLC-MS can work well. However, there are large differences in response between different fatty acids. So for accurate quantitative analyses, calibration on a large number of pure reference compounds may be necessary. It is also emphasized that the stability of response factors over time has not been investigated in this study, and this can be an issue with LC-MS.

HPLC-MS has one great advantage over GC-MS. In cases where one only want to quantify free fatty acids in the presence of other lipids; FFA can be analyzed with negative ionization using the same samples, columns and solvents typically used for analyses of other lipids. GC will in such cases require a laborious pre-separation step, and there is often doubt whether the fractions are pure. Regarding LC-MS as a complementary technique to GC-MS the strength of the methodology is that negative

ionization gives zero fragmentation and no adducts, which means that reliable information about molecular mass is available. This is information that is often missing in GC-MS analyses of FAME because of the large degree of fragmentation.

- ***Pointing out some discoveries:***

- The ionization efficiency was different for the different FFAs, being the lowest for short chain FA, and this efficiency decreases in presence of water.

- The choice of the apolar solvent is the most influential factor in order to generate changes in retention patterns in LC, and THF was clearly different from the other more polar solvents (MeOH, ACN and ACO).

- Higher efficiency and low running times are obtained with MeOH. Efficiency can be improved by decreasing temperature or increasing gradient times.

- The gradient time (steepness of the gradient) showed to have almost no effect in selectivity (ECL) but affects efficiency.

- THF gave much higher responses (peak area) than the other solvents.

- It is possible to predict ECL values in HPLC-MS from chromatographic conditions, from chemical structure and from GC data, where the lowest errors found were for models based on chromatographic conditions with ACN and MeOH.

- Considering double bonds, the Δ-position is the most important factor in the retention of FAs by RP-LC.

- ECN rule is not a god predictor of the retention of FAs in RP-HPLC under programmed conditions.

- ***Recommendations for future works***

According to the differences observed between the different solvents, it would be interesting to test ternary mixtures of H_2O, THF and one of the other three solvents (MeOH, ACN, ACO) to see if it is possible to fine-tune the retention patterns, and if it gives better efficiency than achieved with the THF/H_2O mixtures. It also would be interesting to try this ternary mixture with other lipid classes, like triglycerides and phospholipids.

To perform quantitative studies, the HPLC-MS method developed must be validated, determining parameters like LOD, LOQ, selectivity, precision, accuracy, etc. It also would be good to investigate another MS ionization method like APCI to check if the differences in response observed between the FAs in ESI are still present. The analysis of FAs in presence of other lipids (like in natural samples) would also be interesting.

Conditions with better chromatographic efficiency may be found by finding the A, B and C terms in the van Deemter equation and solving for optimal mobile phase velocity.

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6. Appendix

a)

Table 16 - Concentrations in the mixtures for calibration study

FFA	Concentrations injected µg/ml							
	D1	D2	D3	D4	D5	D6	D7	D8
18:0 (IS)	25.4	33.8	34.7	26.1	29.4	32.9	32.7	28.8
8:0	0	6.4	13.8	18.9	30.8	52.2	73.2	96.0
10:0	0	7.0	15.7	22.8	32.5	53.2	80.7	105.1
12:0	0	6.4	14.3	20.8	29.6	48.4	73.4	95.5
14:0	0	5.9	15.6	22.2	28.5	50.1	72.1	91.1
16:0	0	6.0	12.8	24.7	31.2	49.2	70.9	92.7
20:0	0	6.5	14.8	23.8	32.5	54.8	78.3	98.5
22:0	0	6.0	13.8	21.2	27.3	47.2	73.2	93.6
24:0	0	5.4	12.8	22.8	31.2	47.7	71.6	89.3

b)

Table 17 - p-values from ANOVA test.

Source	p-value
Model	0.4162
Fragmentor V	0.5699
Nebulizer	0.0677
Needle voltage	0.0501
1x1	0.852
1x2	0.9789
1x3	0.9585
2x2	0.6033
2x3	0.9013
3x3	0.9546

c)

