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Title: Appraisal of the suitability of two-stage extraction process by combining compressed fluid technologies of polar lipid fractions from chia seed

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Abstract

Although triacylglycerols (TAG) are the major constituents of chia oil, it also contains minor lipid fractions that include phospholipids (PL) among other desirable components. Its amphiphilic character and excellent biocompatibility make PL appropriate for numerous applications with technological and nutritional significance and potential health benefits. Given the difficulties entailed by the PL isolation, the efficiency for extracting such compounds of two environmental friendly techniques, pressurized liquid extraction (PLE) and supercritical fluid extraction (SFE) was evaluated. By using PLE with food-grade ethanol (EtOH), an oil recovery close to 100% was achieved in just 10 min. This oil extract was particularly rich in α-linolenic acid (ALA; 70%) as compared to the oil extracted by SFE (56%). In this case the oil recovery was only 87% but addition of EtOH to CO_2 enhanced the extraction yield, which reached 99%. However the use of co-solvent did not affect the fatty acid profile of the supercritical extracts or their TAG composition, where the high molecular weight TAG species were the predominant in all cases. With the exception of SFE without co-solvent, all methods applied were capable of extracting the PL fraction, although the content and distribution of the individual components present in this fraction differed markedly depending on the extraction conditions used. In this context, the use of a sequential extraction process, combining SFE and PLE was particularly interesting. The re-extraction by PLE of the chia cake, previously defatted by SFE, allowed to obtain an oil extract highly enriched in PLs, whose content exceeds 16% and with a higher PL species than the rest of the oil extracts.

Keywords: Chia seed; Phospholipids; Supercritical fluid extraction (SFE); Pressurized liquid extraction (PLE); Triacylglycerol composition; FAME composition.

1. INTRODUCTION

Chia (*Salvia hispanica L*) is a plant native to Central America whose seed was used as a staple food and for medicinal purposes by Mesoamerican cultures. Currently, chia seeds are being incorporated into western diet and its consumption have been increasing due to their recognized nutritional benefits (Kulczyński, Kobus-Cisowska, Taczanowski, Kmiecik & Gramza-Michałowska, 2019), which mainly result from the high content of valuable compounds, such as dietary fibers, proteins, antioxidants, vitamins, carotenoids, minerals and, especially essential fatty acids (Grancieri, Martino & Gonzalez de Mejia, 2019; Dąbrowski, Konopka & Czaplicki 2018; Ullah et al., 2016; Ayerza & Coates et al., 2011). There is a growing interest in food industry towards chia seed as a promising source of bioactive compounds for the development of functional foods (Fernández-López, Lucas-González, Viuda-Martos, Sayas-Barberá & Pérez-Alvarez, 2018; Oliveira-Alves et al., 2017; Reyes-Caudillo, Tecante & Valdivia-López, 2008) and the improvement of food formulations (Capitani, Nolasco & Tomas, 2016; Julio et al., 2016; Coorey, Tjoe, & Jayasena, 2014; Olivos-Lugo, Valdivia-Lopez & Tecante, 2010).

Chia seeds stand out for its high-quality oil, which represents the largest vegetable source of α-linolenic (C18:3, ALA) and linoleic (C18:2, LA) acids, and provide a good equilibrium between the concentrations of these two essential fatty acids (Dubois, Breton, Linder, Fanni & Parmentier, 2007). Authorization for placing chia oil on the European market as a novel food was achieved in 2014 (2014/890/EU), but only in the case of oil produced by cold-pressing. Nevertheless, several studies have looked into obtaining oil from chia seeds and different extraction technologies have been explored. Supercritical fluid extraction (SFE) based on the use of $SCCO_2$ has been successfully applied (Villanueva-Bermejo, Calvo, Castro-Gómez, Fornari & Fontecha, 2019;

Guindani et al., 2016; Ixtaina et al., 2010 ; Ixtaina et al., 2011; Rocha Uribe, Novelo Pérez, Castillo Kauil, Rosado Rubio & Alcocer, 2011). Equally, methods using organic liquid solvents, such as ultrasound-assisted extraction (Rosas-Mendoza, Coria-Hernández, Meléndez-Pérez & Arjona-Román, 2017; de Mello, dos Santos García & da Silva, 2015), Soxhlet procedure (Amato et al., 2015; Tolentino et al., 2014; Segura-Campos, Ciau-Solís, Rosado-Rubio, Chel-Guerrero & Betancur-Ancona, 2014), subcritical propane extraction (Zanqui et al., 2015) or pressurized liquid extraction (PLE), (Villanueva-Bermejo et al., 2019; Castejón, Luna & Señorans, 2017) have been frequently studied for chia oil extraction.

