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Corresponding Author: Dr. Soledad Prats Moya, Ph. D.

Corresponding Author's Institution: University of Alicante

First Author: Silvia Carballo Marrero, Ph.D.

Order of Authors: Silvia Carballo Marrero, Ph.D.; Soledad Prats Moya, Ph. D.; Salvador E Maestre Pérez, Ph.D.; Jose L Todolí Torro, Ph.D.

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**Cover Letter**

Maria Soledad Prats Moya
Analytical Chemistry, Nutrition and Food Science Department
University of Alicante
P.O. Box 99
03080 Alicante (Spain)
Maria.prats@ua.es

Editor-in-Chief
*Food Chemistry*

November 27th, 2018

Dear Dr Pegg:

Ms. Ref. No.: FOODCHEM-D-19-00281
Title: Microwave assisted high-performance liquid chromatography for the separation of triacylglycerols in vegetable oils using an evaporative light scattering detector Food Chemistry

Thank you for the opportunity to revise our manuscript especially after the difficulties in finding reviewers. We submit a response to the comments of the two reviewers hoping that a new consideration for publication of the article title: Title: Microwave assisted high-performance liquid chromatography for the separation of triacylglycerols in vegetable oils using an evaporative light scattering detector Food Chemistry and with ref. FOODCHEM-D-19-0028 can be given. The editor should take into account that some modifications of the original manuscript have been done trying to give response to the reviewers and also justifying its novelty and interest in analytical chemistry.

This manuscript has not been published and is not under consideration for publication elsewhere.

Thank you for your consideration!

Sincerely,

María Soledad Prats Moya
Senior Lecturer, Department of Analytical Chemistry, Nutrition and Food Science

# Microwave assisted high performance liquid chromatography for the separation of triacylglycerols in vegetable oils using an evaporative light scattering detector

Silvia Carballo-Marrero, Maria.Soledad Prats-Moya,* Salvador E. Maestre-Pérez, and José-Luis Todolí-Torró

Analytical Chemistry, Nutrition and Food Science Department, University of Alicante, Alicante, Spain

Corresponding author:

María Soledad Prats Moya. University of Alicante, Analytical Chemistry, Nutrition and Food Science Department. P.O. Box 99, 03080 Alicante. E-mail: maria.prats@ua.es

**Abstract**

Microwave (MW) radiation was applied to perform the separation of triacylglycerols (TGs) in oil samples. The novelty of the work lies in the application of MW radiation to assist the separation of several non-polar compounds employing a totally organic mobile phase employing ~~-~~an ~~An~~ evaporative light scattering detector (ELSD). ~~was used throughout.~~ Once the influence of the detector variables on the sensitivity was~~as-~~ optimized~~characterised~~, the TGs separation was compared~~optimised~~ conditioning the column with either ~~a~~ conventional HPLC ~~HPLC system~~ or ~~a~~ MW ~~microwave~~ oven ~~to assist the elution~~. ~~Retention times shortened by about 50 % when applying microwave radiation at a minimum power of 170 W with respect to the results found at room temperature.~~ Contrary to previous applications in which the mobile phase contained water, the improvement in sensitivity using MW was not as significant in comparison with conventional heating but it allowed a shortening in retention times of several TGs in about 50 % respect elution at room temperature and similar to the elution at 40 ºC. The method was finally applied to the quantification of most common TGs in almond, tiger nut, and argan oil. ~~The results showed higher sensitivity for TGs determination when working with conventional heating at 40 ºC and MW radiation than when the column was set at room temperature. Finally, a comparison of the content of triolein and trilinolein in different vegetable oils allowed to verify that, a double logarithm model seemed to be a more accurate calibration model than the linear one when using microwave HPLC.~~

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**Keywords:** MW-HPLC; triacylglycerols; vegetable oils; almonds; tiger nuts, triolein, trilinolein.

## 1. Introduction

Recently, microwave assisted HPLC (MW-HPLC) has emerged as a possible~~n~~ alternative separation method to those in which the column temperature is controlled by means of a conventional oven ~~,~~

(Galinada & Guiochon, 2005; Galinada & Guiochon, 2007; Stone & Taylor, 2003). The microwave heating mechanism is based on molecule dipole rotation and ionic migration processes. ~~The first one occurs when the molecules dipoles rotate in their attempt to align with the applied electromagnetic field. The field alignment and dipole rotation lag produce molecule collisions thus releasing energy in the form of heat. Similarly, ionic species tend to align with the alternating field which generates a current flow producing friction of ions hence increasing the temperature of the medium. The ability of a dielectric compound to absorb energy and to convert it into heat is governed by three properties: dielectric constant, dissipation factor and relaxation time. The greater the values of the three parameters, the greater the compound heating rates (Kingston & Haswell, 1997).~~ The ability exhibited by organic solvents to convert microwave energy into heat depends on their composition, polarity and other dielectric solvent properties such as dielectric constant, dissipation factor and relaxation time (Gabriel, Gabriel, Grant, Halstead, & Mingos, 1998). ~~The greater the values of the three parameters, the greater the compound heating rates (Kingston & Haswell, 1997).~~ Thus, for instance, when the mobile phase contains only substances having a lower dielectric constant than water, the heating caused by MW radiation is assumed to be less intense than in the case of aqueous phases and consequently, a priori, with total organic

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mobile phases little effect of MW radiation on the separation process would be expected. However, heating also depends on the loss factor value that gives an indication of the ability of solvents to convert microwave energy into heat (Lidström, Tierney, Wathey, & Westman, 2001). It is known that t~~(Lidström, Wathey, Tierney, & Westman, 2001). T~~he higher the value of the loss factor the higher the capability to generate thermal energy.

Until now there are few primary studies highlighting the possibilities~~benefits~~ of using microwave irradiation to perform the separation of organic species because of the combined effect of mobile phase heating and a possible influence of the microwave radiation on the analyte partition equilibrium between the two chromatographic phases (~~W. A.~~ Galinada & Guiochon, 2005; Stone & Taylor, 2003; Turner, Laurence, Conner, & Yngvesson, 2000)~~–~~. The first studies revealed an enhancement of chromatographic efficiency when microwave radiation was applied compared with the use of conventional heating (i.e., based on a conduction-convection mechanism). Nonetheless, i~~I~~t was not until 2012 when microwave radiation was applied for the first time to assist the separation of water-soluble vitamins (Terol, Maestre, Prats, & Todolí, 2012). Later, the set up was employed for fat-soluble vitamins using a less polar mobile phase (Carballo, Prats, Maestre, & Todolí, 2015)~~(Carballo, Prats, Maestre, & Todolí, 2015)~~. In both cases, a general shortening in retention times and more efficient separations were observed~~, as compared to the results found when the separation was conducted at room temperature.~~ Initially, this trend could be assigned to the increase in the temperature of the mobile phase caused by the presence of a highly polar solvent such as water and/or dissolved ions. Nevertheless, the MW-HPLC chromatographic results were also improved with respect to those found when the mobile phase was conventionally heated at the same temperature as that reached in the presence of the MW field. Therefore, an additional phenomenon related to the action of the microwaves on the analytes partition between mobile and stationary phases could be likely responsible for these observations. Alternately, it could be interesting to study the behaviour of MW-HPLC when less polar solvents than water are used as mobile phases. This is the case, for example, in the separation of TGs by HPLC.