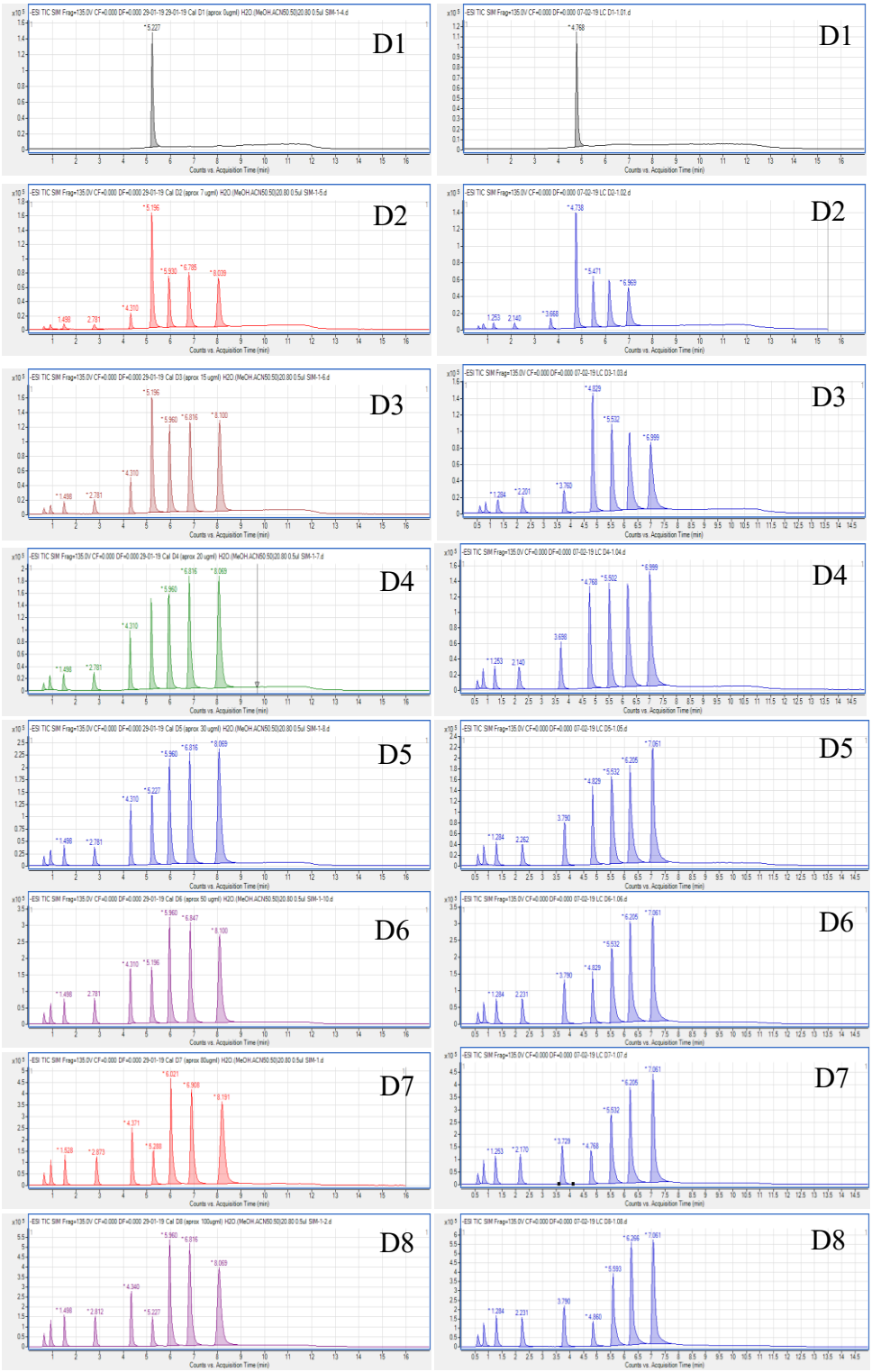

Figure 45 - Chromatograms from calibration curves. Left 26 ºC. Right 40 ºC

d)

Table 18 - DI MeOH:ACN 50:50, concentrations in µg/ml.