Chia oil is mainly composed of triacylglycerols (TAG) (> 90%), but also contains an unsaponifiable fraction which not always is properly extracted. That fraction includes desirable components such as tocopherols, phytosterols, polyphenols, hydrocarbons, pigments, vitamins or phospholipids (PL), which despite being considered minor constituents present important nutritional features. In chia seed, as occurs in other oil plants, large amounts of PL are located in the membrane of the oleosomes, the cell organelles storing TAG reserves (Sreedhar, Kumari, Rupwate, Rajasekharan & Srinivasan, 2015), where they play an important role. Besides to contribute, in association with oleosin proteins, to stabilize the oleosome structures, they are actively involved in TAG biosynthesis (Bates, Durrett, Ohlrogge & Pollard, 2009). During industrial processing of seeds, the membranes degradation occurs and as a result the released PL can freely migrate to the extracted oil. The presence of those PL may have unfavorable effects during the oil refining process, since negatively affects its flavor, odor and appearance and also contribute to numerous technological problems. Consequently, new alternatives for the "oil degumming" in order to achieve an efficient removal of PL are constantly being sought (Ayerdi-Gotor & Rhazi, 2017;

Ambrosewicz-Walacik, Tańska & Rotkiewicz, 2015). Nevertheless, because of its amphiphilic character, oil seed PL are also desirable ingredients for multiple industrial purposes (Herchi et al., 2012). Hence, these compounds are widely used as emulsifiers, stabilizers, controlled-crystallization agents, viscosity modifiers, antioxidants, and reducers or replacers of fat in food products (Guiotto, Cabezas, Diehl & Tomás, 2013). In addition, PL are well-established pharmaceutical excipients and they have a wide range of applications in cosmetic and drug delivery systems (Li et al., 2015; van Hoogevest & Wendel, 2014). On the other hand, since PL participate in a variety of indispensable biological processes, they appear to play important a key role in human health and the relevance of dietary PL as potential nutraceuticals has been recently suggested (Castro-Gómez, García-Serrano, Visioli & Fontecha, 2015).

PL fraction in oils from sources like soybean (Liu & Ma, 2011), rapeseed (Woodfield et al., 2018; Ambrosewick-Walacik et al., 2015), flaxseed (Herchi et al., 2012) or sunflower (Guiotto et al., 2013; Ayerdi-Gotor & Rhazi, 2017) has been well characterized and numerous data about its content as well as composition can be found in the literature. However, there is scarce information available about the presence of PL in chia seed oil. Only two studies have reported estimations of the total PL content calculated on the basis of the total phosphorus present in the oils (Segura-Campos et al., 2014; Ixtaina et al., 2011).

In view of the above, the aim of this study was to evaluate the efficiency of two environmental-friendly methods, PLE and SFE for extracting these minor compounds from chia seeds. In all extracts, lipid composition was thoroughly assessed, with particular emphasis on the content and distribution of individual PL.

2. Materials and methods

2.1 Sample and reagents

2.1.1 Sample preparation

Chia seeds (*Salvia hispanica* L.) from Mexico were supplied by Primaria Premium Raw Materials, S.L. Valencia, Spain). Clean seeds were ground in a knife mill cooled by liquid nitrogen to minimize oil oxidation and any nascent lipase activity, and then were sieved in an electromagnetic digital sieve shaker using 200 mm diameter stainless steel mesh sieves (CISA Cedaceria Industrial S.L. Barcelona, Spain) to finally obtain a particle size ranging from 250 μm to 500 μm. Samples were kept at -20ºC until their use.

2.1.2 Chemicals

All solvents were HPLC and MS grade when available. Dichloromethane, chloroform, hexane, isooctane, isopropanol, dimethylformamide, sulphuric acid 98%, methanol, tetrahydrofuran and acetonitrile were purchased from Labscan (Dublin, Ireland) and food grade ethanol 96%, v/v (F.C.C.) for PLE was obtained from Alcoholes Montplet (Barcelona, Spain). Absolute ethanol (≥98.5%), employed for SFE, sea sand used as dispersant, sodium sulphate anhydrous and sodium carbonate were obtained from Panreac (Barcelona, Spain). Formic acid (98%), triethylamine (99.5%), sodium methoxide 95%, the TAG standards trinanoin and tridecanoin, and the FFA standards, as well as phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE) and sphingomyelin (SM) were supplied by Sigma-Aldrich (St. Louis, MO, USA). Reference samples with known composition butterfat BCR-164 and BCR-519 (EU Commissions; Brussels, Belgium) were from Fedelco Inc. (Madrid, Spain).