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~~A possible way of better studying this latter effect would be to carry out separations in which the mobile phase would consist of pure organic species with low polarity. A good example is the separation of triacylglycerols (TGs). Although Gas Chromatography (GC) offers good peak resolution for the separation of triacylglycerols (TGs), reliable TGs quantification can be difficult due to reproducibility issues. In contrast, HPLC allows triacylglycerols separation with poorer resolution with respect to GC. Despite these considerations,~~ Rreversed HPLC (RP-HPLC) is the most common technique used for the determination of TGs mixtures. In this case, the organic solvents employed in the elution of TGs have dielectric constant values significantly lower than water. In the last decade, the Aerosol Charge Detector (CAD) or the Evaporative Light Scattering Detector (ELSD) has found its way into HPLC applications portfolio due to their compatibility with gradient elution separations, which is an important limitation of the refractive index detector (RID). Additionally, ELSD is more sensitive than RID although it gives a nonlinear calibration response particularly when organic solvents are used. This nonlinear relation between peak area (A) and concentration (m) is due to the variation of the particle size as a function of the analyte concentration (Righezza & Guiochon, 1988), the mobile phase flow rate and its physical properties, the type of nebuliser, nebulising gas velocity, the temperature of both the spray chamber and the drift tube and the analyte chemical structure. ~~As, in general practice, nonlinear relationships are not desirable for quantification purposes, linearization can be approached by applying a logarithmic function as shown in equation 1.~~

When the ELSD is used, it is essential to optimise experimental variables such as the composition and temperature of the mobile phase, the nebulization gas flow rate and the temperatures of the drift tube and the spray chamber to obtain the greatest signal. In some of the published papers, a chemometric comparison of TGs profiles for different vegetable oils is made, but no experimental optimisation is quoted. Meanwhile, in a few others, the optimisation of the experimental working conditions in terms of sensitivity is mentioned (Bosque-Sendra, Cuadros-Rodríguez, Ruiz-Samblás, & de la Mata, 2012; Cunha & Oliveira, 2006; Lecoeur, Simon, Sautou, Decaudin, & Vaccher, 2014; Rombaut, De Clercq, Foubert, & Dewettinck, 2009) ~~(Bosque-Sendra et al., 2012), (Lecoeur et al., 2014), (Rombaut et al., 2009), (Cunha & Oliveira, 2006).~~

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The aim of the present work was thus to evaluate ~~for the first time~~ the contribution of MW-HPLC- for performing the quantification~~determination~~ of TGs in edible oil samples using an ELSD~~Evaporative Light Scattering Detector.~~ and comparing the results with a conventional HPLC separation at room temperature. ~~Data achieved when the column was operated at room temperature were taken as reference.~~

~~The influence of the column heating mechanism was also studied.~~ The hypothesis of the work was that as using an organic mobile phase, the effect of the MW radiation on the heating of the mobile phase might be negligible and maybe less significant changes in the retention process will be observed than with water mobile phases unless other effects than efficient local heating of MW irradiation can also have influence on the process.~~a reduction in the analysis time can be reached without significatively increasing the mobile phase temperature and reducing the durability of the HPLC column. Furthermore, attention was paid to the optimisation of the ESLD detector performance and calibration models useful for the TGs separation and quantitative determination in oil samples. Data achieved when the column was operated at room temperature were taken as reference.~~

## 2. Materials and methods

### 2.1. Reagents~~, samples,~~ and solutions

Acetone (Panreac Química, Barcelona, Spain) and acetonitrile (ACN) (Scharlab, Barcelona, Spain) of HPLC quality grade were used. Pure trilinolein (LLL), triolein (OOO) and trilaurin (LaLaLa) purchased from Sigma Aldrich (Sant Louis, MO, USA). The Triacylglycerol standard 17810 distributed from Supelco (Bellefonte, PA, USA) that contained tricaprylin (CyCyCy), tricaprin (CCC), trimyristin (MMM), trilaurin (LaLaLa) and tripalmitin (PPP) were also employed to prepare the standards. ~~Finally, certified reference material of cocoa butter (IRMM-801) certified in the relative mass content of 1,3-dipalmitoyl-2-oleyl-glycerol (POP), 1-palmitoyl-2-oleoyl-3-stearoyl-~~

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glycerol (POS), 1-palmitoyl-2,3-dioleoyl-glycerol (POO), 1,3-distearoyl-2-oleoyl-glycerol (SOS) and 1-stearoyl-2,3-dioleoyl-glycerol (SOO), from Sigma-Aldrich (Sant Louis, MO, USA), was also utilised. Stock solutions of triacylglycerols (500 mg $L^{-1}$) were diluted in propan-1-ol and then sonicated in an ultrasonic bath for 5 min to ensure total dissolution. This solution was adequately diluted in the mobile phase to obtain standards having from 10 to 120 mg $L^{-1}$ of each compound.

Samples of soybean oil, argan oil, kernel peeled almonds and tiger nuts were obtained in a local supermarket. Tiger nuts and almond kernels from the Desmayo Llargueta cultivar were ground in an electric grinder to a fine powder. Particles which passed through a 1.5 mm sieve were used for oil extraction. The oil extraction was carried out using methanol: chloroform mixture according to the Folch method (Folch, Lees, & Solane Stanley, 1957). The oil obtained was then dried with a nitrogen stream and kept sealed in an amber vial at -18 ºC in a freezer until its analysis.

2.2. Samples and Standards and sample preparation

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Vegetable oils were diluted in propan-1-ol in proportions that ranged from 1/400 to 1/750 depending on the sample. Once prepared, samples were kept in the fridge at 4 ºC until their analysis. A quality control standard containing five TGs was daily analysed to assure that the

variability of the results was included within the range of uncertainty of the method calculated using control charts (Masson, 2007). Before the chromatographic analysis, standards and samples were adequately filtered through a 0.45-µm nylon syringe filter (Millipore, Massachusetts, USA).

2.3. Chromatographic conditions of HPLC-ELSD

The chromatographic system employed was an HPLC pump Model PU-2089 (Jasco Inc., Tokyo, Japan) and a Rheodyne valve model 7725(i) (Cotati, CA, USA) equipped with a 10 µL sample loop. The signal of the triacylglycerols was registered with a SoftA Corporation (Tokyo, Japan) 300s ELSD. The separation of the compounds was carried out using an Inertsil ODS-2 column of 250 mm x 4.6 mm I.D with 5 µm particle size (GL Sciences, Eindhoven, Netherlands) and hardware of peek, meanwhile the mobile phase consisted of a mixture of acetone/acetonitrile (65/35) flowing at 1 mL $min^{-1}$~~. These conditions were previously selected for the determination of TGs in almond samples~~ (Prats Moya, Grané Teruel, Berenguer Navarro, & Martín Carratalá, 1999). Standards and samples were injected in triplicate. The chromatography data processor employed was ChromPass (Jasco, Deutschland).

For conventional heating, an HPLC column oven from Gecko-2000 (CIL Cluzeau Info Labo, Sainte-Foy-La-Grande, France) was employed. Meanwhile, for MW-HPLC~~ assisted with microwaves~~, the column was placed inside a microwave oven in a hanging position in the same way as in previous work (Terol et al., 2012)~~ (Terol, Maestre, Prats, & Todolí, 2012a)~~. The heating system consisted of a conventional LG microwave oven (LG MB4047C) at the standard 2450 MHz frequency)(see Supplementary Figure 1). In order to study the effect of MW radiation over the separation process but avoiding~~avoid column~~ overheating of the mobile phase, the nominal power employed was the minimum allowed by the oven, i.e. 170 W. However, the column did not wholly absorb the radiation. Therefore, to avoid magnetron damage, a water container (2100 mL total volume) at a temperature of 20 ºC was placed inside the oven as a radiation well (Carballo et al., 2015)~~ (Carballo, Prats, Maestre, & Todolí, 2015)~~.

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Furthermore, an optimisation study was performed to select both the 300 S ELSD spray chamber and drift tube temperatures. The flow of argon was kept fixed at ~~–~~65 ± 5psi to ensure optimal droplet size distribution. ~~The pressure of the nebulising gas (argon) was set at 4.5 bars, yielding a gas flow rate of 1.21 L min$^{-1}$.~~ The mobile phase temperature at the exit of the column was measured using a digital thermometer TL-1 (ThermoProbe, USA).

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## 2.4. Identification of the chromatographic peaks

Chromatographic peak Identification of vegetable oils was achieved by comparison of the relative retention times obtained with known TG profiles such as soybean (Endo et al., 2011), argan, olive (Cunha, Casal, & Oliveira, 2005) and almond oil (Barreira et al., 2012). Identification of triacylglycerols not present in the standards was made by plotting log $\alpha$ vs the number of double bonds (n) in the TGs (European Commission, 2008) being $\alpha$ the rate between the retention time of the TG with respect to the retention time of OOO.

## 2.5. Aerosol particle size distribution measurement

The aerosols generated by the nebuliser of the detector, i.e. primary aerosols, were measured using a particle size analyser based on the Fraunhofer diffraction of a laser (model 2600c, Malvern Instruments, Malvern Worcestershire, UK). The sizer was equipped with a 63 mm lens focal length, which enabled the system to measure droplets with diameters included within the 1.2 to 118 µm range. The nebuliser tip was set at 30 mm from the lens and 15 mm from the laser beam centre.

## 2.6. Method validation

Method validation was performed according to Eurachem, 2014 (Magnusson & Örnemark, 2014). Precision was expressed as the percentage of the relative standard deviation (RSD).