FFA 8:0												
Mixture	Conc. Sample	Conc. IS	C. Sam/ C. IS	A sample x 10^5			A IS x 10^5			A sample/ A IS		
				Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
1	0.00	25.43	0.00	0.00	0.04	0.04	43.14	27.15	24.14	0.00	0.00	0.00
2	6.40	33.82	0.19	1.51	5.18	4.68	23.44	31.46	28.67	0.06	0.16	0.16
3	13.79	34.73	0.40	6.36	8.35	7.62	21.72	26.55	25.29	0.29	0.31	0.30
4	18.85	26.12	0.72	8.67	10.46	9.08	16.93	18.69	18.01	0.51	0.56	0.50
5	30.81	29.35	1.05	12.82	13.61	12.82	18.57	17.60	17.62	0.69	0.77	0.73
6	52.17	32.88	1.59	17.08	17.71	16.50	16.51	15.99	15.29	1.03	1.11	1.08
7	73.22	32.65	2.24	20.37	20.74	18.17	13.36	13.04	11.65	1.53	1.59	1.56
8	96.02	28.76	3.34	22.27	21.93	21.11	9.89	9.99	9.69	2.25	2.20	2.18
FFA 10:0												
Mixture	Conc. Sample	Conc. IS	C. Sam/ C. IS	A sample x 10^5			A IS x 10^5			A sample/ A IS		
				Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
1	0.00	25.43	0.00	0.00	0.04	0.04	43.14	27.15	24.14	0.00	0.00	0.00
2	7.01	33.82	0.21	2.19	6.78	6.25	23.44	31.46	28.67	0.09	0.22	0.22
3	15.69	34.73	0.45	8.16	10.63	9.94	21.72	26.55	25.29	0.38	0.40	0.39
4	22.83	26.12	0.87	15.47	18.64	16.76	16.93	18.69	18.01	0.91	1.00	0.93
5	32.53	29.35	1.11	19.26	19.65	18.90	18.57	17.60	17.62	1.04	1.12	1.07
6	53.24	32.88	1.62	25.71	25.84	24.79	16.51	15.99	15.29	1.56	1.62	1.62
7	80.74	32.65	2.47	32.13	31.70	28.13	13.36	13.04	11.65	2.41	2.43	2.41
8	105.06	28.76	3.65	34.33	33.47	32.11	9.89	9.99	9.69	3.47	3.35	3.31
FFA 12:0												
Mixture	Conc. Sample	Conc. IS	C. Sam/ C. IS	A sample x 10^5			A IS x 10^5			A sample/ A IS		
				Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
1	0.00	25.43	0.00	0.00	0.03	0.03	43.14	27.15	24.14	0.00	0.00	0.00
2	6.38	33.82	0.19	2.83	7.34	6.66	23.44	31.46	28.67	0.12	0.23	0.23
3	14.26	34.73	0.41	10.33	13.00	12.41	21.72	26.55	25.29	0.48	0.49	0.49
4	20.76	26.12	0.79	14.95	17.77	16.10	16.93	18.69	18.01	0.88	0.95	0.89
5	29.58	29.35	1.01	20.76	20.95	20.20	18.57	17.60	17.62	1.12	1.19	1.15
6	48.41	32.88	1.47	27.24	27.13	25.86	16.51	15.99	15.29	1.65	1.70	1.69
7	73.41	32.65	2.25	34.71	33.88	30.38	13.36	13.04	11.65	2.60	2.60	2.61
8	95.53	28.76	3.32	38.60	37.99	36.06	9.89	9.99	9.69	3.90	3.80	3.72
FFA 14:0												
Mixture	Conc. Sample	Conc. IS	C. Sam/ C. IS	A sample x 10^5			A IS x 10^5			A sample/ A IS		
				Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
1	0.00	25.43	0.00	0.00	0.02	0.03	43.14	27.15	24.14	0.00	0.00	0.00
2	5.91	33.82	0.17	2.94	6.50	5.93	23.44	31.46	28.67	0.13	0.21	0.21
3	15.58	34.73	0.45	12.01	13.92	13.43	21.72	26.55	25.29	0.55	0.52	0.53
4	22.17	26.12	0.85	16.41	18.67	17.08	16.93	18.69	18.01	0.97	1.00	0.95
5	28.45	29.35	0.97	20.32	19.79	19.68	18.57	17.60	17.62	1.09	1.12	1.12
6	50.13	32.88	1.52	28.17	27.84	26.53	16.51	15.99	15.29	1.71	1.74	1.74
7	72.14	32.65	2.21	33.76	32.85	29.34	13.36	13.04	11.65	2.53	2.52	2.52
8	91.10	28.76	3.17	36.31	35.95	34.83	9.89	9.99	9.69	3.67	3.60	3.60
FFA 16:0												
Mixture	Conc. Sample	Conc. IS	C. Sam/ C. IS	A sample x 10^5			A IS x 10^5			A sample/ A IS		
				Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
1	0.00	25.43	0.00	0.09	0.09	0.09	43.14	27.15	24.14	0.00	0.00	0.00
2	6.02	33.82	0.18	3.60	6.77	6.26	23.44	31.46	28.67	0.15	0.22	0.22
3	12.77	34.73	0.37	9.69	11.42	11.14	21.72	26.55	25.29	0.45	0.43	0.44
4	24.71	26.12	0.95	18.65	20.78	19.52	16.93	18.69	18.01	1.10	1.11	1.08
5	31.17	29.35	1.06	23.83	22.83	22.08	18.57	17.60	17.62	1.28	1.30	1.25
6	49.16	32.88	1.50	28.75	27.88	26.53	16.51	15.99	15.29	1.74	1.74	1.74
7	70.87	32.65	2.17	33.80	32.80	29.20	13.36	13.04	11.65	2.53	2.52	2.51
8	92.66	28.76	3.22	37.30	37.39	36.03	9.89	9.99	9.69	3.77	3.74	3.72
FFA 20:0												
Mixture	Conc. Sample	Conc. IS	C. Sam/ C. IS	A sample x 10^5			A IS x 10^5			A sample/ A IS		
				Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
1	0.00	25.43	0.00	0.03	0.01	0.01	43.14	27.15	24.14	0.00	0.00	0.00
2	6.52	33.82	0.19	5.59	5.48	5.01	23.44	31.46	28.67	0.24	0.17	0.17
3	14.82	34.73	0.43	7.53	9.72	9.55	21.72	26.55	25.29	0.35	0.37	0.38
4	23.77	26.12	0.91	12.32	13.53	12.78	16.93	18.69	18.01	0.73	0.72	0.71
5	32.49	29.35	1.11	16.05	15.95	14.86	18.57	17.60	17.62	0.86	0.91	0.84
6	54.83	32.88	1.67	21.28	20.83	19.90	16.51	15.99	15.29	1.29	1.30	1.30
7	78.27	32.65	2.40	24.35	24.45	20.94	13.36	13.04	11.65	1.82	1.88	1.80
8	98.51	28.76	3.43	25.82	26.65	24.70	9.89	9.99	9.69	2.61	2.67	2.55
FFA 22:0												
Mixture	Conc. Sample	Conc. IS	C. Sam/ C. IS	A sample x 10^5			A IS x 10^5			A sample/ A IS		
				Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
1	0.00	25.43	0.00	0.01	0.00	0.00	43.14	27.15	24.14	0.00	0.00	0.00
2	5.96	33.82	0.18	3.22	4.71	4.46	23.44	31.46	28.67	0.14	0.15	0.16
3	13.81	34.73	0.40	6.42	7.72	7.37	21.72	26.55	25.29	0.30	0.29	0.29
4	21.72	26.12	0.83	8.84	10.00	9.45	16.93	18.69	18.01	0.52	0.53	0.52
5	27.28	29.35	0.93	10.91	10.03	10.25	18.57	17.60	17.62	0.59	0.57	0.58
6	47.18	32.88	1.43	13.89	13.71	13.05	16.51	15.99	15.29	0.84	0.86	0.85
7	73.19	32.65	2.24	17.70	17.34	15.90	13.36	13.04	11.65	1.33	1.33	1.36
8	93.56	28.76	3.25	18.87	18.73	18.34	9.89	9.99	9.69	1.91	1.88	1.89
FFA 24:0												
Mixture	Conc. Sample	Conc. IS	C. Sam/ C. IS	A sample x 10^5			A IS x 10^5			A sample/ A IS		
				Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
1	0.00	25.43	0.00	0.30	0.08	0.08	43.14	27.15	24.14	0.01	0.00	0.00
2	5.38	33.82	0.16	4.68	4.08	4.07	23.44	31.46	28.67	0.20	0.13	0.14
3	12.82	34.73	0.37	5.12	6.65	6.29	21.72	26.55	25.29	0.24	0.25	0.25
4	22.79	26.12	0.87	7.65	9.54	8.46	16.93	18.69	18.01	0.45	0.51	0.47
5	31.22	29.35	1.06	10.88	10.05	10.17	18.57	17.60	17.62	0.59	0.57	0.58
6	47.72	32.88	1.45	12.76	12.13	11.65	16.51	15.99	15.29	0.77	0.76	0.76
7	71.55	32.65	2.19	16.54	14.64	14.45	13.36	13.04	11.65	1.24	1.12	1.24
8	89.33	28.76	3.11	17.07	17.64	17.38	9.89	9.99	9.69	1.73	1.77	1.79

