

Analysis of linoleoyl and oleoyl macroglycerides by high performance liquid chromatography coupled to the atmospheric pressure photoionization mass spectrometry

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Abstract – Linoleoyl macroglyceride (LM) and oleoyl macroglyceride (OM) are pharmaceutical ingredients, obtained from corn and apricot kernel oils respectively. This study aims to know the detailed chemical composition of LM and OM, in order to understand their roles in pharmaceutical formulations. These two products were analyzed by non-aqueous reversed phase high-performance liquid chromatography (NARP-HPLC), using Vintage Series KR C18 column (250 × 4.6 mm, 5 μm) and non-aqueous acetonitrile/acetone mixture as mobile phase. The ionization source used was atmospheric pressure photoionization (APPI) and the analyzer was LTQ-Orbitrap[®] (hybrid analyzer: double linear ion trap coupled to a Fourier transform orbital trap). LM and OM consist of complex mixtures, constituted of mono-(MG), di-(DG) and triglycerides (TG) and mono-(MPEGE) and di PEG-6 esters (DPEGE) of linoleic acid (18:2) for LM and of oleic acid (18:1) for OM. NARP-HPLC-APPI method allowed the separation and the identification of the glyceride classes (MGs, DGs and TGs) and the PEG esters of different chain lengths (PEG-chain lengths of fatty acid moieties and number of units of ethylene oxide), at the same time and in one single run, for both products LM and OM. The comparative study between LM and OM showed that, a higher presence of linoleic esters for LM, and a higher presence of oleic esters for OM.

Keywords: monoglycerides / diglycerides / triglycerides / PEG-6 esters / reverse phase high-performance liquid chromatography / atmospheric pressure photoionization

Résumé – **Analyse des macroglycérides de linoléoyle et d'oléoyle par chromatographie liquide à haute performance couplée à la spectrométrie de masse par photoionisation à pression atmosphérique.** Les macroglycérides de linoléoyle (LM) et oléoyle (OM) sont des ingrédients pharmaceutiques, obtenus respectivement à partir d'huiles de maïs et de noyaux d'abricot. Cette étude vise à connaître la composition chimique détaillée des LM et OM, afin de comprendre leur rôle dans les formulations pharmaceutiques. Ces deux produits ont été analysés par chromatographie liquide haute performance en phase inverse non aqueuse (NARP-HPLC), en utilisant une colonne KR C18 de la série Vintage (250 × 4,6 mm, 5 μm) et un mélange acétonitrile/acétone non aqueux comme phase mobile. La source d'ionisation utilisée était la photoionisation à la pression atmosphérique (APPI) et l'analyseur était le LTQ-Orbitrap[®] (analyseur hybride : double piège à ions linéaire couplé à un piège à mouvement orbital utilisant la transformée de Fourier). LM et OM sont des mélanges complexes, constitués de mono-(MG), di-(DG) et triglycérides (TG) et de mono-(MPEGE) et di esters de PEG-6 (DPEGE) d'acide linoléique (18:2) pour LM et d'acide oléique (18:1) pour OM. La méthode NARP-HPLC-APPI a permis la séparation et l'identification des classes de glycérides (MG, DG et TG) et des esters de PEG de différentes longueurs de chaîne (PEG-chaînes d'acide gras et nombre d'unités d'oxyde d'éthylène), simultanément et en un seul passage, pour les deux produits LM et OM. L'étude comparative entre LM et OM a montré qu'il y avait une plus d'esters linoléiques pour LM et plus d'esters oléiques pour OM.

Mots clés : monoglycérides / diglycérides / triglycérides / esters PEG-6 / chromatographie liquide haute performance en phase inverse / photoionisation à pression atmosphérique

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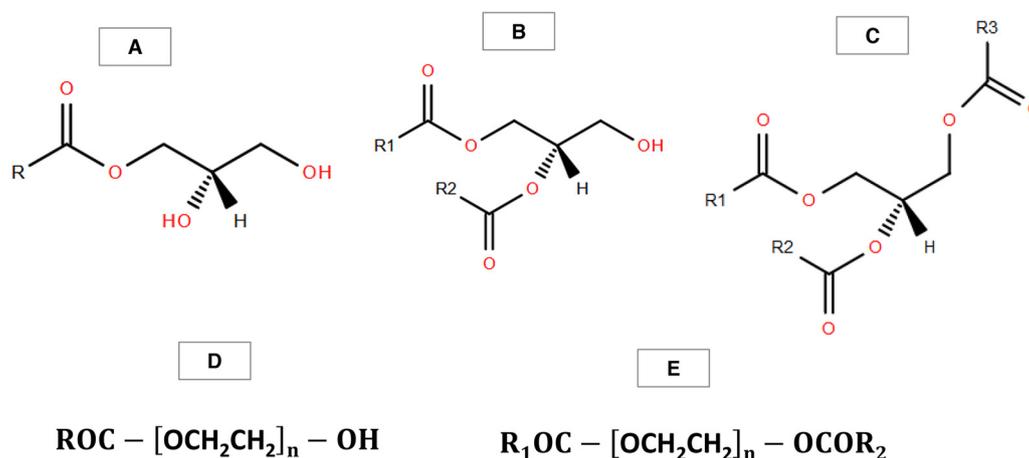


Fig. 1. Chemical structures of MG (A); DG (B); TG (C); MPEGE (D); and DPEGEs (E) (LIPID MAPS, 2003; Abdullah *et al.*, 2017).

1 Introduction

Vegetable oils are products used for food and therapeutic purposes. They are used as raw material in the pharmaceutical, cosmetic, detergent industries... as active ingredient or excipient in the crude or transformed state. The transformation of vegetable oils allows to obtain compounds with improved physicochemical and/or organoleptic characteristics for examples: stability during storage, odor, color... (Soumanou *et al.*, 2005).

Linoleoyl macroglyceride (LM) and oleoyl macroglyceride (OM) are pharmaceutical ingredients with various roles: dissolution enhancers; emulsifying agents; nonionic surfactants; penetration agents; solubilizing agents; sustained release agents. They are recommended for use in pharmaceutical formulations administered by oral, dermal, rectal and vaginal routes. LM is constituted of mono-, di- and triglycerides of linoleic acid (MG (18:2), DG (18:2/18:2) and TG (18:2/18:2/18:2)) and mono- and di-PEG-6 esters of linoleic acid (MPEGE (18:2) and DPEGE (18:2/18:2)) (Labrafil® M 2125 CS). OM is constituted of mono-, di- and triglycerides of oleic acid (MG (18:1), DG (18:1/18:1) and TG (18:1/18:1/18:1)) and mono- and di-PEG-6 esters of oleic acid (MPEGE (18:1) and DPEGE (18:1/18:1)) (Labrafil® M 1944 CS) (Fig. 1).

These two ingredients could exhibit some differences of behavior during the manufacturing of complex pharmaceutical formulations such as nanocarriers. One hypothesis is that the physical stability of these formulations could be affected by prevailing fine chemical composition of these two ingredients.

The purpose of this paper was to develop a separative analytical method using an optimized gradient of elution that will allow the separation of each compound within glycerides subclasses and PEG-esters of LM and OM, to be able to establish a detailed identification of the composition of each one.

Non-aqueous reversed-phase high performance liquid chromatography (NARP-HPLC) is very used for mono-, di-, and triglycerides analysis (Mottram and Evershed, 1996; López-López *et al.*, 2001; Holčapek *et al.*, 2003, 2005; Salghi *et al.*, 2014). Especially due to the very low volatility and insolubility in aqueous solutions of triglycerides (TGs), NARP-HPLC is the most suitable technique for their analysis

(Maloumbi *et al.*, 2015). The C18 grafted columns are the most suitable for the analysis of TGs with fatty acids in the range C16 to 22. A stationary phase with chain lengths similar to those of TG acyl chains maximizes the interactions and offers the greatest efficiency (Rezanka *et al.*, 2017). Regarding the mobile phase, acetonitrile/acetone or acetonitrile/isopropanol mixtures are commonly used.

The glycerides do not have chromophores, therefore, UV detection is rarely used. However, the glycerides absorb at low wavelengths (200–210 nm), but some solvents also absorb at these wavelengths. For this reason, the universal detectors, evaporative light-scattering detector (ELSD) and charged aerosol detector (CAD) are commonly used for the detection.