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Quantification of TGs was tested using two calibration models: (a) linear, i.e. plotting peak areas versus TGs concentration; and, (b) double logarithmic (i.e., log A vs log m according to equation 1) (Megoulas & Koupparis, 2005).

log A = b log m + log a (Equation 1)

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where m is the concentration of the target analyte; A, the peak area and a and b are numerical coefficients that depend on concentration, nature and droplet size of analytes but also on gas and liquid flow rates.

Finally, the accuracy of the tested methods was expressed as the relative error percentage considering the TG actual concentration ($C_{actual}$) in the sample and the TG experimentally obtained concentration ($C_{calculated}$). (equation 2).

$$Error\ percentage = \frac{C_{actual} - C_{calculated}}{C_{actual}} \times 100 \text{ (Equation 2)}$$

~~where $C_{actual}$ is the TG actual concentration in the sample and $C_{calculated}$ is the experimentally obtained concentration.~~ Accuracy was also estimated from a recovery test adding to a~~. A~~n oil sample ~~was spiked with~~ known concentrations of OOO and LLL~~, which were initially present in the sample, and the recoveries were obtained using equation 3:~~

~~$$Recovery\ percentage = \frac{C_{Spk} - C_{Unspk}}{C_{ad}} \times 100$$ (Equation 3)~~

~~where $C_{Spk}$ is the concentration of the TG spiked sample, $C_{Unspk}$ is the concentration of the TG unspiked sample (both experimentally determined) and $C_{ad}$ is the concentration of standards added to the spiked sample.~~

*2.7 Statistical data analysis*

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Desirability function calculations were performed by using the Design-Expert trial version 7.0.0. (Stat-Ease, Minneapolis, USA). Calibration curves and regression coefficients were calculated using Microsoft Excel 2010 (Microsoft Corporation, Redmond, Washington, United States). A one-way analysis of variance (ANOVA) was applied to the TG concentration data; means were compared using the Tukey's test at the 5 per cent level of significance to know whether there were significant differences among the data obtained using conventional heating HPLC and MW-HPLC. This analysis was carried out using the IBM SPSS Statistics 23 (Armonk, New York, United States) software.

## 3. Results and Discussion

### *3.1 Optimisation of ELSD signal*

~~In the last decade, the Aerosol Charge Detector (CAD) or the Evaporative Light Scattering Detector (ELSD) has found its way into HPLC applications portfolio due to their compatibility with gradient elution separations, which is an important limitation of the refractive index detector (RID). Additionally, ELSD is more sensitive than RID although it gives a nonlinear calibration response particularly when organic solvents are used. This nonlinear relation between peak area and concentration is due to the variation of the particle size as a function of the analyte concentration (Righezza & Guiochon, 1988), the mobile phase flow rate and its physical properties, the type of nebuliser, nebulising gas velocity, the temperature of both the spray chamber and the drift tube and the analyte chemical structure. As, in general practice, nonlinear relationships are not desirable for quantification purposes, linearization can be approached by applying a logarithmic function as shown in equation 1.~~

~~When the ELSD is used, it is essential to optimise experimental variables such as the composition and temperature of the mobile phase, the nebulization gas flow rate and the temperatures of the drift tube and the spray chamber to obtain the greatest signal. In some of the published papers, a chemometric comparison of TGs profiles for different vegetable oils is made,~~

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In order to achieve appropriate~~good~~ results, the mobile phase should entirely evaporate from the aerosol while avoiding thermal decomposition of analytes (Megoulas & Koupparis, 2005). ~~As a starting point, T~~the ELSD employed in this application was a ~~-~~SofTA ELSD 300 S.~~ manual recommends employing~~ The flow of argon was kept fixed as this detector uses a special concentric flow nebulizer and the manual recommends to work at a constant flow of Argon (65 ± 5psi) to ensure a narrow droplet size distribution~~psi for a correct nebulization of the sample,.working~~ with temperatures lower than 65 °C for the spray chamber, whereas higher drift tube temperatures could~~should~~ be used. Because the boiling point of the two pure components of the mobile phase was~~as~~ 56.3 ºC for acetone and 81.6 ºC for acetonitrile, the spray chamber temperature was first set at 35 ºC while drift tube temperature was varied from 55 to 105 ºC. In these experiments, the peak area for OOO remained virtually unaltered regardless of the selected drift tube temperature. The mixture of acetonitrile and acetone is a non-azeotropic mixture, which means that, upon heating, the most volatile component boiled off before the least volatile one. Based on the obtained results, an intermediate drift tube temperature of 75 ºC was chosen to ensure complete evaporation of acetone thus avoiding variations in the composition of the aerosol interacting with the ELSD beam (~~L. E.~~ Magnusson, Risley, & Koropchak, 2015)~~(L. E. L. E. Magnusson, Risley, & Koropchak, 2015)~~.

Additionally, fixing~~using~~ 75 ºC for the drift tube, the spray chamber temperature was varied from 25 to 65 ºC. It was found that a 2.6-fold increase ~~of~~ in the spray chamber temperature led to a 3.6-fold signal (peak area) enhancement factor. This trend was probably due to enhanced aerosol solvent evaporation that promoted the transport of droplets through the chamber. Finally, 55 ºC was the selected temperature, because higher values of this variable led to a degradation of the signal-to-noise ratio.

### 3.2 Influence of the flow rate and temperature of the mobile phase

Experiments at flow rates from 0.6 to 1.4 mL $min^{-1}$ were carried out using an acetone/acetonitrile (65/35) mixture and the previously selected ELSD spray chamber and drift tube temperatures. The obtained peak areas showed no significant differences for OOO and LLL up to flow rates of 1-1.1 mL $min^{-1}$. At higher flow rates peak area slowly decreased. This trend was probably due to a higher loss of particles reaching the detector as they impacted against the walls of the spray chamber (Lecoeur et al., 2014). Therefore, 1 mL $min^{-1}$ was the selected flow rate.

Another important variable that could affect the nebulization process was the mobile phase temperature. Temperatures of room temperature, 30, 35 and 40 ºC were studied. For each experiment, the container with the solvent was kept in a thermostated water bath. Progressive enhancement in signal-to-noise ratio was observed as the temperature of the mobile phase increased. The increase in the peak area with respect to room temperature was 0, 35 and 45 % at 30, 35 and 40 ºC, respectively.

To test whether a slight mobile phase heating had an impact on the aerosol characteristics, the aerosol generated by the nebulizer was measured. The median of the aerosol volume drop size distribution did not change significantly as the mobile phase temperature went up (*i.e.*, 7.77 ± 0.04; 7.75 ± 0.04; and 7.71 ± 0.02 µm at 30, 35 and 40ºC, respectively). Therefore, the differences in signal-to-noise ratio mentioned above should be due to the increase in the number of particles reaching the detector during their transport to the detector.

### *3.3. Selection of the HPLC column*

Once the best ELSD experimental conditions were selected, the TGs separation was evaluated using three different octadecylsilyl silica columns with 5 µm stationary phase particle diameters: Luna C18 (4.6 mm ID x 250 mm), Inert Sustain C18 (4.6 mm ID x 100 mm) and InertSil ODS-2 (4.6 mm ID x 250 mm). The mobile phase was the same as used for optimising the ELSD response, whereas the flow rate was 1 mL $min^{-1}$, the amount of sample injected was 10 µL and the

temperature of the column was set at 30 ºC. Soybean oil was used as a reference sample to identify the peaks in the chromatogram as suggested by reference (European Commission, 2008). The criteria used to evaluate the chromatographic performance of each column was based on obtaining the number of theoretical plates (N), the separation index (SI), the resolution (Rs) for the critical peaks pairs (OLL/PLL), (POL+SLL/OOL), and the total number of peaks (TNP) detected.

According to the data obtained, the column which gave the best resolution and separation index of peaks (OLL/PLL) and (POL+SLL/OOL) was the Inertsil ODS-2. Meanwhile, Luna C18 provided higher N values. Therefore, the Derringer´s desirability function was used to objectively make a selection of the column (Lesellier, Latos, & de Oliveira, 2014). According to this methodology, several variables were combined into a function (D) (Derringer & Suich, 1980) that involved the transformation of each criterion to a desirability value d, where $0 \leq d \leq 1$, being 0 an utterly undesirable value and 1 an utterly desirable value. Next, the individual desirability's values were combined using the geometric mean, which gave the overall desirability (D) of the combined response levels (Dean & Lewis, 2006). For example, considering the resolution (Rs) between peaks POL+SLL/OOL, the d value was 1 for Inertsil ODS-2 (best resolution), whereas it was 0 for InertSustainC18 which showed the worst Rs. Meanwhile, the value for Luna C18 was 0.217. Finally, the column which showed the highest value of D was the Inertsil C18.