Table 19 - DI H_2O:(MeOH:ACN 50:50) 20:80, concentrations in µg/ml.

FFA 8:0												
Mixture	Conc. Sample	Conc. IS	C. Sam/ C. IS	A sample x 10^5			A IS x 10^5			A sample/ A IS		
				Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
1	0.00	25.43	0.00	0.00	0.00	0.00	6.16	6.07	6.00	0.00	0.00	0.00
2	6.40	33.82	0.19	0.45	0.39	0.38	8.57	7.62	7.33	0.05	0.05	0.05
3	13.79	34.73	0.40	0.91	0.79	0.76	6.54	6.76	6.75	0.14	0.12	0.11
4	18.85	26.12	0.72	1.12	1.06	1.05	4.34	4.67	4.67	0.26	0.23	0.22
5	30.81	29.35	1.05	1.90	1.73	1.71	5.56	5.19	5.21	0.34	0.33	0.33
6	52.17	32.88	1.59	3.14	2.87	2.79	5.57	5.38	5.19	0.56	0.53	0.54
7	73.22	32.65	2.24	4.21	4.02	3.95	5.02	4.68	4.65	0.84	0.86	0.85
8	96.02	28.76	3.34	5.11	5.36	5.27	4.02	4.05	4.05	1.27	1.32	1.30
FFA 10:0												
Mixture	Conc. Sample	Conc. IS	C. Sam/ C. IS	A sample x 10^5			A IS x 10^5			A sample/ A IS		
				Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
1	0.00	25.43	0.00	0.00	0.00	0.00	6.16	6.07	6.00	0.00	0.00	0.00
2	7.01	33.82	0.21	0.77	0.65	0.63	8.57	7.62	7.33	0.09	0.09	0.09
3	15.69	34.73	0.45	1.36	1.22	1.18	6.54	6.76	6.75	0.21	0.18	0.18
4	22.83	26.12	0.87	2.38	2.27	2.27	4.34	4.67	4.67	0.55	0.49	0.49
5	32.53	29.35	1.11	3.26	2.97	2.92	5.56	5.19	5.21	0.59	0.57	0.56
6	53.24	32.88	1.62	5.22	4.85	4.75	5.57	5.38	5.19	0.94	0.90	0.92
7	80.74	32.65	2.47	7.32	6.94	6.88	5.02	4.68	4.65	1.46	1.48	1.48
8	105.06	28.76	3.65	8.46	8.81	8.66	4.02	4.05	4.05	2.10	2.17	2.14
FFA 12:0												
Mixture	Conc. Sample	Conc. IS	C. Sam/ C. IS	A sample x 10^5			A IS x 10^5			A sample/ A IS		
				Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
1	0.00	25.43	0.00	0.00	0.00	0.00	6.16	6.07	6.00	0.00	0.00	0.00
2	6.38	33.82	0.19	0.95	0.80	0.77	8.57	7.62	7.33	0.11	0.11	0.11
3	14.26	34.73	0.41	1.85	1.70	1.66	6.54	6.76	6.75	0.28	0.25	0.25
4	20.76	26.12	0.79	2.44	2.40	2.36	4.34	4.67	4.67	0.56	0.51	0.51
5	29.58	29.35	1.01	3.75	3.42	3.37	5.56	5.19	5.21	0.67	0.66	0.65
6	48.41	32.88	1.47	5.79	5.46	5.27	5.57	5.38	5.19	1.04	1.01	1.02
7	73.41	32.65	2.25	8.32	7.93	7.80	5.02	4.68	4.65	1.66	1.69	1.68
8	95.53	28.76	3.32	10.06	10.19	10.24	4.02	4.05	4.05	2.50	2.52	2.53
FFA 14:0												
Mixture	Conc. Sample	Conc. IS	C. Sam/ C. IS	A sample x 10^5			A IS x 10^5			A sample/ A IS		
				Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
1	0.00	25.43	0.00	0.00	0.00	0.00	6.16	6.07	6.00	0.00	0.00	0.00
2	5.91	33.82	0.17	1.05	0.88	0.84	8.57	7.62	7.33	0.12	0.11	0.11
3	15.58	34.73	0.45	2.31	2.11	2.10	6.54	6.76	6.75	0.35	0.31	0.31
4	22.17	26.12	0.85	2.98	2.86	2.86	4.34	4.67	4.67	0.69	0.61	0.61
5	28.45	29.35	0.97	4.12	3.67	3.68	5.56	5.19	5.21	0.74	0.71	0.71
6	50.13	32.88	1.52	6.70	6.34	6.07	5.57	5.38	5.19	1.20	1.18	1.17
7	72.14	32.65	2.21	8.78	8.45	8.12	5.02	4.68	4.65	1.75	1.80	1.74
8	91.10	28.76	3.17	10.34	10.54	10.46	4.02	4.05	4.05	2.57	2.60	2.58