Tamba Sompila *et al.* (2014) used NARP-HPLC for the analysis of TGs, they demonstrated that the use of the retention laws allows to identify TGs of equatorial African oils (Tamba Sompila *et al.*, 2014). Nevertheless, their approach requires a first step to determine the fatty acid composition of the oils, and to possess standard compounds. However, HPLC coupling with mass spectrometry (MS) is generally used. HPLC-MS is a powerful tool in lipid analysis; it provides structural information with great sensitivity. Atmospheric pressure chemical ionization (APCI) is the most frequently used ionization technique for glycerides analysis. This technique allows easy coupling to NARP-HPLC and high ionization efficiency for non-polar molecules (Jakab *et al.*, 2002; Holčapek *et al.*, 2003; Castilho *et al.*, 2004; Fasciotti and Pereira Netto, 2010; Rezanka *et al.*, 2010). Atmospheric pressure photoionization (APPI) and electrospray ionization (ESI) were also used for the analysis of vegetable oils (Gómez-Ariza *et al.*, 2006). Gómez-Ariza *et al.* (2006) showed that APPI and ESI sources are complementary modes. APPI allows more sensitive detection of MG and DG fragment ions than ESI and ESI is more suitable for the detection of TGs. However, Imbert *et al.* (2012) demonstrated that APPI has a higher ionization capacity than APCI and ESI for lipids.

As for the characterization of PEG and PEG esters the methods used are: – for PEG, matrix-assisted laser desorption ionization-Fourier transform mass spectrometry (MALDI-FTMS) (Pastor and Wilkins, 1998) and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) (Jackson *et al.*, 1997)–, for PEG esters, low

energy collision-induced dissociation in ESI mass spectrometry (ESI-CID) with direct injection into the system (Chen *et al.*, 2002). Chen *et al.* (2002) showed that low energy dissociation of PEG esters strongly dependent on the type of cation used for ionization. The structural information on the polymer chain and end groups is best obtained using Li^+ , Ag^+ , and other transition metal ions (Chen *et al.*, 2002).

In the present work, we developed an analytical method by reversed phase high performance liquid chromatography for the simultaneous separation of all chemical entities within glycerides sub-classes and PEG esters of LM and OM. First, NARP-HPLC-CAD was performed using LM to optimize the experimental chromatographic conditions to reach the best selectivity. Afterwards, the developed method was applied to OM. Then, a mixture of standards molecules of MG, DG and TG was injected to check the reliability of the HPLC method. Second, NARP-HPLC-APPI-LTQ-Orbitrap was performed to allow the identification of each constituent. The same mixture of standards molecules was injected to determine the behavior fragmentation of MG, DG and TG. Then, the identification of main peaks of LM and OM was established. Finally, a comparative study between LM and OM was conducted.

2 Materials and methods

2.1 Chemical compounds

1-oleoyl-rac-glycerol (MG 18:1), 1,2-dioleoyl-sn-glycerol (1,2-DG (18:1/18:1)), 1,3-dilinoleoyl-sn-glycerol (1,3-DG (18:2/18:2)) and 1,2,3-trilinoleoyl-sn-glycerol (TG (18:2/18:2/18:2)) were purchased from Avanti Polar Lipids (Alabama, United States). Labrafil M 2125 CS[®] (Linoleoyl macroglyceride) And Labrafil M 1944 CS[®] (Oleoyl macroglyceride) were obtained from Gattefossé (Saint-Priest, France). N-Lauroyl-D-sphingosine; dichloromethane, acetone and acetonitrile HPLC quality were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France).

2.2 Chromatographic method

HPLC instrumentation and the charged aerosol detector were from Thermo Fisher Scientific (Bremen, Germany). The nebulization temperature was set at 35 °C and nitrogen pressure at 5 bars.

Vintage Series KRC18 column (250 × 4.6 mm, 5 μm) was from Interchim (Montluçon, France). The column was thermostated at 25 °C. The mobile phase was a mixture of acetonitrile and acetone. A mixture 97/3, acetonitrile/acetone, v/v, was applied in the first ten minutes, an automatic gradient was applied from 10 min to 100 min until reaching 60/40 of acetonitrile/acetone, v/v, then maintained at 60/40 from 100 to 200 min. The flow rate was set at 1 mL/min with automatic injection of 20 μL. All samples were diluted at 1/100 in a mixture of acetonitrile and dichloromethane 50/50, v/v.

In addition, to compare between LM and OM, two batches were analyzed, with three injections for each batch. In each sample the same chemical internal standard (ISTD) was added, N-Lauroyl-D-sphingosine at 1 mg/mL.

2.3 Mass spectrometry method

HPLC is coupled with LTQ-Orbitrap velos Pro mass spectrometer from Thermo Fisher Scientific (Bremen, Germany). The signal was acquired with Xcalibur software from Thermo Fisher Scientific. The spectrometer is a hybrid device incorporating two analyzers, a double linear ion trap (at high and low pressure) and a Fourier Transform orbital trap. The ionization source used was atmospheric pressure photoionization (APPI), using the positive-ion mode. Vaporizer temperature of the probe was set at 350 °C. Sheath gas, auxiliary gas, and sweep gas flow rates were set at 40, 20, and 0 (arbitrary unit) respectively. Capillary temperature was set at 325 °C and S-lens RF level at 60%. The data was acquired in the mass range m/z 150.00–1100.

2.4 Chemometrics

2.4.1 Processing data

Raw data file was imported in MZmine software (MZmine 2, 2010). Displaying the total ion chromatogram (TIC) permitted to take note of the baseline level and the height of the smallest significant peak. The peak detection is a three-step process: mass detection, chromatogram building and peak deconvolution. For mass detection, we used centroid mode and noise level was set to 10^5 and MS level to 1. For chromatogram building, selected minimum time span was 1 min and minimum height 10^6 with a m/z tolerance of 0.001 m/z or 5 ppm. Then, a deconvolution was conducted using the “Local minimum search” algorithm with a chromatographic threshold of 1%, a search minimum in retention time (RT) range of 1 min, a minimum relative height of 5%, a minimum absolute height of 10^6 , a minimum ratio of peak top/edge of 20 and a peak duration ranging from 0 to 5 min. After peak deconvolution, MZmine produces a resolved peak list where the most intense isotope was considered. Peak alignment is done with join aligner method with m/z tolerance set to 0.001, weight for m/z set to 20, and the maximum allowed relative distance between two retention time values was 5%, and score for perfectly matching RT values was 10. After, gap filling was applied using the option same RT and m/z range with m/z tolerance of 0.001 m/z or 5 ppm. As we chose to add a chemical standard to all samples, we normalized our data on peak area using the weighted contribution of standard compound with a m/z vs. RT balance of 3. Our chemical internal standard of exact mass 481.449 appears at m/z : 464.443–464.448 corresponding to ion fragment $[\text{M} + \text{H} - \text{H}_2\text{O}]^+$.

2.4.2 Data analysis: Descriptive statistics and multivariate analysis

From the matrix obtained after data processing by MZmine software, a new matrix was constructed, by the calculating the mean and standard deviation of m/z corresponding to each RT, for LM and OM.

In addition, principal component analysis (PCA) and orthogonal partial least square – discriminant analysis (OPLS-DA) were performed using Simca P+ (version 12.0.1.0, Umetrics). The analysis was carried out on 12 samples and 1327 variables (variables represent the couples RT and m/z).

Prior to analysis, our data set was centered and normalized. The variation of each variable was centered so that the mean of each column is equal to 0 and scaled to unit variance (1/SD). Principal component analysis (PCA) is a multivariate technique that analyses a data table in which observations (here different Labrafils) are described by several variables (RT and m/z). The goal is to extract the important information from the data matrix and to find a set of new orthogonal axes called principal components. However, PCA remains a descriptive chemometric method. Therefore, OPLS-DA is used to highlight the discriminant variables explaining the difference between the groups.

3 Results and discussion

3.1 Optimization of chromatographic conditions

The optimization of chromatographic conditions was performed with LM. It was not performed with a mixture of standards as it was not possible to have all the standards because of the richness of the chemical composition of LM and OM.

The gas chromatography was not considered because of the presence of the polymers (MPEGEs and DPEGEs). Non-aqueous phase mobile was used because LM and OM are not soluble in water.

Due to the low volatility, the presence of polymers and the no water solubility of LM and OM, liquid chromatography and non-aqueous phase mobile were selected for the analysis of the two ingredients. The initial operating conditions were based on the method of the TGs separation developed by [Tamba Sompila *et al.* \(2014\)](#). The first run was 60 min with an isocratic system 70/30, acetonitrile/acetone, v/v, using vintage Series KRC18 column (250 × 4.6 mm, 5 μm) and charged aerosol detector

In NARP-HPLC, acetonitrile is the weak solvent. It allows better resolutions and facilitates the separation of unsaturated glycerides through its interactions with electrons π of double bonds. Acetone (or isopropanol) is the strong solvent, added in a smaller amount than the weak solvent, it allows to modulate the analysis time and the polarity of the mobile phase ([Maloumbi *et al.*, 2015](#); [Rezanka *et al.*, 2017](#)).