### *3.4 Optimisation of the MW-HPLC-ELSD system for separation of TGs*

~~So far, microwave assisted HPLC has been successfully applied for vitamin separation using a polar mobile phase containing water (Terol et al., 2012), (Silvia Carballo et al., 2015).~~ Once the detection conditions and the column were selected, experiments were done in the presence of a microwave field for a fully organic mobile phase. All measurements were carried out at 170 W, the minimum microwave power that allowed the device employed. This MW power was selected to avoid both excessive heating of the mobile phase and components of the sample ~~column~~ that could damage the stationary phase and ~~the~~ overlapping of some chromatographic peaks due to the complex profiles of TGs in vegetable oils. For the acetone: acetonitrile mobile phase, stable

chromatographic conditions were quickly reached after the first chromatographic run. These facts can be appreciated in Figure 1a where the system pressure during the first injection is presented as a discontinuous line. The starting pressure was around 7.3 MPa and during the first 30 minutes irradiating the column, the pressure system suffered a slight drop. After that time the pressured stabilized at about 6.5 MPa and kept steady during the successive injections of samples as shown in Figure 1b ~~(Figures 1a and 1b)~~. This was likely due to the low specific heat of both solvents. Furthermore, stable retentions times were also observed after the first chromatographic run (Figure 1b). This fact indicates that equilibrium conditions should have been reached inside of the HPLC column. Previous studies using microwave radiation to assist an HPLC separation also showed a similar ~~this~~ trend (Carballo et al., 2015; Terol et al., 2012). It should be mentioned that, although the MW radiation was quickly absorbed by the mobile phase, moderate heating inside of the column was produced in this application. A measurement of the mobile phase temperature at the exit of the column was attempted but a considerable fluctuance of it was observed being the maximum registered temperature ~~. In fact, the reached temperature at the exit of the column was~~ 32 ±1 ~~± 1 º~~ºC. This difficulty could be explained on the basis that the main component of the mobile phase (acetone) is a volatile solvent so it absorbs much heat when evaporating producing a cooling of the solvent at the exit of the column.

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Additionally, in order to verify the impact of the microwave radiation on the optimum ELSD operating conditions, the spray chamber temperature was varied, Figure 2. The average noise when the spray chamber temperature was set at 35, 45, 55 and 65 ºC was 0.9, 2.8, 1.3, and 10.3 mV, respectively. Therefore, in agreement with the studies described in section 3.1.2, 55 ºC was the selected spray chamber temperature. A study of the influence of the mobile phase flow rate was also done for MW-HPLC and the highest signal to noise ratio was also found at 1 mL $min^{-1}$. Therefore, it was concluded that the use of an MW-HPLC system at 170 W prior to the ELSD did not affect the detector operating conditions suitable for TGs analysis.

### *3.5* Comparison of chromatographic TGs separation obtained with conventional heating HPLC-ELSD and MW-HPLC-ELSD

In this study, the column was conditioned at three different temperatures using a conventional HPLC oven (room temperature, 30 and 40 ºC) and a mixture of 7 TGs was injected. As expected, shorter retention times were obtained as the temperature went up (Figure 3). This effect was especially significant for the most retained triglycerides in the column. Increasing column temperature usually speeds up the HPLC analysis due to a reduction in the viscosity of the mobile phase, which increases the analyte diffusivity (Teutenberg, 2009). Interestingly retention times obtained when using MW-HPLC were very similar to those obtained for HPLC at 40 º C. Both methods allowed decreasing the analysis time by around 50 % as compared to the separation at room temperature (see Figure 3). The maximum temperature registered of the mobile phase at the exit of the column was 32 ~~± 1~~ ºC when the microwave was employed and about 35 ºC ~~± 1 ºC~~when ~~for~~ the conventional oven set at 40 ºC, but as commented above there were fluctuations on the mobile phase temperature of nearly 2 ºC at the exit of the column due to the volatilization of acetone. Even though the difficulties in measuring the temperature it seems that the temperature of the mobile phase when the column was heated with a conventional oven at 40 ºC was slightly higher than the temperature found when MW radiation was employed. ~~C.~~ Therefore, the present study suggested that microwave radiation did influence the analyte kinetic diffusion and repartition processes as a virtually transparent mobile phase was selected for TGs separation in comparison to other mobile phases with higher polarity. A possible explanation could be based on the effect of the oscillating field on the preferential orientation of the analyte molecules with respect to the stationary phase that could promote the rapid establishment of the partition equilibria of TGs between the mobile and stationary phases.

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In an earlier application where MW radiation was applied at a power of 170 W to assist the separation of water-soluble vitamins in a C18 column using a polar mobile phase consisting of a mixture of 0.05 M perchloric acid and acetonitrile/water (90/10, v/v) a 30 and 50 % reduction in retention times was obtained for the last peaks eluted (riboflavin and biotin), respectively, in comparison to the elution at room temperature. In that case, an increase in the temperature of the mobile phase by nearly 20 ºC was observed. This significant heating was due to the composition of

the mobile phase. Additionally, a 40 and 65 % reduction in peak width was registered for riboflavin and biotin, respectively (Terol et al., 2012)~~(Terol et al., 2012a)~~. In a different application involving separation of fat-soluble vitamins, the application of MW radiation at 170 W resulted in a 36 % shortening in analysis time with respect to conventional HPLC. Furthermore, peak height increased by nearly 100 % and band broadening decreased by 51 % for tocopherol homologues (Carballo et al., 2015). In that~~is~~ application, the mobile phase consisted of an acetonitrile/tetrahydrofuran/water (87.5/ 5 /7.5 v/v/v) mixture. The mobile phase temperature at the exit of the column was 32 ± 1 ºC. It was verified that, because of~~of~~ the absence of ions in the mobile phase, the increase in its temperature was considerably lower than in the former application.

However, in the present work, even though the application of microwave radiation to the column induced a reduction in retention times similar to conventional heating at 40 ºC with respect to HPLC at room temperature, the obtained peak widths were not reduced accordingly~~similar for all four tested situations~~. The obtained results suggested that peak width was slightly lower for HPLC at 40 ºC than for MW-HPLC and similar to that obtained when working at 30 ºC (Table 1). This effect was not expected considering the previous studies in which MW-HPLC usually produced narrower peaks than conventional heating in HPLC (~~W. A.~~ Galinada & Guiochon, 2005; Terol et al., 2012). That phenomenon was explained mainly base in a more homogeneous distribution of the heat inside of the HPLC column.~~(Terol, Maestre, Prats, & Todolí, 2012a).~~

Although MW favoured the intraparticle diffusion of analytes and directly narrowed peaks, this beneficial effect could be blurred (Galinada & Guiochon, 2005) depending on the nature of the mobile phase and analytes. As the column was inside the MW oven, the so-called “hot spots” could appear inside the column during MW irradiation where the mobile phase was overheated and probably partially evaporated causing local flow rate changes (thermal runaway instability). This phenomenon could promote the dispersion of the peaks (Jerby, 2017). The presence of a solvent in the mobile phase with a low boiling point and a high loss factor could partially account for this behaviour that was not observed in other studies before where the mobile phase did not contain volatile solvents (Galinada et al., 2005), (Stone & Taylor, 2003). Consequently, even though a lower apparent separation efficiency could be found with MW-HPLC with respect to conventional

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heating at 40 ºC, it was better than room temperature (25-26 ºC) and ~~still~~ similar to that obtained at 30ºC, with the advantage that the retention times were shorter.

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### *3.6 Validation of an MW-HPLC-ELSD method for the determination of TGs*

3.6.1 Comparison of sensitivity and linearity of the calibration with conventional heating and with MW radiation

Although the ELSD is considered as a universal detector, it is known that its response depends on several variables previously mentioned~~including physicochemical properties of the analytes, concentration and interaction with the stationary phase.~~. Entirely different response factors were obtained depending on both the type of TGs determined and the way the column was conditioned. Table 1 shows a comparison of the response factors (RF) under~~for~~ the assayed chromatographic conditions. The lowest RF values were obtained at room temperature whereas both conventional heating and MW-HPLC provided higher values of these parameters. It was also observed that for every TG there was a specific response that varied in the working range of concentrations (Holčapek, Lísa, Jandera, & Kabátová, 2005). Additionally, it was found that RF decreased as retention time increased except for LLL and in a smaller extent for OOO. For both TGs lower RF were obtained than expected according to their retention times.