FFA 16:0												
Mixture	Conc. Sample	Conc. IS	C. Sam/ C. IS	A sample x 10^5			A IS x 10^5			A sample/ A IS		
				Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
1	0.00	25.43	0.00	0.00	0.02	0.02	6.16	6.07	6.00	0.00	0.00	0.00
2	6.02	33.82	0.18	1.40	1.21	1.14	8.57	7.62	7.33	0.16	0.16	0.16
3	12.77	34.73	0.37	2.28	2.18	2.20	6.54	6.76	6.75	0.35	0.32	0.33
4	24.71	26.12	0.95	3.88	3.86	3.90	4.34	4.67	4.67	0.89	0.83	0.84
5	31.17	29.35	1.06	5.44	4.97	5.00	5.56	5.19	5.21	0.98	0.96	0.96
6	49.16	32.88	1.50	7.71	7.41	7.08	5.57	5.38	5.19	1.38	1.38	1.37
7	70.87	32.65	2.17	10.09	9.55	9.21	5.02	4.68	4.65	2.01	2.04	1.98
8	92.66	28.76	3.22	11.98	12.23	12.13	4.02	4.05	4.05	2.98	3.02	2.99
FFA 20:0												
Mixture	Conc. Sample	Conc. IS	C. Sam/ C. IS	A sample x 10^5			A IS x 10^5			A sample/ A IS		
				Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
1	0.00	25.43	0.00	0.01	0.01	0.00	6.16	6.07	6.00	0.00	0.00	0.00
2	6.52	33.82	0.19	1.97	1.84	1.77	8.57	7.62	7.33	0.23	0.24	0.24
3	14.82	34.73	0.43	2.92	3.37	3.28	6.54	6.76	6.75	0.45	0.50	0.49
4	23.77	26.12	0.91	3.83	4.49	4.60	4.34	4.67	4.67	0.88	0.96	0.98
5	32.49	29.35	1.11	6.52	6.17	6.21	5.56	5.19	5.21	1.17	1.19	1.19
6	54.83	32.88	1.67	9.13	9.40	9.07	5.57	5.38	5.19	1.64	1.74	1.75
7	78.27	32.65	2.40	11.58	10.69	11.34	5.02	4.68	4.65	2.31	2.28	2.44
8	98.51	28.76	3.43	13.72	13.22	13.74	4.02	4.05	4.05	3.41	3.27	3.39
FFA 22:0												
Mixture	Conc. Sample	Conc. IS	C. Sam/ C. IS	A sample x 10^5			A IS x 10^5			A sample/ A IS		
				Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
1	0.00	25.43	0.00	0.01	0.00	0.00	6.16	6.07	6.00	0.00	0.00	0.00
2	5.96	33.82	0.18	1.92	1.84	1.80	8.57	7.62	7.33	0.22	0.24	0.25
3	13.81	34.73	0.40	2.71	3.38	3.23	6.54	6.76	6.75	0.41	0.50	0.48
4	21.72	26.12	0.83	3.48	4.45	4.43	4.34	4.67	4.67	0.80	0.95	0.95
5	27.28	29.35	0.93	5.62	5.50	5.50	5.56	5.19	5.21	1.01	1.06	1.05
6	47.18	32.88	1.43	8.52	8.14	7.81	5.57	5.38	5.19	1.53	1.51	1.50
7	73.19	32.65	2.24	11.58	10.26	10.58	5.02	4.68	4.65	2.31	2.19	2.27
8	93.56	28.76	3.25	12.50	13.20	12.45	4.02	4.05	4.05	3.11	3.26	3.07
FFA 24:0												
Mixture	Conc. Sample	Conc. IS	C. Sam/ C. IS	A sample x 10^5			A IS x 10^5			A sample/ A IS		
				Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
1	0.00	25.43	0.00	0.03	0.04	0.03	6.16	6.07	6.00	0.01	0.01	0.01
2	5.38	33.82	0.16	1.94	1.89	1.85	8.57	7.62	7.33	0.23	0.25	0.25
3	12.82	34.73	0.37	2.65	3.44	3.31	6.54	6.76	6.75	0.41	0.51	0.49
4	22.79	26.12	0.87	3.84	5.07	5.05	4.34	4.67	4.67	0.89	1.09	1.08
5	31.22	29.35	1.06	7.00	6.82	6.82	5.56	5.19	5.21	1.26	1.31	1.31
6	47.72	32.88	1.45	9.33	9.03	9.20	5.57	5.38	5.19	1.67	1.68	1.77
7	71.55	32.65	2.19	12.03	10.91	11.60	5.02	4.68	4.65	2.40	2.33	2.49
8	89.33	28.76	3.11	13.22	13.42	13.06	4.02	4.05	4.05	3.29	3.31	3.22

Table 20 - LC H_2O:(MeOH:ACN 50:50) 20:80, concentrations in µg/ml.