The isocratic system acetonitrile/acetone 70/30 v/v was not efficient for our purpose as PEG esters overlapped with MGs between 0 to 20 min.

A first experimental plan was established to optimize the separation of the peaks in isocratic mode, increasing the percentage of the weak solvent: a pitch of 5% of acetonitrile was considered (*i.e.* the mobile phase mixtures tested were: 75/25, 80/20, 85/15, 90/10, 95/5, acetonitrile/acetone, v/v). Indeed, acetonitrile, in larger quantities, allowed a good resolution of less apolar products but delayed the release of highly apolar products.

Then, a gradient mode was considered to improve the analysis time and elute all constituents. Several combinations of the mobile phase and slope of eluent strength were tested. Finally, the best gradient profile was the following, for the first 10 minutes, an isocratic step acetonitrile/acetone 97/3 v/v was set. Following by an automatic gradient applied from 10 min to 100 min, until reaching acetonitrile/acetone 60/40 v/v. Then another isocratic step was applied from 100 min to 200 min,

with this last mobile phase composition acetonitrile/acetone 60/40 v/v.

As LM and OM as both mixtures of glycerides and PEG-esters, this method had been applied for LM as well for OM. The major peaks for LM and OM were separated under these improved experimental conditions ([Fig. 2](#)).

3.2 Reliability of the developed method: relationships chemical structure/chromatographic behavior

To highlight the relationships chemical structure/chromatographic behavior of glycerides, the same HPLC conditions retained for the analysis of LM and OM were used to analyze a mixture of standard molecules. First, MG (18:1), 1,2-DG (18:1/18:1), 1,3-DG (18:2/18:2), TG (18:2/18:2/18:2) standards were injected separately to make sure of the retention time (RT) of each one. Second, a mixture of all the standards was injected, the obtained chromatogram is shown in [Figure 3](#). MG was eluted first followed by DGs then TGs. RT increased with increasing molecular weight. For molecules of near molecular weight, as DG (18:1/8:1) and DG (18:2/18:2), the molecule with more unsaturated bonds would have a shorter retention time.

Comprehensively, the retention increased with increasing of the total carbon number (CN). For the same CN, the retention decreased according to the number of double bonds (DB). It is known that NARP-HPLC mainly separates the different glycerides according to their equivalent carbon number (ECN), which is calculated as following, $ECN = CN - (2 \times DB)$ ([Holčapek *et al.*, 1999](#); [Lísa *et al.*, 2007, 2011](#); [Rezanka *et al.*, 2017](#)).

To highlight the relationships chemical structure/chromatographic behavior of PEG-esters, the NARP-HPLC-APPI-MS was applied to LM and OM as no standards of pure PEG-esters were available. Using mass spectrometry (see [Sect. 3.3.2](#)), it has been possible to determine the elution order of the polymers. Mainly it depends first on the number of esters groups (mono-PEG-esters eluted before di-PEG-esters), second the number of double bonds (DB) on the fatty acid moieties (monounsaturated compounds eluted after diunsaturated compounds). Thirdly, MPEGEs with various number of ethyleneglycol units were eluted under the same chromatographic peak, while for the DPEGEs, the retention decreased according to the number of ethyleneglycol units.

3.3 Characterization of mono-, di- and triglycerides as well as PEG esters by HPLC-APPI-LTQ-Orbitrap

3.3.1 Characterization of mono-, di- and triglycerides

APPI is recently introduced technique, where a highly energetic photon is used to effect generation of charged ion species. In comparison to APCI, APPI offered lower detection limits, highest sensitive and the same fragmentation pattern for mono, di and triacylglycerols ([Holčapek *et al.*, 1999](#); [Gómez-Ariza *et al.*, 2006](#); [Rezanka *et al.*, 2010](#)).

Mass spectra of MG (18:1), 1,2-DG (18:1/18:1), 1,3-DG (18:2/18:2) and TG (18:2/18:2/18:2) are shown in [Figure 5](#). The APPI mass spectra exhibited the protonated molecular ion $[M + H]^+$ for each glyceride. So, the molecular masses of the different compounds can be easily determined. The APPI-MS

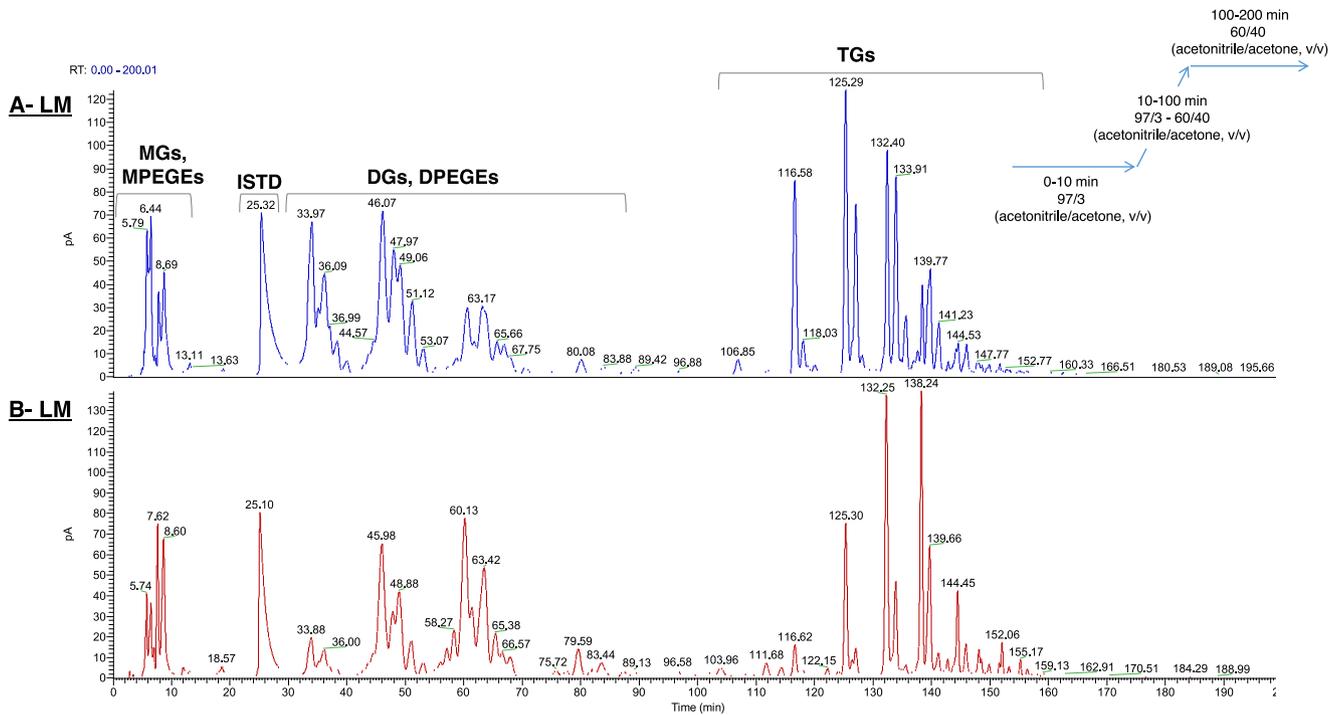


Fig. 2. NARP-HPLC-CAD analysis of LM and OM with optimized chromatographic conditions. 97/3, acetonitrile/acetone, v/v was set for the first 10 minutes, from 10 min to 100 min an automatic gradient was applied until reaching 60/40, acetonitrile/acetone, v/v, and an isocratic system was applied from 100 min to 200 min with a mixture 60/40, acetonitrile/acetone v/v.