Due to the differences in the response factor among the TGs studied, mainly for those that eluted later in the chromatogram, it was necessary to obtain a calibration line for each ~~particular compound~~compound. Therefore, the linearity of the response was studied to select the best quantification method. With that purpose, a minimum of four ~~or five~~ standards with concentrations between 10 and 120 mg $L^{-1}$ were analysed and the corresponding linear regression equations and correlation coefficients ($R^2$) were calculated.

As expected, when using ELSD the peak areas varied non linearly with the analyte concentration in most of the cases (Mathews et al., 2004). This was due to the occurrence of different detector beam scattering mechanisms as a function of the concentration. This effect was

especially marked for the TGs eluting later in the chromatograms and for concentrations higher than 80 mg $L^{-1}$. Consequently, after applying a simple linear curve fit good $R^2$ values (*i.e.*, higher than 0.99) were obtained only for CyCyCy and CCC, working at 30, 40 ºC and with MW in the range of concentration from 10-80 mg $L^{-1}$. Table 2 shows the calibration coefficients for MW-HPLC experiments.

The TGs response was successfully linearly modelled by applying a double logarithmic regression in a range of concentrations between 10 and 120 mg $L^{-1}$ ~~-~~for all conditions employed. The constants of equation 1, i.e. log a and b, are shown in Figure 4. Log ~~a~~ values (*i.e.*, the intercept of the log-log regression curve) were all negative. A higher positive log a indicates that a higher amount of particles interacted with the ELSD light beam and induced light scattering (Lesellier, Valarché, West, & Dreux, 2012). Taking into account the obtained results, it emerged that the increase in the temperature of the mobile phase improved the transport of all particles to the detector. Moreover, ~~and consequently~~ the variability of the log a values as a function of the TGs nature diminished~~was~~ ~~lower~~ at 40 ºC and using microwave radiation. ~~than at room temperature and at 30 ºC. At these temperatures, for saturated TGs, the higher the retention time, the higher the log a value.~~ However, the structure of the components and number of unsaturation present in the fatty acids may have an influence especially for elution at 30 ºC and at room temperature. ~~-~~ This effect was evident for LLL, except when working at room temperature (Figure 4). It seems that unsaturations could have an important effect on both the retention time and the dispersion of the laser light in the detector and hence on the analytical signal.

Regarding the slope of the curves (*i.e.*, b values), ~~it should be pointed out that a~~a decrease and homogenization of this magnitude was found when the column was conditioned at 40 ºC and with MW-HPLC. These results suggested that, for these two situations, the size of particles that intercepted the ELSD beam became independent of the chemical nature of the analyte. This is a positive effect as for a certain range of concentration a unique b value could be assumed for all TGs and so a dequantitated determination should be done without the need of obtaining all calibration equations.

Finally, the sensitivity (S) of the quantification method based on log-log calibration is given by:

S = a X b (Mitchell, Bao, Benz, & Zhang, 2009) (equation 3) ~~equation 4~~

A comparison of the S values obtained for seven TGs included in the study at different column temperatures with conventional HPLC and with MW-HPLC is supplied as supplementary table 1. In general, sensitivities were higher working at 40 ºC followed by the sensitivities obtained with MW radiation. This result is more significant for TGs that elute late in the chromatogram. Moreover, it is also remarkable to indicate that the sensitivity of saturated TGs gradually decreased as the number of carbons increased whereas the dispersion of the peaks followed the opposite trend (see Table 1). Holcapek et al. described similar trends, only for the ELSD, since for other detectors such as an ultraviolet absorption spectrophotometer or a mass spectrometer (MS), the sensitivity was not dependent on the TGs fatty acid saturation degree (Holčapek et al., 2005).

3.6.2 MW-HPLC precision and accuracy

The precision regarding retention times, peak heights and areas was estimated according to both the repeatability and the intermediate precision (Table 2). The repeatability was calculated considering the chromatographic parameters obtained after analysing a TG standard five times in the same day, while the intermediate precision was obtained analysing the same standard three times a day in five different days over a total of ten days. Both the repeatability and intermediate precision were acceptable as, for all analytes, their values were lower than 5 and 6%, respectively. PPP was an exception to this rule because it showed a precision (RSD) of nearly 15 % in terms of peak area. The worse repeatability of PPP was due to the higher dispersion of the generated peak as it eluted at rather long retention times.

Additionally, the accuracy was assessed in two different ways. Initially, by analysing a mixture of TGs of known concentrations and comparing the calculated concentration value with the actual value as error percentage (bias) according to equation 2. Table 2 compares the error percentage for some TGs when linear calibration and double logarithm calibration was used. Linear calibration led to an underestimation in the concentration for most of the TGs at intermediate concentrations and overestimation for analytes present at the lowest concentrations

(< 10 mg $L^{-1}$). Double logarithm regression allowed an adequate quantification of the TGs in all the range of concentrations here considered (i.e., error percentage < 4 %). Finally, a recovery test was performed by spiking a solution of argan oil with 25 and 60 mg $L^{-1}$ of LLL and OOO, respectively. The recoveries obtained when using the double logarithm model were comprised in the acceptable range of 80-110 % (see table 2) (Bressolle, Bromet-Petit, & Audran, 1996).

### *3.7 TGs quantification in vegetable oils*

The optimised MW-HPLC-ELSD method was applied to the determination of TGs in vegetable oils. Supplementary figure 2~~1~~ shows, as an example, the comparison of chromatograms obtained for an almond oil eluted at 30 ºC and with microwave radiation. An important reduction in the retention time was obtained applying MW radiation where the resolution of the peaks was still acceptable for quantification purposes. OOO and LLL triacylglycerols were quantified in the samples using linear regression, and double logarithm regression. The determination of these two triacylglycerols in vegetable oils is quite interesting as these TGs are constituted by unsaturated fatty acids that are relevant in healthy diets. (Ursoniu, Sahebkar, Serban, & Banach, 2018).

It is interesting to note that in all the cases the average concentrations for both TGs obtained with linear regression were significantly different with a probability of 95% to the average values obtained using a double logarithm model. More specifically, the concentration obtained for an argan oil expressed as average value ± standard deviation (n=3) for OOO was 133 ± 5 and 155 ± 5 mg $g^{-1}$ oil, for linear, and double logarithm calibrations, respectively. The corresponding LLL concentration was: 96 ± 2 and 113 ± 2 mg $g^{-1}$ oil. Additionally, the respective values obtained for LLL in an almond oil were 35 ± 3 and 25 ± 3 mg $g^{-1}$ oil. Meanwhile, the content in OOO was 370 ± 6 and 319 ± 4 mg $g^{-1}$ in almond oil and 356 ± 22 and 309 ± 16 mg $g^{-1}$ of tiger nut oil, respectively. It could be observed that the linear calibration led to different concentrations than those provided by non-linear methods. This result proved that double logarithm calibration was appropriate in a broader concentration range thus minimising sample dilution.

As there is a great number of TGs in oils and the number of individual standards is limited and expensive, the most frequent quantification method is the peak area percentage. In this way, some studies have been published using the relation of areas of some chromatographic peaks to detect adulterations in oils (Ursoniu et al., 2018), (Ping, Aziz, & Idris, 2018) (*e.g.*, argan oil with olive oil (Cunha & Oliveira, 2006)). Unfortunately, only in very few articles, the concentration of some of the TGs present has been given (Heron et al., 2010), (Héron, Maloumbi, Dreux, Verette, & Tchapla, 2006), (Wei, Hu, Lv, Dong, & Chen, 2015). Due to the lack of available quantitative data, it was not possible to compare the argan oil and tiger nut oil TGs content with literature data as they were expressed as peak area percentage in all the cases. Almond oil TGs were studied by Holcapek et al. using HPLC with APCI-MS detection and they found a value of 24 mg of LLL $g^{-1}$ and 213 mg of OOO $g^{-1}$ almond oil (Holčapek et al., 2005). The value of OOO was lower than the value found in the present work, but it was not surprising as it has been claimed that the fatty acid content varies depending on almond cultivars and agricultural practices (Grane-Teruel, Prats-Moya, Berenguer-Navarro, & Martin-Carratala, 2001).

## 4. Conclusions

This work has demonstrated the usefulness of the MW-HPLC-ELSD technique to shorten the analysis time for the determination of TGs as compared to conventional HPLC-ELSD. Even though that the differences in the mobile phase temperature are not very important in any of the conditions assayed results suggest that additionally to the thermal effect of microwave radiation and additional effect related to partition equilibria of the analytes between the stationary and the mobile phase is produced that allow reducing the TGs retention times similar to the elution with conventional oven at 40 ºC. ~~The results also suggest that microwave radiation may directly affect the established partition equilibria of the analytes between the stationary and mobile phases.~~ In this application, it was not possible to change the mobile phase nature as the separation of compounds was worse. But in an upcoming study another separation should be chosen that allows the employment of a completely organic mobile phase but with not so low boiling point and maybe

a lower loss factor as acetone. That hypothetical mobile phase would allow obtaining additionally to a reduction the retention time an improvement on sensitivity.