FFA 8:0												
Mixture	Conc. Sample	Conc. IS	C. Sam/ C. IS	A sample x 10^5			A IS x 10^5			A sample/ A IS		
				Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
1	0.00	25.43	0.00	0.00	0.00	0.00	7.61	5.94	6.07	0.00	0.00	0.00
2	6.40	33.82	0.19	0.18	0.13	0.14	9.41	8.15	7.82	0.02	0.02	0.02
3	13.79	34.73	0.40	0.35	0.33	0.28	9.72	8.41	7.67	0.04	0.04	0.04
4	18.85	26.12	0.72	0.47	0.45	0.39	8.02	6.78	6.39	0.06	0.07	0.06
5	30.81	29.35	1.05	0.77	0.82	0.67	8.40	7.77	6.99	0.09	0.11	0.10
6	52.17	32.88	1.59	1.34	1.33	1.13	9.15	8.37	7.69	0.15	0.16	0.15
7	73.22	32.65	2.24	2.17	1.72	1.66	8.95	7.82	7.66	0.24	0.22	0.22
8	96.02	28.76	3.34	2.65	2.55	2.18	8.12	7.74	6.96	0.33	0.33	0.31
FFA 10:0												
Mixture	Conc. Sample	Conc. IS	C. Sam/ C. IS	A sample x 10^5			A IS x 10^5			A sample/ A IS		
				Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
1	0.00	25.43	0.00	0.00	0.00	0.00	7.61	5.94	6.07	0.00	0.00	0.00
2	7.01	33.82	0.21	0.33	0.29	0.23	9.41	8.15	7.82	0.04	0.04	0.03
3	15.69	34.73	0.45	0.55	0.53	0.46	9.72	8.41	7.67	0.06	0.06	0.06
4	22.83	26.12	0.87	1.12	1.15	1.05	8.02	6.78	6.39	0.14	0.17	0.16
5	32.53	29.35	1.11	1.43	1.55	1.34	8.40	7.77	6.99	0.17	0.20	0.19
6	53.24	32.88	1.62	2.67	2.66	2.28	9.15	8.37	7.69	0.29	0.32	0.30
7	80.74	32.65	2.47	4.52	3.74	3.60	8.95	7.82	7.66	0.51	0.48	0.47
8	105.06	28.76	3.65	5.33	5.37	4.61	8.12	7.74	6.96	0.66	0.69	0.66
FFA 12:0												
Mixture	Conc. Sample	Conc. IS	C. Sam/ C. IS	A sample x 10^5			A IS x 10^5			A sample/ A IS		
				Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
1	0.00	25.43	0.00	0.00	0.00	0.00	7.61	5.94	6.07	0.00	0.00	0.00
2	6.38	33.82	0.19	0.40	0.41	0.37	9.41	8.15	7.82	0.04	0.05	0.05
3	14.26	34.73	0.41	0.88	0.83	0.77	9.72	8.41	7.67	0.09	0.10	0.10
4	20.76	26.12	0.79	1.32	1.33	1.14	8.02	6.78	6.39	0.16	0.20	0.18
5	29.58	29.35	1.01	1.88	2.04	1.75	8.40	7.77	6.99	0.22	0.26	0.25
6	48.41	32.88	1.47	3.40	3.38	3.13	9.15	8.37	7.69	0.37	0.40	0.41
7	73.41	32.65	2.25	6.05	5.09	5.08	8.95	7.82	7.66	0.68	0.65	0.66
8	95.53	28.76	3.32	7.69	7.43	6.84	8.12	7.74	6.96	0.95	0.96	0.98
FFA 14:0												
Mixture	Conc. Sample	Conc. IS	C. Sam/ C. IS	A sample x 10^5			A IS x 10^5			A sample/ A IS		
				Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
1	0.00	25.43	0.00	0.00	0.00	0.00	7.61	5.94	6.07	0.00	0.00	0.00
2	5.91	33.82	0.17	0.46	0.42	0.42	9.41	8.15	7.82	0.05	0.05	0.05
3	15.58	34.73	0.45	1.19	1.14	1.05	9.72	8.41	7.67	0.12	0.14	0.14
4	22.17	26.12	0.85	1.75	1.79	1.59	8.02	6.78	6.39	0.22	0.26	0.25
5	28.45	29.35	0.97	2.14	2.42	2.16	8.40	7.77	6.99	0.25	0.31	0.31
6	50.13	32.88	1.52	4.50	4.52	4.17	9.15	8.37	7.69	0.49	0.54	0.54
7	72.14	32.65	2.21	7.40	6.27	6.24	8.95	7.82	7.66	0.83	0.80	0.82
8	91.10	28.76	3.17	9.08	9.02	8.33	8.12	7.74	6.96	1.12	1.17	1.20
FFA 16:0												
Mixture	Conc. Sample	Conc. IS	C. Sam/ C. IS	A sample x 10^5			A IS x 10^5			A sample/ A IS		
				Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
1	0.00	25.43	0.00	0.00	0.00	0.00	7.61	5.94	6.07	0.00	0.00	0.00
2	6.02	33.82	0.18	0.99	0.71	0.68	9.41	8.15	7.82	0.11	0.09	0.09
3	12.77	34.73	0.37	2.24	1.54	1.41	9.72	8.41	7.67	0.23	0.18	0.18
4	24.71	26.12	0.95	4.43	3.44	3.07	8.02	6.78	6.39	0.55	0.51	0.48
5	31.17	29.35	1.06	5.59	4.62	4.05	8.40	7.77	6.99	0.67	0.59	0.58
6	49.16	32.88	1.50	8.84	7.10	6.57	9.15	8.37	7.69	0.97	0.85	0.85
7	70.87	32.65	2.17	12.79	9.47	9.57	8.95	7.82	7.66	1.43	1.21	1.25
8	92.66	28.76	3.22	15.35	13.49	12.53	8.12	7.74	6.96	1.89	1.74	1.80
FFA 20:0												
Mixture	Conc. Sample	Conc. IS	C. Sam/ C. IS	A sample x 10^5			A IS x 10^5			A sample/ A IS		
				Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
1	0.00	25.43	0.00	0.00	0.00	0.00	7.61	5.94	6.07	0.00	0.00	0.00
2	6.52	33.82	0.19	4.20	3.41	3.44	9.41	8.15	7.82	0.45	0.42	0.44
3	14.82	34.73	0.43	7.41	6.73	6.12	9.72	8.41	7.67	0.76	0.80	0.80
4	23.77	26.12	0.91	10.45	9.02	8.77	8.02	6.78	6.39	1.30	1.33	1.37
5	32.49	29.35	1.11	13.59	11.98	10.81	8.40	7.77	6.99	1.62	1.54	1.55
6	54.83	32.88	1.67	18.57	17.11	15.90	9.15	8.37	7.69	2.03	2.04	2.07
7	78.27	32.65	2.40	29.21	19.87	20.40	8.95	7.82	7.66	3.27	2.54	2.66
8	98.51	28.76	3.43	33.08	26.45	24.87	8.12	7.74	6.96	4.07	3.42	3.57
FFA 22:0												
Mixture	Conc. Sample	Conc. IS	C. Sam/ C. IS	A sample x 10^5			A IS x 10^5			A sample/ A IS		
				Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
1	0.00	25.43	0.00	0.00	0.00	0.00	7.61	5.94	6.07	0.00	0.00	0.00
2	5.96	33.82	0.18	5.44	4.25	4.30	9.41	8.15	7.82	0.58	0.52	0.55
3	13.81	34.73	0.40	9.71	8.67	7.86	9.72	8.41	7.67	1.00	1.03	1.02
4	21.72	26.12	0.83	13.63	11.00	12.05	8.02	6.78	6.39	1.70	1.62	1.88
5	27.28	29.35	0.93	17.62	14.27	14.32	8.40	7.77	6.99	2.10	1.84	2.05
6	47.18	32.88	1.43	22.24	22.92	22.48	9.15	8.37	7.69	2.43	2.74	2.92
7	73.19	32.65	2.24	36.50	27.57	33.35	8.95	7.82	7.66	4.08	3.53	4.35
8	93.56	28.76	3.25	42.03	40.76	39.13	8.12	7.74	6.96	5.17	5.26	5.62
FFA 24:0												
Mixture	Conc. Sample	Conc. IS	C. Sam/ C. IS	A sample x 10^5			A IS x 10^5			A sample/ A IS		
				Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
1	0.00	25.43	0.00	0.00	0.00	0.00	7.61	5.94	6.07	0.00	0.00	0.00
2	5.38	33.82	0.16	6.35	4.31	4.13	9.41	8.15	7.82	0.67	0.53	0.53
3	12.82	34.73	0.37	12.58	8.38	8.21	9.72	8.41	7.67	1.29	1.00	1.07
4	22.79	26.12	0.87	18.89	12.79	14.23	8.02	6.78	6.39	2.35	1.89	2.22
5	31.22	29.35	1.06	24.74	18.26	18.52	8.40	7.77	6.99	2.95	2.35	2.65
6	47.72	32.88	1.45	27.86	25.79	28.73	9.15	8.37	7.69	3.05	3.08	3.74
7	71.55	32.65	2.19	46.45	33.50	40.98	8.95	7.82	7.66	5.19	4.29	5.35
8	89.33	28.76	3.11	49.02	48.11	47.62	8.12	7.74	6.96	6.03	6.21	6.84