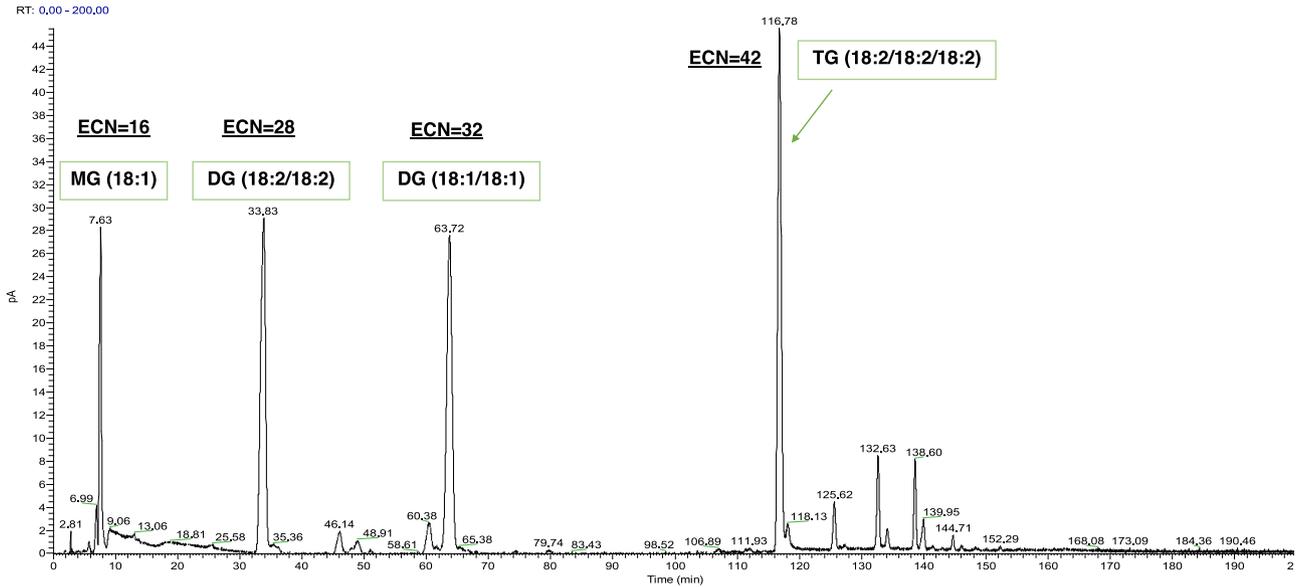


Fig. 3. NARP-HPLC-CAD analysis of a mixture of MG (18:1), 1,2-DG (18:1/18:1), 1,3-DG (18:2/18:2) and TG (18:2/18:2/18:2) standards. MG, DGs and TG were eluted in order of increasing ECN.

ionization and fragmentation patterns of MG, DG and TG are as described by RP-HPLC-APCI (Holčapek *et al.*, 1999; Gómez-Ariza *et al.*, 2006; Rezanka *et al.*, 2010).

– APPI-MS spectrum of MG (18:1) presents the protonated molecular ion $[M + H]^+$ (m/z 357.299) and fragment ions: the protonated molecule loses a molecule of water $[M + H - H_2O]^+$ (m/z 339.289). Then, we see its typical

fatty acid (18:1) *i.e.* $[RCO]^+$ (m/z 265.252) and which then loses a molecule of water $[RCO - H_2O]^+$ (m/z 247.241) (Fig. 4A);

– Similarly, APPI-MS spectra of 1,2-DG (18:1/18:1) and 1,3-DG (18:2/18:2) give the protonated molecular ions $[M + H]^+$ (m/z 621.540 and m/z 617.509 respectively) and fragment ions: the protonated molecules lose a

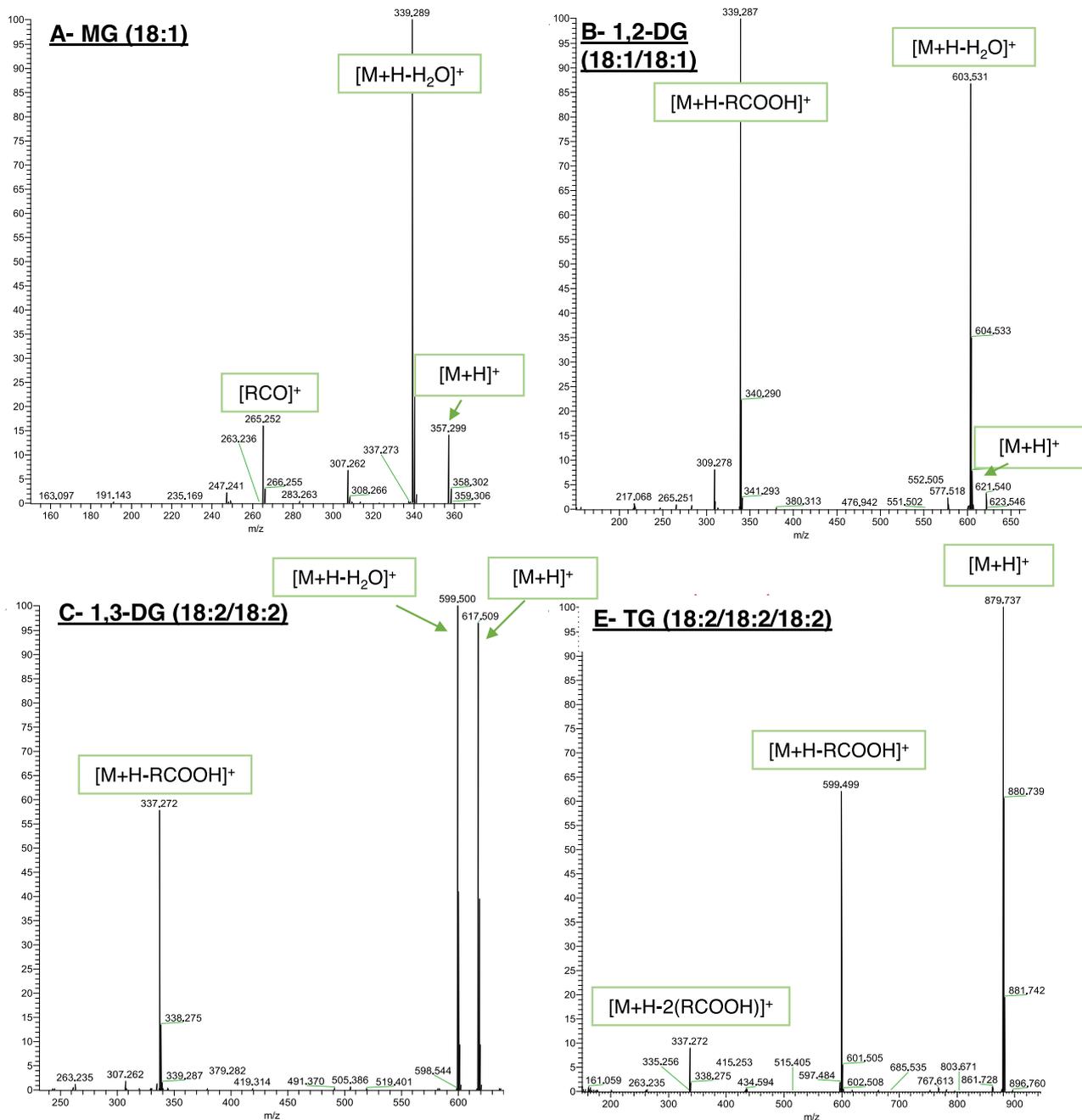


Fig. 4. APPI-MS spectra in positive-ion mode of MG (18:1) (A); DG (18:1/18:1) (B); DG (18:2/18:2) (C); and TG (18:2/18:2/18:2) (D).

molecule of water $[M+H-H_2O]^+$ (m/z 603.531 of 1,2-DG (18:1/18:1) and m/z 599.500 of 1,3-DG (18:2/18:2)). Then by losing a fatty acid (18:1 for 1,2-DG (18:1/18:1) and 18:2 for 1,3-DG (18:2/18:2)), their characteristic MG (18:1) and MG (18:2) are obtained *i.e.* $[M+H-RCOOH]^+$ (m/z 339.287 and m/z 337.272). Their fatty acids (18:1 and 18:2) *i.e.* $[RCO]^+$ (m/z 265.251 and m/z 263.235) and $[RCO-H_2O]^+$ (m/z 247.240 and m/z 245.226) are also observed (Figs. 4B and 4C).

It was possible to detect and identify the other DGs present at low quantities in the standard mixture, DG (18:1/18:2) at 46.1 min and 1,3-DG (18:1/18:1) at 60.4 min (Fig. 4). The

isomeric 1,2-DG (18:1/18:1) was distinguished from 1,3-DG (18:1/18:1), with the same ECN, 1,3-DG was eluted before 1,2-DG, as it has been shown by Holčapek *et al.* (Holčapek *et al.*, 1999). The hydrophobic interactions with the stationary phase for the positional isomer 1,2-DG are stronger compared to those of the 1,3-isomer, because of the position of the central hydroxy group (Holčapek *et al.*, 1999).