~~The achieved data also reveal that additional fundamental studies are required to unravel de mechanisms responsible for shortening retention times in the presence of a microwave field. On this subject, alternative non-polar organic mobile phases with no acetone should be assayed in the future so as to minimize the likelihood for hot spot generation inside the column. Note that the heat transfer when using microwave radiation depends on the solvent and not only on the analytes dielectric constants but also on the dissipation factor, the relaxation time and the boiling point.~~

Finally, s~~S~~ince MW-HPLC affords good results in terms of retention times and sensitivity at fairly low temperatures, it could be considered as a potential technique to separate thermolabile compounds when a completely organic mobile phase has to be used. ~~.~~

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Ms. Ref. No.: FOODCHEM-D-19-00281
Title: Microwave assisted high performance liquid chromatography for the separation of triacylglycerols in vegetable oils using an evaporative light scattering detector Food Chemistry

Dear Dr Pegg,

Thank you for the opportunity to revise our manuscript especially after the difficulties in finding reviewers. Bellow, we attach a response to the comments of the two reviewers.

**Answer to Reviewer #2:**

Abstract:
Line 1 of abstract: change "microwave radiation" by "microwave (MW) radiation".
Ok, thank you
I think the names of the oils analyzed should be included in the summary (for example on line 9 of the abstract).
Yes, you are right, we have included them.

Introduction:
In general, the introduction seems too long and includes concepts too basic, so it should be summarized.
We have shortened it trying to eliminate those things that are not necessary as they are related to microwave radiation properties. The changes included in the document can be seen with the tool track changes in the WORD processor.
Line 7: references are not well cited in the text. I think that you should review the rest of the references cited in the text because there are enough errors.
Line 27: delete the name of the first author.
Thank you, we have modified references accordingly.

Materials and methods:
Sections 2.1 and 2.2 should be reorganized. In section 2.1 would be Reagents and solutions (lines 65-74 and 85-87) and in section 2.2 will be samples and sample preparation (lines 75-81 and 88-93).
Ok, both sections have been reorganized as suggested.

Line 141: reference is not well cited in the text. OK, we have corrected it.

Lines 150-156: You should reorganize the text in the following way: "Finally, the accuracy of the tested methods was expressed as the relative error percentage considering the TG actual concentration in the sample and the TG experimentally obtained concentration".
Lines 157-164: it should be removed from ", and the recoveries ..." until the end (line 164).
We agree with your suggestion of reorganization of the text. Thank you.

Results and discussion:
lines 181-199 and 267-268: I think that these paragraphs should be moved to the introduction section.
This information has been resumed and translated to introduction.

Line 392: rename this equation. OK, it has been renamed to equation 3.
Table 2: the units (mg/L) are represented differently than in the text.
Ok, Changes have been done.
Section 3.4 Optimisation of the…: in the text does not explain all the data contained in Figure 1a and 1b, please clarify this.
- because you include the pressure in the figure? What was the best irradiating injection?
We have tried to explain better figure 1 (lines 253-262). After the first injections, the retentions times were stable so only the first injection would have to be discarded.

- Figure 1b: in the figure foot should indicate which line of the two drawn corresponds to the second and the third injection irradiating Figure 3. In this figure, the important is to see how the retention times decrease, so you should remove the scale because it does not correspond to the heights of the peaks.

You are right the Y-axis scale has been deleted.

**Answer to reviewer 1**

We agree with R(1) that a lot of works can be found in the bibliography in relation to the determination of triacylglycerols in oils, most of them are applications of a previously established method to a great number of samples. But authors do not agree with the assertion that the work lacks novelty. In the manuscript herein considered, the aim of the work was centred in two main things which we think they add novelty to the work: a study of the best experimental conditions for obtaining the most convenient S/N response and also the study of the influence of MW radiation on the separation process.

In the optimization of the ELSD conditions several variables related to the detection were considered but the gas supply was maintain fixed as at least in the model that we used is not easy to change flow and the manual of the equip says that: “the 300 S ELSD uses a special concentric flow nebulizer and a constant flow (65 psi) of inert gas to ensure a narrow droplet size distribution” (https://www.teledyneisco.com/en-us/liquidChromatography/Chromatography%20Documents/Manuals/SofTA%20300S%20ELSD

%20User%20Manual.pdf). It is true that this was not explained in the text. An explanation of it has been included in lines 175-177 (yellow) in the text.

The second aim of our work was centred in assaying the behaviour of the application of microwave radiation to assist the separation of non-polar compounds with an organic mobile phase. You are right that maybe the assembly of the MW-HPLC system is not clear and we are going to include a figure with a description of it as supplementary material (Supplementary Figure 1, line 110). It is also true that implementing an MW-HPLC system has the disadvantage that the hardware of the HPLC column should be of peek and not of stainless steel as it is more usual but on the other hand, it is also true that alternatives to conventional heating in HPLC are interesting and using a conventional microwave oven could be cheaper and more efficient energetically than using a convention HPLC oven. This will depend on the application in which it is employed and for that reason in this work we assayed a different situation in which a totally organic mobile phase was used. This application was not published before for a complete separation of compounds and the information should be of considerable importance for the chromatographic community.

For this application, the retention times obtained applying the minimum MW power of 170 W were very similar to the retention times obtained when a conventional oven at 40 ºC was employed. But even though the increase in the mobile phase temperature was moderate the reduction in retention times was important. We have been trying to measure the temperature of the mobile phase at the exit of the column to compare it but we found some difficulties as it fluctuates several degrees along with the measurement. This difficulty could be explained on the basis that the main component of the mobile phase (acetone) is a volatile solvent so it absorbs much heat when evaporating. Despite these fluctuations, it was observed that the temperature of the mobile phase at the exit of the column shows approximatively a difference of about 2 $^{o}$C when MW-HPLC was used in comparison with conventional HPLC at 40 ºC. Considering that in this application the differences in the mobile phase temperature are low among all the experiences done an additional influence on the interaction of

the TGs with the stationary phase could happen. A clarification of this phenomenon in the manuscript has been included in lines 285-288.

It is true that the sensitivity obtained for TGs was not improved using MW-HPLC for this application as happened in other applications where the mobile phase contained water and the reason should be in the properties of the components of the mobile phase, especially on acetone due to its low boiling point but the work demonstrates that for other application in which a non-volatile mobile phase can be used the separation can be improved using MW-HPLC with the advantage that as the temperatures of work are moderate the stationary phase can be kept safe.

***Response to Reviewers**

**Table 1.** Comparison of width at half peak ($W_{0,5}$) and retention factors (RF) for seven triglycerides eluted under HPLC-ELSD at room temperature, 30 ºC and 40 ºC and using MW-HPLC-ELSD at 170 W.

| | **Room T** | | **HPLC 30°C** | | **HPLC 40°C** | | **MW-HPLC** | |
|---|---|---|---|---|---|---|---|---|
| | *RF* | $w_{0.5}$ | *RF* | $w_{0.5}$ | *RF* | $w_{0.5}$ | *RF* | $w_{0.5}$ |
| **CyCyCy** | 0.66 ± 0.01 | 0.093 ± 0.005 | 0.9 ± 0.2 | 0.084 ± 0.001 | 0.8 ± 0.3 | 0.086 ± 0.006 | 0.71 ± 0.04 | 0.096 ± 0.006 |
| **CCC** | 0.596 ± 0.005 | 0.122 ± 0.002 | 0.8 ± 0.2 | 0.106 ± 0.004 | 0.8 ± 0.1 | 0.10 ± 0.01 | 0.71 ± 0.07 | 0.112 ± 0.001 |
| **LaLaLa** | 0.50 ± 0.02 | 0.187 ± 0.002 | 0.6 ± 0.1 | 0.15 ± 0.01 | 0.78 ± 0.07 | 0.125 ± 0.006 | 0.6 ± 0.01 | 0.16 ± 0.01 |
| **LLL** | 0.17 ± 0.02 | 0.266 ± 0.007 | 0.21 ± 0.05 | 0.204 ± 0.002 | 0.27 ± 0.02 | 0.163 ± 0.006 | 0.21 ± 0.02 | 0.22 ± 0.01 |
| **MMM** | 0.270 ± 0.001 | 0.32 ± 0.01 | 0.39 ± 0.07 | 0.235 ± 0.002 | 0.46 ± 0.02 | 0.19 ± 0.02 | 0.36 ± 0.02 | 0.26 ± 0.04 |
| **OOO** | 0.147 ± 0.009 | 0.49 ± 0.02 | 0.32 ± 0.08 | 0.36 ± 0.04 | 0.29 ± 0.03 | 0.28 ± 0.03 | 0.22 ± 0.02 | 0.39 ± 0.03 |
| **PPP** | 0.152 ± 0.009 | 0.59 ± 0.05 | 0.17 ± 0.07 | 0.40 ± 0.04 | 0.30 ± 0.01 | 0.32 ± 0.07 | 0.24 ± 0.03 | 0.43 ± 0.07 |