e)

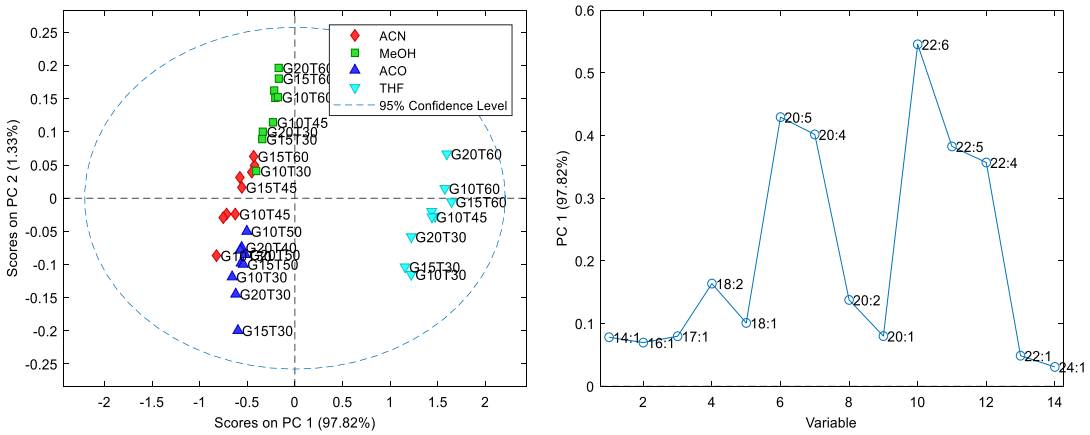

Figure 46 - PCA of ECL for ACN, MeOH, ACO and THF without FFA18:3 and 20:3.(A) PC1 vs PC2 scores plot. (B) PC1 loading plot.

f)