– APPI-MS spectrum of TG (18:2/18:2/2) provides the protonated molecular ions $[M+H]^+$ (m/z 879.737) and also fragment ions: the protonated molecule loses a fatty acid (18:2) and gives its typical DG (18:2/18:2) *i.e.* $[M+H-RCOOH]^+$ (m/z 599.499). The protonated molecule loses two fatty acid (18:2) and gives its typical

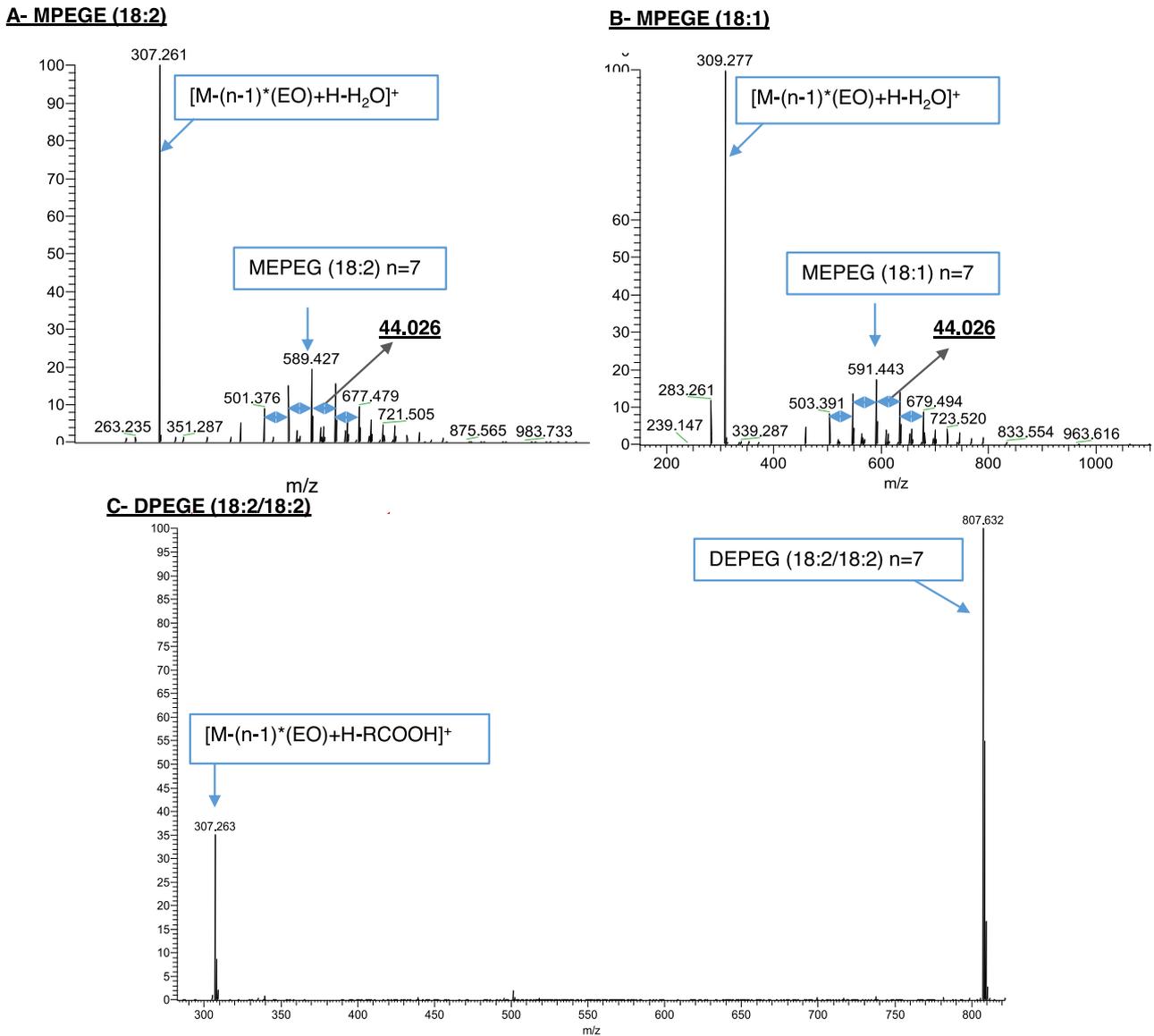


Fig. 5. APPI-MS spectra in positive-ion mode of MPEGE (18:2) (A); MPEGE (18:1) (B); and DPEGE (18:1/18:2) (C).

MG (18:2) *i.e.* $[M+H-2(RCOOH)]^+$ (m/z 337.272) (Fig. 5D).

Other triglycerides were present in the standard mixture, peaks at 125.6, 132.6 and 138.6 min (Fig. 4). Using protonated molecular and fragment ions, the TGs were identified and are TG (18:2/18:2/18:1), TG (18:1/18:2/18:1) and TG (18:1/18:1/18:1). The TGs are eluted in order of increasing ECN (44, 46 and 48).

The position of the substitution of fatty acids in TGs was determined from fragmentation of TG, considering the diglyceride fragments m/z. It has been demonstrated that the ratio of the abundance of these fragment ions could be used to identify the sn-2 and sn-1 (or sn-3) positions (Mottram and Evershed, 1996). The relative abundance of $[M+H-RCOOH]^+$ ion fragment corresponding to loss of fatty acid from sn-2 position is less than the relative abundance of $[M+H-RCOOH]^+$ ion fragment due to loss fatty acid from sn-1 (or sn-3) (Mottram and Evershed, 1996).

3.3.2 Characterization of PEG esters

APPI-MS ionization and fragmentation profiles of PEG esters are highlighted as follows:

For each mono-PEG ester, the protonated molecular ion $[M+H]^+$ and the fragment ion $[M-(n-1)(EO)+H-H_2O]^+$ were observed. The MPEGEs mass distribution was exhibited as adjacent protonated molecular ions $[M+H]^+$ separated by 44.026 Da, consistent to the mass of the repeat unit, ethylene oxide (EO), as observed with ESI mass spectrometry (Chen *et al.*, 2002).

For example, mass spectrum of MPEGEs (18:2) presents: the different protonated molecular ions corresponding to MPEGE with different repeat units (m/z 633.453, m/z 589.427 and m/z 545.401... corresponding to $n=8$, $n=7$ and $n=6$... respectively), and m/z 307.261 typical to (MPEGE) $n=1$ with C18:2 fatty acid moiety *i.e.* $[M-(n-1)(EO)+H-H_2O]^+$ (Fig. 5A). Similarly, for MPEGE (18:1), mass spectrum

provides: the protonated molecular ions, m/z 635.469, m/z 591.443 and m/z 547.417... corresponding to $n=8$, $n=7$ and $n=6$... respectively; and m/z 309.277 typical to MPEGEs with C18:1 fatty acid moiety *i.e.* $[M-(n-1)(EO)+H-H_2O]^+$ (Fig. 5B).

For each di-PEG ester, the protonated molecular ion $[M+H]^+$ and fragment ion $[M-(n-1)(EO)+H-RCOOH]^+$ typical to di-ethylene glycol esters *i.e.* (DPEGE) $_{n=1}$ were observed.

For example, DPEGE (18:2/18:2), mass spectrum provides: the protonated molecular ion, m/z 807.632 corresponding to DPEGE with 7 repeat units; and m/z 307.263 characteristic to (MPEGE) $_{n=1}$ with C18:2 fatty acid moiety *i.e.* $[M-(n-1)(EO)+H-RCOOH]^+$ (Fig. 5C).

3.4 Identification of constituents of linoleoyl and oleoyl macroglycerides

MGs, DGs, TGs, MPEGEs and DPEGEs were identified for LM and OM as described above, exploiting the retention time, protonated molecular ion and typical fragment ions for each molecule.

The chromatograms of LM and OM can be divided into three segments, the first one being from 0 to 10 min, the second from 30 min to 70 min and the third from 115 min to 150 min. For both products, the first package represents MGs and MPEGEs, the second DGs and MPEGEs and the third TGs. Retention times were obtained after alignment and normalization of all peaks of LM and OM chromatograms (for more details see [Supplementary data 1, 2 and 3](#)).

The chemical species identified in the first segment (from 0 to 10 min) were MG (18:1) and MG (18:2) as well as MPEGE (18:1) and MPEGE (18:2). As expected, MG (18:2) is eluted before MG (18:1). Regarding mono-PEG esters, MPEGE (18:2) is eluted after MG (18:2) and MPEGE (18:1) after MG (18:1). The mass distributions with repeat units from $n=4$ to $n=11$ were identified on the mass spectra for MPEGE (18:1) and MPEGE (18:2).