**Table 2.** Repeatability, intermediate precision, accuracy and error observed when using different fitting data models for quantification purposes with MW-HPLC-ELSD.

| | | CyCyCy | CCC | LaLaLa | LLL | MMM | OOO | PPP |
|---|---|---|---|---|---|---|---|---|
| Calibration range (mg/L) | | 10 - 80 | 10 - 80 | 10 – 80 | 10 - 120 | 10 - 120 | 10 - 120 | 10 - 120 |
| n | | 4 | 4 | 4 | 5 | 5 | 5 | 5 |
| $R^2$ linear fitting | | 0.994 | 0.990 | 0.987 | 0.974 | 0.980 | 0.975 | 0.979 |
| $R^2$ double-log fitting | | 0.997 | 0.999 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |
| **Repeatability[a] (% RSD)** | | | | | | | | |
| Time | | 0.1 | 0.1 | 0.1 | 0.2 | 0.3 | 0.6 | 0.7 |
| Area | | 2.1 | 4.0 | 0.9 | 3.0 | 2.1 | 3.1 | 4.8 |
| Height | | 2.0 | 4.1 | 3.2 | 1.4 | 5.7 | 1.2 | 1.0 |
| **Intermediate precision[b] (% RSD)** | | | | | | | | |
| Time | | 0.3 | 0.6 | 1.2 | 1.9 | 1.5 | 2.4 | 2.9 |
| Area | | 3.5 | 2.7 | 5.0 | 3.9 | 1.8 | 4.4 | 14.7 |
| Height | | 3.1 | 5.6 | 2.6 | 2.6 | 3.4 | 0.8 | 25.6 |
| **Accuracy** | | | | | | | | |
| Amount added (mg/L) | | - | - | - | 25.0 | - | 60.0 | - |
| % Recovery | | | | | 114 ± 11 | - | 93.5 ± 0.8 | |
| **Error (%)** | | | | | | | | |
| *10 mg $L^{-1}$* | Linear fitting | 26 | 30 | 36 | 68 | 65 | 66 | 62 |
| | Double-log fitting | -3.8 | -2.5 | -1.0 | -0.2 | -0.6 | 1.3 | -1.2 |
| *40 mg $L^{-1}$* | Linear fitting | -7.5 | -9.9 | -10.9 | -16.3 | -15.9 | -14.2 | -13.8 |
| | Double-log fitting | 4.1 | 1.8 | 2.5 | -1.8 | -0.1 | 0.4 | 2.0 |
| *80/120 mg $L^{-1}$* | Linear fitting | 1.8 | 2.3 | 2.6 | 6.1 | 5.2 | 6.1 | 5.4 |
| | Double-log fitting | -3.9 | -2.2 | -1.7 | 1.8 | -0.1 | 2.0 | -0.1 |

[a]: Repeatability was calculated for n=3.

[b]: Intermediate precision was obtained after three consecutive injections per day, two days in a row.

[c]: Recovery has been calculated as the mean for n=3 ± $\sqrt{n}$ where t is t student at a 95% confidence level and s the standard deviation.

## Figures

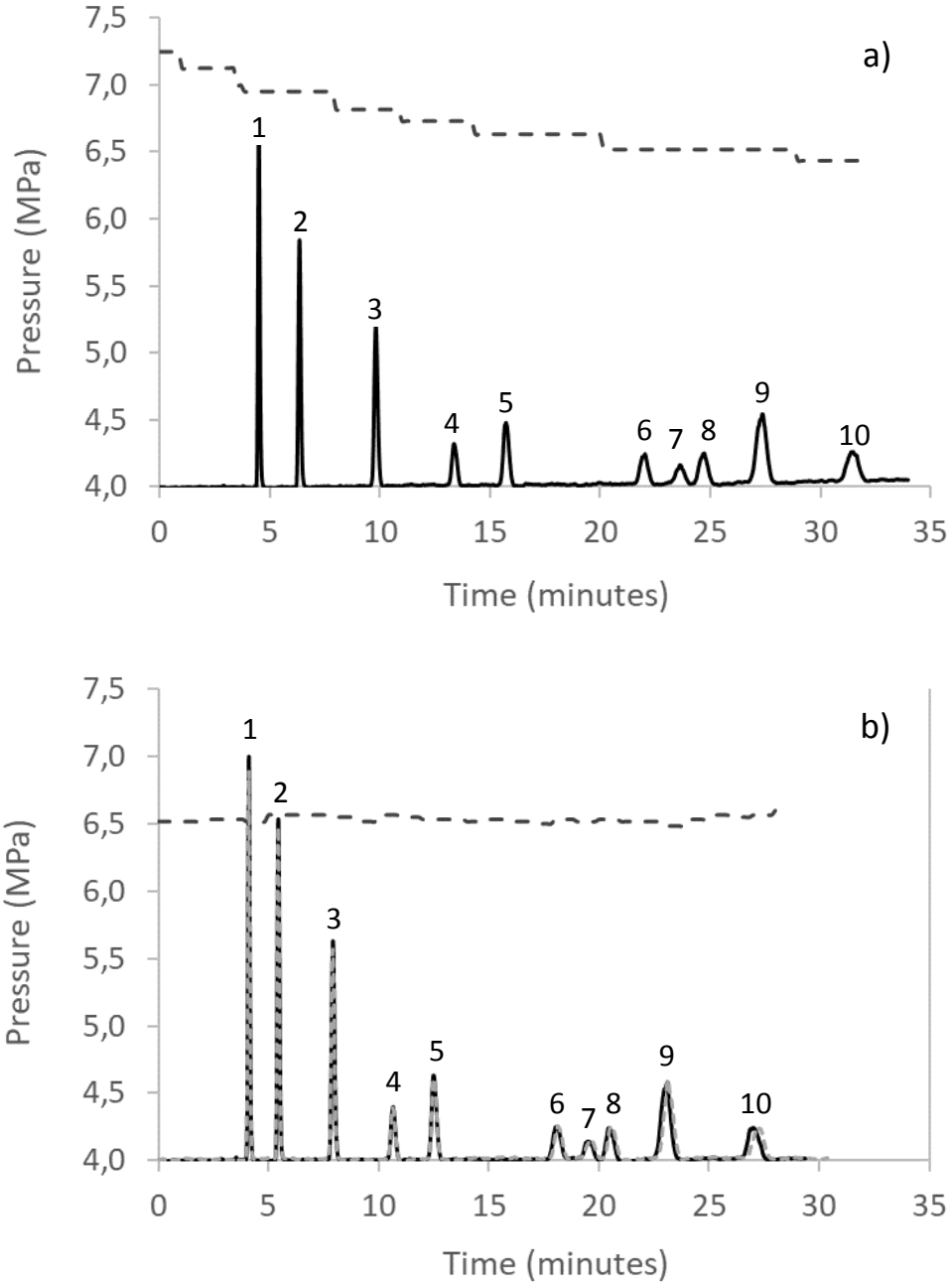



**Figure 1**. MW-HPLC chromatograms obtained after (a) the first injection irradiating the column at the minimum power of 170 W; and (b) obtained after the second (dark continuous line) and third injection (discontinuous line) irradiating the column for a mixture of ten triglycerides. Changes on system pressure are presented in black dashed lines. (1) CyCyCy, (2) CCC, (3) LaLaLa, (4) LLL, (5) MMM, (6) OOO, (7) POP, (8) PPP, (9) POS and (10) SOS.