Table 21 - PLSR models including different variables

M	Variables included	Description	RMSEC	RMSECV	R^2 C	R^2 CV	LV
1	A,B,C,D,E,F,G,H,I	all (C db Δ Δ^4 Δ^3 Δ^2n n^3n^2)	0.0915	0.1383	0.9986	0.9970	4
2	A,B,C,D,E,G,H,I	without Δ^4	0.1055	0.1389	0.9982	0.9969	4
3	A,B,C,D,G,H,I	without $\Delta^4\Delta^3$	0.0853	0.1137	0.9988	0.9982	4
4	A,B,C,G,H,I	without $\Delta^4\Delta^3\Delta^2$	0.0917	0.1336	0.9986	0.9971	4
5	A,B,G,H,I	without $\Delta^4\Delta^3\Delta^2\Delta$	0.0951	0.1502	0.9985	0.9964	4
6	A,B,C,D,E,F,G,H	without n^3	0.0676	0.1042	0.9993	0.9983	4
7	A,B,C,D,E,F,G	without n^3n^2	0.1076	0.1408	0.9981	0.9968	4
8	A,B,C,D,E,F	without n^3n^2 n	0.1297	0.1885	0.9973	0.9944	3
9	A,B,C,D,E,G,H	without Δ^4 n^3	0.0812	0.1165	0.9989	0.9978	4
10	A,B,C,D,G,H	without $\Delta^4\Delta^3n^3$	0.0827	0.1095	0.9989	0.9981	4
11	A,B,C,G,H	without $\Delta^4\Delta^3\Delta^2$ n^3	0.0943	0.1324	0.9986	0.9972	3
12	A,B,C,G	without$\Delta^4\Delta^3\Delta^2$ n^3 n^2	0.1242	0.1484	0.9975	0.9966	2
13	A,B,C,D,G	without $\Delta^4\Delta^3$ n^3n^2	0.1100	0.1324	0.9980	0.9972	3

g)

UNIVERSITY OF BERGEN	Record: Use of chromatographic columns	RE-GC-001-01 Issue date: 20/02/2019 Page 1 of 1

Column: ZORBAX SB-C8, Rapid Resolution HD 2,1 x 50 mm 1,8-Micron Agilent (P.N 857700-906; S.N USDB702079)

Date	Compounds analyzed	Mobile phase	Equipment	Flow rate (ml/min)	Temp. (°C)	Pressure (Bar)	Operator	Observations
23.02.19	Fatty acids	H_2O: (ACN: MeOH 50:50) 20:80	6420 QQQ LC/MS	0,4	26	219	Inés	Initial washing 3hs with ACN
25.02.19	"	(H_2O/ACN/MeOH)+ NH_4COOH + HCOOH	"	0,4	"	211	Inés	—
27.02.19	"	(H_2O: MeOH 40:60): (ACN: MeOH 50:50)	"	0,3	"	137	Inés	(45°C: P 117 Bar), (0,4 ml/min: P 194 Bar)
01.03.19	"	"	"	0,35	28	184	Inés	—
04.03.19	"	"	"	"	"	187	Inés	—
13.03.19	"	"	"	0,35	"	183	Inés	—
14.03.19	"	"	"	"	"	183	Inés	—
19.03.19	"	"	"	"	"	186	Inés	—
20.03.19	"	ACN / MeOH	"	"	45	119 / 155	Inés	—
21.03.19	"	H_2O: ACN 20:80 / H_2O: MeOH 20:80	"	"	"	118 / 229	Inés	—
22.03.19	"	H_2O: MeOH 20:80	"	"	"	223	Inés	—
23.03.19	"	H_2O: ACN 44:56	"	"	30/45/60	202/165/138	Inés	—
~~12.04.19~~ 23.03.19	"	H_2O: MeOH 25:75	"	"	30/45/60	307/244/198	Inés	—
12.04.19	"	H_2O: ACO (acetone) 38:62	"	"	30/40/50	295/250/215	Inés	—

UNIVERSITY OF BERGEN	Record: Use of chromatographic columns	RE-GC-001-01 Issue date: 20/02/2019 Page 1 of 1

Column: ZORBAX SB-C8, Rapid Resolution HD 2,1 x 53mm 1,8 µm Agilent (P.N 857700-906; S.N USDB702074)

Date	Compounds analyzed	Mobile phase	Equipment	Flow rate (ml/min)	Temp. (°C)	Pressure (Bar)	Operator	Observations
30/05/19	Fatty acids	H_2O: THF 55:45	6420 QQQ LC/MS	0,35	45	360	Inés	—
31/05/19	" "	H_2O: THF 55:45	"	"	30/45/60	459/359/292	Inés	The limit of Pressure was increased to 500 bar to perform the analyses at 30°C
06/06/19	" "	H_2O: MeOH 25:75	"	"	30	312	Inés	—
07/06/19	" "	"	"	"	"	311	Inés	—
11/06/19	" "	"	"	"	"	310	Inés	—
14/06/19	" "	H_2O: ACN 44:56	"	"	30	200	Inés	—
15/06/19	" "	H_2O: Acetone 38:62	"	"	30	287	Inés	—
16/06/19	" "	H_2O: MeOH 25:75	"	"	30	309	Inés	—
17/06/19	" "	H_2O: THF 55:45	"	"	30	445	Inés	—
18/06/19	" "	H_2O: THF 55:45	"	"	30	477	Inés	Problems with pumping (Pump B) Broken connector.
20/06/19	" "	H_2O: THF 55:45	"	"	30	450	Inés	—

Figure 47 - Use of chromatographic columns record.