In the second segment (from 30 min to 70 min) six DGs were identified. 1,2 and 1,3-DG (18:2/18:2), 1,2 and 1,3-DG (18:1/18:2), and 1,2 and 1,3-DG (18:1/18:1). DGs are eluted in order of increasing ECN (DG (18:2/18:2) < DG (18:1/18:2) < DG (18:1/18:1)) and DGs with the same ECN, 1,2-isomer is eluted after 1,3-isomer. 30 DPEGEs were identified: DPEGE (18:2/18:2), DPEGE (18:1/18:2) and DPEGE (18:1/18:1), with repeat units from $n=3$ to $n=12$ for each of them. DPEGEs (18:2/18:2) were eluted first followed by DPEGEs (18:1/18:2) then DPEGEs (18:1/18:1) eluted. Then, the different DPEGEs were eluted in order of decreasing repeat units.

APPI allowed the identification of ten TGs in the last package (from 115 min to 150 min). TGs separation followed ECNs and TGs with the same ECN, called critical pairs were also separated, as TG (18:2/18:2/18:1) and TG (18:2/18:2/16:0) (ECN=44), TG (18:1/16:0/18:2) and TG (18:2/16:0/16:0) (ECN=46), and TG (18:1/18:1/18:1) and TG (18:1/16:0/18:1) (ECN=48). Indeed, under optimized conditions, NARP-HPLC separates some critical pairs. 9,12,24 NARP-HPLC can also separate some regioisomers. 16, it was found that symmetrical TGs elute before the corresponding asymmetrical TGs. 28 In our study, the peak at 132.4 min correspond to TG

(18:1/18:2/18:1) is well separated from the peak at 133.6 min, correspond to its regioisomer TG (18:1/18:1/18:2) (Fig. 6).

3.5 Comparative study between linoleoyl and oleoyl macroglycerides

MG, DGs and TG injected at the same concentration (1 mg/mL), had different response factors ($R(i)$): $R(MG) < R(DG) < R(TG)$. The response factors of DG (18:1/18:1) and DG (18:2/18:2) had the same order. (Tab. 1) Therefore, in the samples of LM and OM, the relative amounts of the different lipid classes (MGs, DGs and TGs) cannot be compared. The comparison can only be done between the lipid sub-classes, within MGs or DGs or TGs.

The peak areas of LM and OM were normalized, to the peak area (ion fragment $[M+H-H_2O]^+$) of ISTD (see the [Sect. 2.4.1](#)). From the matrix obtained after data processing, a new matrix was constructed, by calculating the mean and standard deviation of relative intensities of m/z corresponding to each RT, for LM and OM. The comparison of the two ingredients was performed on the majority ion (m/z) of each constituent (Fig. 7, [Supplementary data 4](#)). For the glycerides, we observed that the majority ion was $[M+H]^+$ above four unsaturations, otherwise it was $[M+H-H_2O]^+$ for MGs and DGs or $[M+H-RCOOH]^+$ for TGs. For the PEG-esters, the majority ion was $[M+H]^+$. ([Supplementary data 4](#))

The [Figure 7](#) shows that LM and OM have the same qualitative composition. The difference between the two ingredients is the relative amounts of each compound within each sub-classes of glycerides and PEG-esters.

LM contains more MG (18:2) and DGs (18:2/18:2) than OM. OM contains more MG (18:1) and DGs (18:1/18:1) than LM. However, DGs (18:1/18:2) are at the same amount for LM and OM. Regarding TGs, LM contains more TG (18:2/18:2/18:2), TG (18:2/18:2/18:1), TG (18:2/18:2/16:0) and TG (18:2/16:0/16:0) than OM. OM contains more TG (18:1/18:1/18:1), TG (18:1/18:2/18:1), TG (18:1/18:0/18:1) and TG (18:1/16:0/18:1) than LM ([Fig. 7A](#)).

LM contains more MPEGEs (18:2) and DPEGEs (18:2) than OM. OM contains more MPEGEs (18:1) and DPEGEs (18:1/18:1) than LM. DPEGEs (18:1/18:2) are at the same amount for LM and OM ([Fig. 7B](#)).

3.6 PCA and OPLS-DA results

PCA was carried out for the two macroglycerides. As mentioned in the [Section 2.2](#), for each macroglyceride, two batches were analyzed, with three injections for each batch. The first two principal components accounted for 66% of the variance. The 1st principal component PC1 explained 53% of variance and the 2nd principal component PC2 13% of variance.

The projection of samples (score plot) on the 1st and 2nd components is presented in [Figure 8](#). 1st component represents the chemical composition, the 2nd component represents the batch. PC1 separated the two macroglycerides into two distinct groups and PC2 separated batch 1 and batch 2 for both LM and OM. These two pharmaceutical ingredients were obtained, from corn oil for LM and from apricot kernel oil for OM, by a manufacturing process standardized by the supplier.

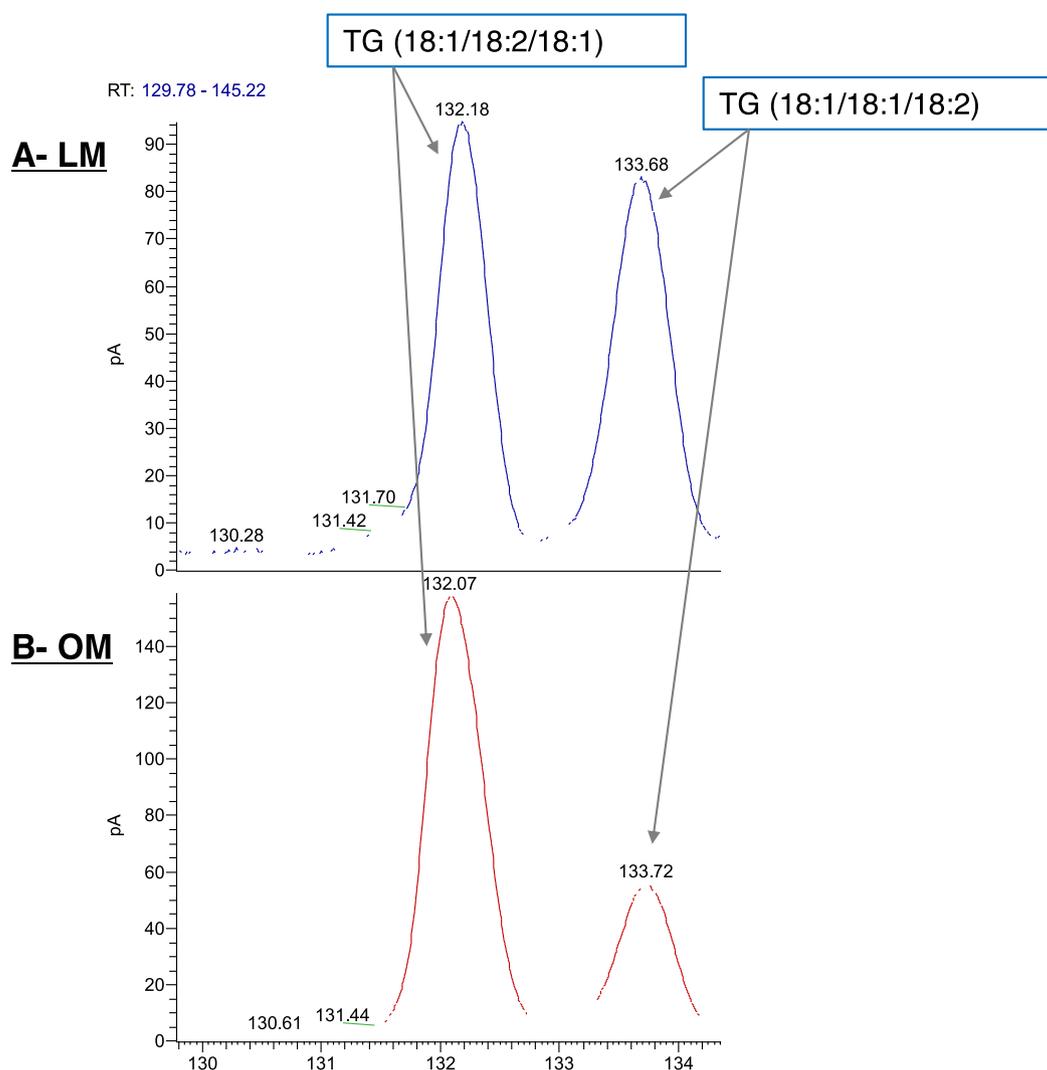


Fig. 6. Separation of two regioisomers TG (18:1/18:2/18:1) and TG (18:1/18:1/18:2) by NARP-HPLC.