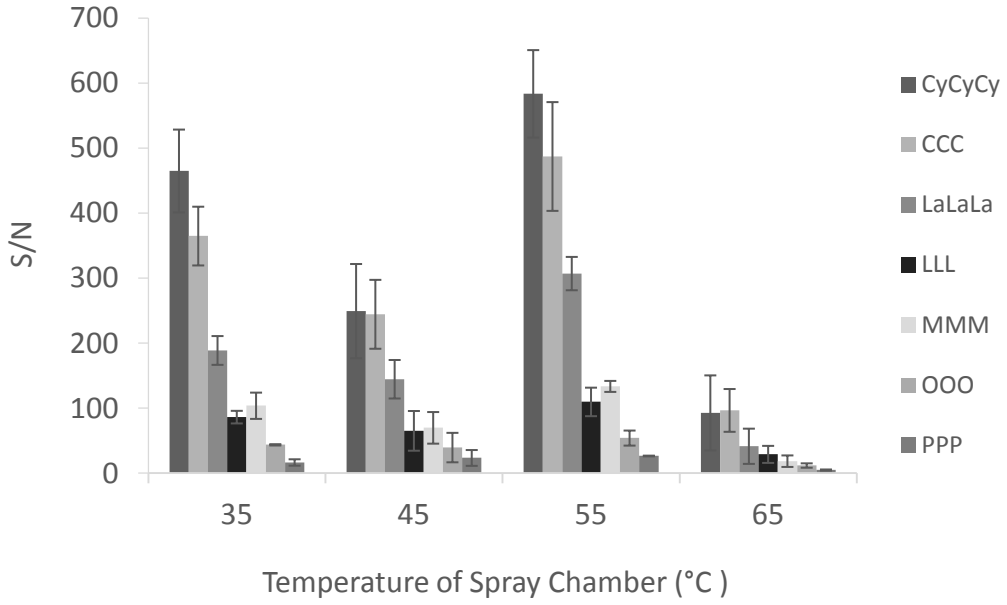



**Figure 2.** Signal to noise (S/N) relation calculated at different ELSD spray chamber temperatures with fixed drift temperature at 75 ºC for several triglycerides when a sample of sunflower oil was injected under the chromatographic conditions selected. Error bars reflect the 95 % confidence interval for n=3.

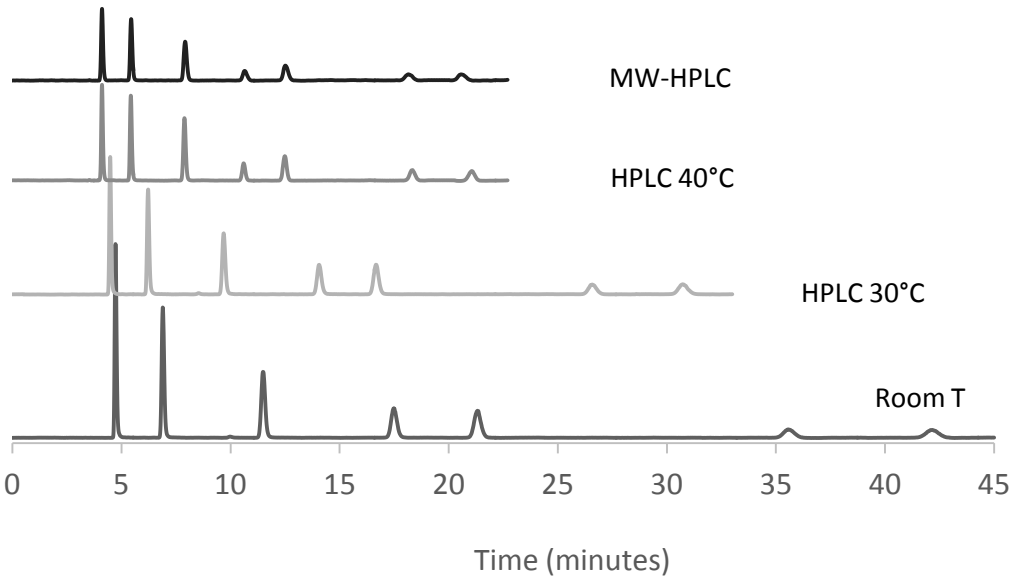
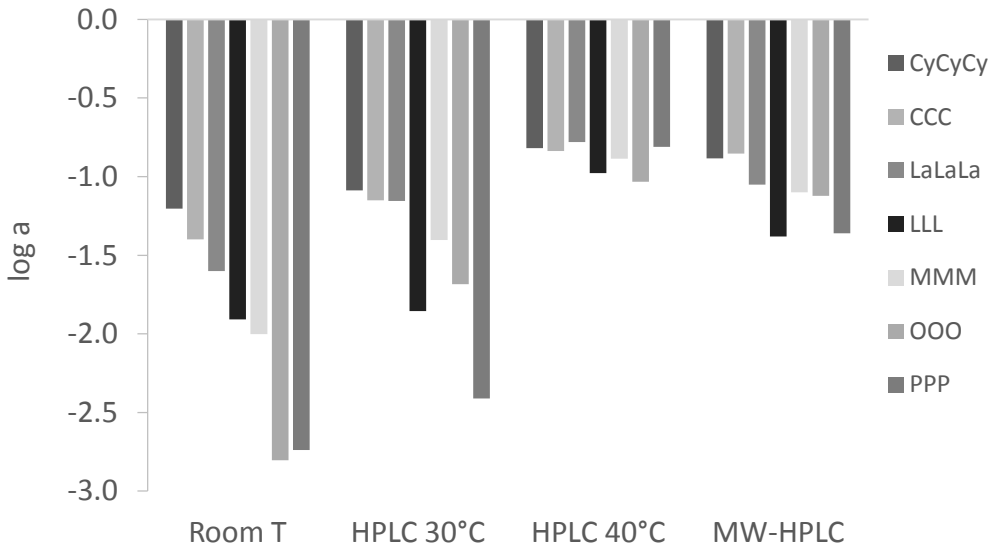
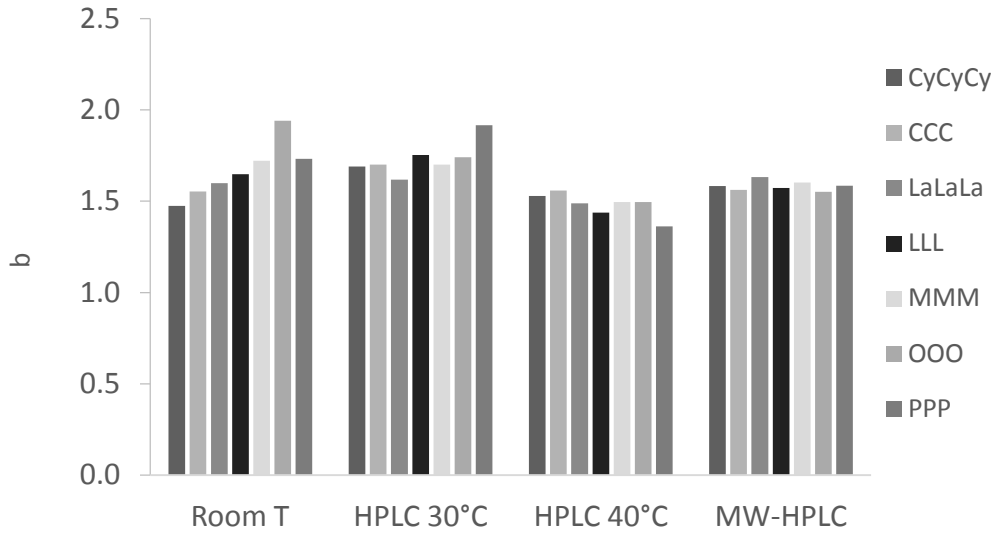



**Figure 3**. Chromatograms obtained under different elution conditions.




(a)




(b)

**Figure 4**. Comparison log a (a) and b (b) values obtained for seven TGs under different elution conditions.

**Supplementary Material**

**Click here to download Supplementary Material: Supplementary figure 1.pptx**

**Supplementary Material**

**Click here to download Supplementary Material: Supplementary figure 2.docx**

**Supplementary Material**

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*Declaration of Interest Statement

**Declaration of interests**

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

PRATS MOYA MARIA SOLEDAD - 41448684Q

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Highlights:

1. A novel high performance liquid chromatography method assisted by a microwave field (MW-HPLC) has been successfully applied for the first time to the determination of triacylglycerols using an evaporative light scattering detector (ELSD).
2. The retention times achieved with MW-HPLC have been shortened with respect to those reported for a conventional HPLC method even when using a mobile phase with low polarity. This fact has suggested that the use of microwave radiation affects directly the analyte partition equilibrium between the mobile and stationary phases.
3. An ELSD under optimised conditions is perfectly indicated as a detector for MW-HPLC