Table 1. Response factors of MG, DGs and TG injected at the same concentration (1 mg/mL).

Molecules	Response factors
MG (18:1)	3.71×10^6
DG (18:1/18:1)	6.81×10^7
DG (18:2/18:2)	3.92×10^7
TG (18:2/18:2/18:2)	1.25×10^8

As the raw materials are of vegetable-origin, then, there is a variability of the composition of corn and apricot kernel oils. Therefore, the batches of finished goods can have a relative variability of their compositions. This explains the separation, in the score plot, of the two batches analyzed for LM and OM.

OPLS-DA was carried out to highlight the discriminant variables explaining the difference between the two groups. S-plot displayed at the lower part an overexpression for LM and

at the upper part an overexpression for OM. Table 2 summarizes the expressed latent variables. For each variable, an identification of the molecule specie was performed. For LM, a higher presence of MEPEG, DG, DEPEG and TG of linoleic acid (18:2 carbon chain) is highlighted and for OM, MEPEG, DG, DEPEG and TG of oleic acid (18:1 carbon chain). In addition, for LM, a higher presence of TG (18:2/18:2/18:1), (18:2/18:2/16:0), TG (18:1/16:0/18:2) and TG (18:2/16:0/16:0) is highlighted and for OM, TG (18:1/18:2/18:1), TG (18:1/18:0/18:1) and (18:1/16:0/18:1).

4 Conclusion

NARP-HPLC method developed in this work allowed not only the separation of mono-, di- and triglycerides, but also the separation of mono- and di-PEG esters. Then, the characterization of the fine composition for both linoleoyl macroglyceride (LM) and oleoyl macroglyceride (OM), two pharmaceutical ingredients widely used, can be accessed at the molecular level, mainly the microheterogeneity of the

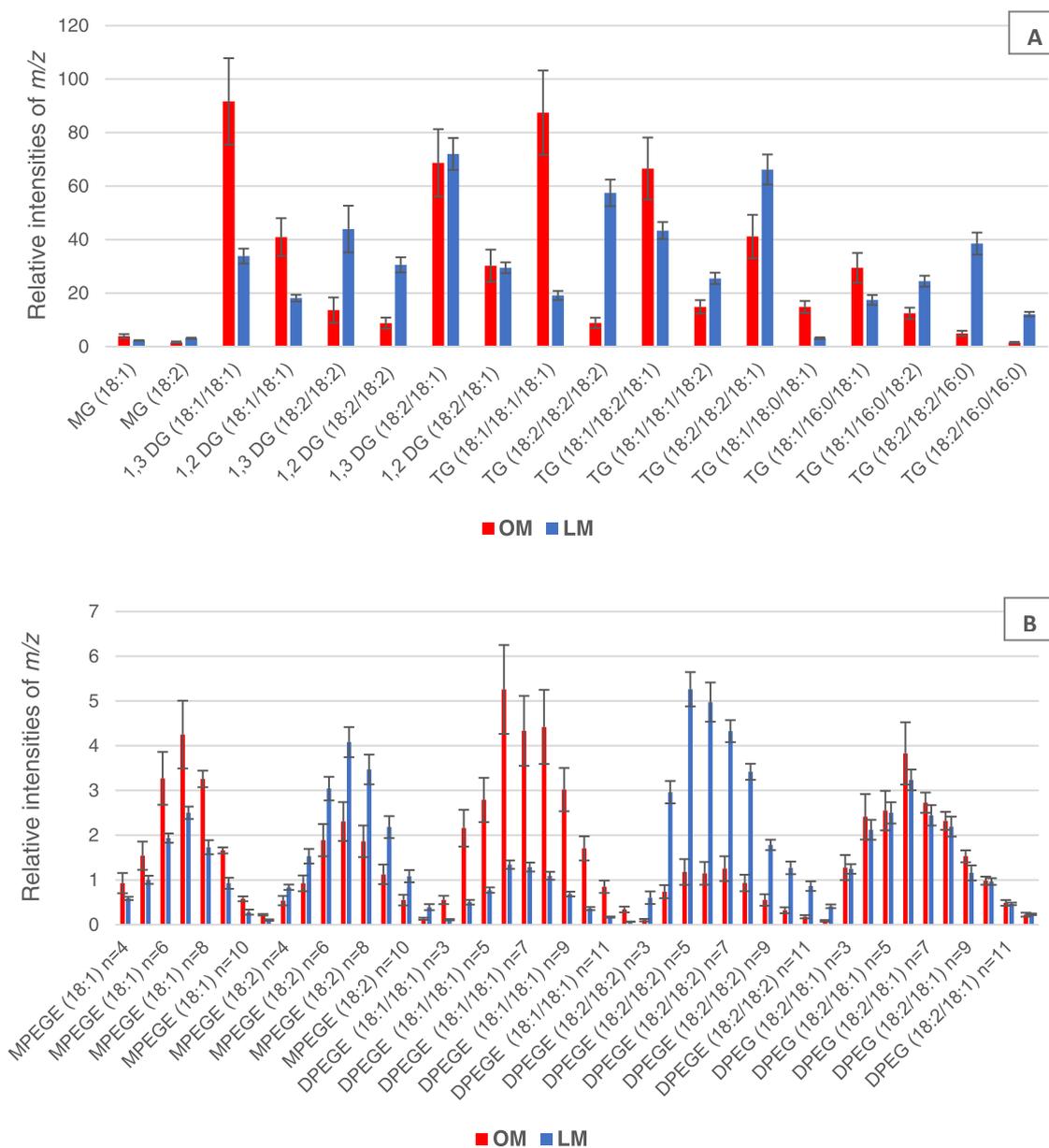


Fig. 7. Comparative profile of LM and OM: relative intensities of majority ion m/z (mean and standard deviation) for each compound. A: corresponds to monoglycerides, diglycerides and triglycerides. B: corresponds to mono- and di-PEG esters. The relative intensities of m/z are the means of the normalized peak areas of the two batches of LM and OM injected in triplicate.

glycerides sub-classes and the number of ethylene glycol units within PEG-esters.

The coupling HPLC-APPI allowed the identification of the most peaks of LM and OM. The constituents identified in LM and OM were MG (18:1) and MG (18:2). 1,2-DG (18:1/18:1) and 1,3-DG (18:1/18:1); 1,2-DG (18:2/18:2) and 1,3-DG (18:2/18:2); 1,2-DG (18:1/18:2) and 1,3-DG (18:1/18:2). TG (18:1/18:1/18:1), TG (18:2/18:2/18:2) and 8 mixed TGs of (18:1), (18:2), (16:0) and (18:0). MPEGEs (18:1) and MPEGEs (18:2), with number of units of ethylene oxide from $n=4$ to $n=11$. DPEGEs (18:1/18:1), DPEGEs (18:2/18:2) and DPEGEs (18:1/18:2), with number of units of ethylene oxide from $n=3$ to $n=12$.

MGs, DGs and TGs were eluted in order of increasing ECN. For the same ECN, some regioisomers and critical pairs were also separated. 1,3-isomers of DG were eluted before 1,2-isomers. Within the TG sub-class, the position and the number of unsaturated bonds affected the retention times. Thus, TG (18:1/18:1/18:2) was eluted after TG (18:1/18:2/18:1). Moreover, 3 critical pairs (TG (18:2/18:2/18:1) and TG (18:2/18:2/16:0), TG (18:1/16:0/18:2) and TG (18:2/16:0/16:0), and TG (18:1/18:1/18:1) and TG (18:1/16:0/18:1)) were well separated, showing the discriminant power of the method.

For PEG esters, MPEGEs were eluted before DPEGEs. Then, PEGEs were eluted by decreasing the number of double bonds (DB) on the fatty acid moieties (monounsaturated

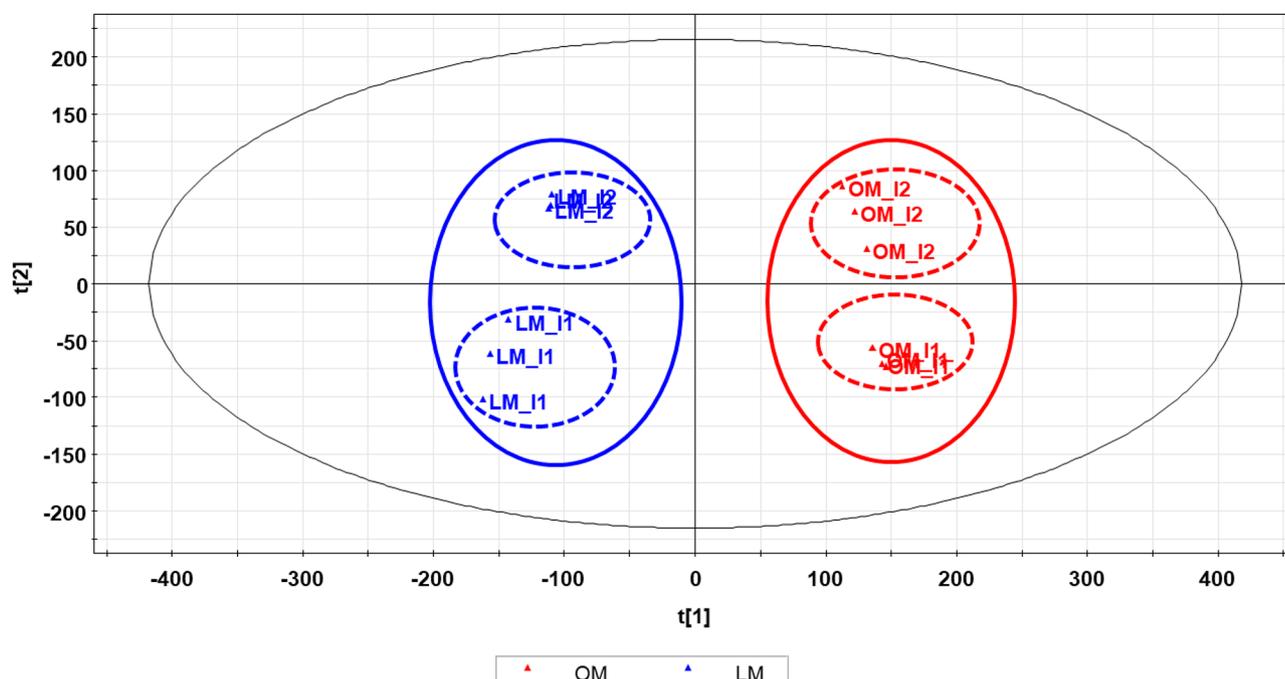


Fig. 8. Score plot of LM (blue) and OM (red). I1 and I2 represent the batch 1 and the batch 2, respectively, for both LM and OM.

Table 2. OPLS-DA: variables expressed in the upper and lower part of the S-plot.

LM (Lower part of S-plot)				OM (Upper part of S-plot)			
RT (min)	m/z	Ion fragment	Assignment	RT (min)	m/z	Ion fragment	Assignment
6.4	307.262	$[M-(n-1)*(EO) + H-H_2O]^+$	MEPEG (18:2)	8.7	309.277	$[M-(n-1)*(EO) + H-H_2O]^+$	MEPEG (18:1)
33.6	599.501	$[M+H-H_2O]^+$	DG (18:2/18:2)	60.2	603.533	$[M+H-H_2O]^+$	DG (18:1/18:1)
35.9	307.262	$[M-(n-1)*(EO) + H-RCOOH]^+$	DEPEG (18:2/18:2)	63.1	309.278	$[M-(n-1)*(EO) + H-RCOOH]^+$	DEPEG (18:1/18:1)
116.5	599.501	$[M+H-RCOOH]^+$	TG (18:2/18:2/18:2)	138.2	603.535	$[M+H-RCOOH]^+$	TG (18:1/18:1/18:1)
125.4	881.757	$[M+H]^+$	TG (18:2/18:2/18:1)	132.3	883.772	$[M+H]^+$	TG (18:1/18:2/18:1)
126.9	855.741	$[M+H]^+$	TG (18:2/18:2/16:0)	139.7	577.517	$[M+H-RCOOH]^+$	TG (18:1/16:0/18:1)
						(DG 18:1/16:0)	
						$[M+H-RCOOH]^+$	
						(DG 18:1/18:1)	
133.8	575.501	$[M+H-RCOOH]^+$	TG (18:1/16:0/18:2)	144.4	605.55	$[M+H-RCOOH]^+$	TG (18:1/18:0/18:1)
	577.517	(DG 18:2/16:0)				(DG 18:1/18:1)	
		$[M+H-RCOOH]^+$				$[M+H-RCOOH]^+$	
		(DG 18:1/16:0)				(DG 18:1/18:0)	
135.6	551.501	$[M+H-RCOOH]^+$	TG (18:2/16:0/16:0)	-	-	-	-
	575.501	(DG 16:0/16:0)					
		$[M+H-RCOOH]^+$					
		(DG 18:2/16:0)					

compounds eluted after diunsaturated compounds). Regarding the mass distribution of PEG esters, it was detected under the same peak for MPEGEs (18:1), as well as for MPEGEs (18:2). This highlights that the number of ethylene glycol units did not impact the retention of MPEGEs. For Di-PEG esters, the presence of the two fatty acid moieties allowed a better separation compared to MPEGEs and the compounds of this sub-class were eluted in order of decreasing of number of units of ethylene oxide.

It can be concluded that NARP-HPLC combined with APPI-MS is a suitable tool to characterize the two macroglycerides. HPLC-APPI allowed us to obtain the detailed chemical composition of LM and OM. These two pharmaceutical ingredients have the same qualitative composition but differ by the relative amounts of each compound, depending on the analyzed macroglyceride.

As expected, the data analysis (descriptive statistics and multivariate analysis) of both the compositions, showed a

higher presence of linoleic esters for LM (mainly expressed in MEPEGs, DGs, DEPEGs and TGs compounds), and a higher presence of oleic esters for OM (mainly expressed in MEPEGs, DGs, DEPEGs and TG). In addition, for LM, a higher presence of TG (18:2/18:2/18:1), (18:2/18:2/16:0), TG (18:1/16:0/18:2) and TG (18:2/16:0/16:0) is highlighted and for OM, TG (18:1/18:2/18:1), TG (18:1/18:0/18:1) and (18:1/16:0/18:1).

The European Pharmacopoeia monographs for the quality control of these ingredients (OM and LM) propose to access the overall fatty acid profile after hydrolysis of all the esters. The purpose of this global approach was to characterize the pharmaceutical raw material through its main fatty acid *i.e.* oleic acid (C18:1) for OM and linoleic acid (C18:2) for LM. Nevertheless, it remains very interesting to have a more detailed molecular approach of OM and LM for a best understanding of their behavior during the manufacturing process of some dosage forms (such as nanocarriers). Indeed, their differences in the molecular distribution of OM and LM could lead to various functionality-related characteristics of these ingredients.

Supplementary Material

Supplementary data 1: RT, ECN and *m/z* values of protonated molecular ion and main fragment ions of MGs and MPEGEs for LM and OM.

Supplementary data 2: RT, ECN and *m/z* values of protonated molecular ion and main fragment ions of DGs and DPEGEs for LM and OM.

Supplementary data 3: RT, ECN and *m/z* values of protonated molecular ion and main fragment ions of TGs for LM and OM.

Supplementary data 4: Majority ions (*m/z*) of some constituents of LM and OM.

The Supplementary Material is available at <http://www.ocl-journal.org/10.1051/ocl/2020020/olm>.

Abbreviations

LM	Linoleoyl macrogolglyceride
OM	Oleoyl macrogolglyceride
NARP	Non-aqueous reversed phase
HPLC	High-performance liquid chromatography
APPI	Atmospheric pressure photoionization
LTQ	Linear trap quadrupole
MG	Monoglyceride
DG	Diglyceride
TG	Triglyceride
PEG	Polyethylene glycol
MPEGE	Mono-PEG esters
DPEGE	Di-PEG esters
ELSD	Evaporative light-scattering detector
CAD	Charged aerosol detector
APCI	Atmospheric pressure chemical ionization
ESI	Electrospray ionization

MALDI	Matrix-assisted laser desorption ionization
FTMS	Fourier transform mass spectrometry
TOF	Time of flight mass spectrometry
CID	Collision-induced dissociation
ISTD	Chemical internal standard
TIC	Ion chromatogram
RT	Retention time
PCA	Principal component analysis
OPLS-DA	Orthogonal partial least square–discriminant analysis
CN	Total carbon number
DB	Double bonds
ECN	Equivalent carbon number
EO	Ethylene oxide
R	Response factor
PC	Principal component

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