2.2 Supercritical fluid extraction (SFE)

SFE were conducted using a pilot-plant supercritical fluid extractor (model SF2000; Thar Technology, Pittsburgh, PA, USA), comprising a 0.273 L cylinder extraction cell and two separators (S1 and S2), each of 0.5 L capacity, with independent control of temperature and pressure. A detailed description of the equipment can be found in the work reported by Villanueva-Bermejo, Zahran, Rodríguez-Risco, Reglero & Fornari (2017). The mass of chia seeds employed for the experiments was 130 g and two subsequent extraction steps were carried out with the aim of fractionating TAG and PL from seeds. The first step was carried out at 45 MPa, 40ºC, 40 g/min CO_2 flow rate and 120 min extraction time. This condition allowed the extraction of most part of TAG and it was selected due to the higher concentration of ALA obtained in the oil, as was previously reported by Villanueva-Bermejo et al. (2019). Then, a second extraction with $SCCO_2$ containing different co-solvent (ethanol) concentrations (15.5%, 25.3% and 36.9% w/w) was performed at the same operational conditions and 60 min extraction time. Upon completion of the first extraction step, the system was depressurized and a partly defatted chia seed cake aliquot (4 g) was removed from the high-pressure vessel before starting the second extraction step. Seed aliquots were finally subjected to PLE (Section 2.3). Chia oils from the first extraction step were collected by depressurization at the system recirculation pressure (5 MPa), whereas ethanol extracts from the second step were collected at ambient pressure. Oil and ethanol extracts obtained in each extraction step precipitated mostly in the first separator and they were mixed in a single fraction with the residual amounts precipitated in the second separator. Ethanol was removed by rotary evaporation (Strike 202 model; Steroglass S.R.L., Perugia, Italy). Finally, oils and ethanol extracts were resuspended in CH_2Cl_2, filtered through a 0.45 μm Millipore filter coupled to a syringe containing approx. 1 g of anhydrous sodium sulphate and stored at -35°C until chromatographic analysis.

2.3 Pressurized liquid extraction (PLE)

The extractions were carried out as described by Villanueva-Bermejo et al. (2019) using an Accelerated Solid Extraction ASE-200 equipment (Dionex Corp., Sunnyvale, CA), and food grade ethanol (EtOH) as extraction solvent. A more detailed description of the procedure and equipment employed can be found in the work reported by Castro-Gómez et al. (2014). Briefly, 2 g of chia seed sample were mixed with 2 g of sea sand, which was used as a dispersant, and loaded into a stainless-steel extraction cell covered with filters on both sides. The partly defatted chia seed cake aliquots from the first SFE step were also subjected to the PLE process under identical experimental conditions. The extraction was done during one static cycle of 10 min at 60ºC and applying a pressure of 10.3 MPa. The oil extract was first concentrated by removing the EtOH in a rotary vacuum evaporator (Strike 202 model; Steroglass S.R.L., Perugia, Italy) and then was fully evaporated under a gentle stream of nitrogen, weighed, stored in amber vials, and frozen at -35ºC until analysis. Each extraction was performed in triplicate.

2.4 Chemical analysis

2.4.1 Fatty acid composition

Derivatization of the lipid extracts from chia seeds was performed according to the method described by Castro-Gómez, Fontecha & Rodríguez-Alcalá (2014). Tritridecanoine (C13:0-TAG) was used as an internal standard (200 µL; 1.3 mg/mL) and added to samples before methylation. Fatty acid methyl esters (FAMEs) were separated in an Agilent chromatograph (model 6890N; Agilent Technologies Inc., Palo Alto, USA) equipped with an MS detector (Agilent 5973N) using a CP-Sil 88 fused-silica capillary column (100 m × 0.25 mm ID, 0.2 µm; Chrompack, Middelburg, The Netherlands). Chromatographic conditions were as follows: the column was held at 100°C for 1 min after injection and then temperature increased 7°C/min up to 170°C,

held there for 55 min, then increased at 10°C/min up to 230°C and held for 33 min. Total time for chromatographic run was 105 min. Helium was used as the carrier gas with a column inlet pressure of 0.2 MPa. The injector temperature was set at 250°C. The injection volume was 1 µL and a split ratio 1:25 was used. MS detector conditions were as follows: transfer line temperature 250°C, source temperature 230°C, and quadrupole temperature 150°C. The mass spectrometer operated under electron impact mode (70 eV), it was used in total ion current (TIC) mode and scanned the mass range from 40 to 500 m/z. For peak identification, mass spectra obtained in our analysis were compared with those in the National Institute of Standards and Technology library (NIST, 2.1.0 version; Gaithersburg, MD, USA). For qualitative and quantitative analysis, response factors were calculated using anhydrous milk fat (reference material BCR-164).

2.4.2 TAG composition

Analysis of TAG molecular species was based on the method described by Fontecha, Mayo, Toledano & Juarez (2006). The oil samples from chia seeds were injected (0.5 µL at 30 mg/mL) using a split ratio 1:20 in a CLARUS 400 gas chromatograph (Perkin Elmer Ltd., Beaconsfield, UK) equipped with an automatic split/splitless injector and a flame ionization detector (FID). A Rtx-65 TAG fused silica capillary column Crossbond™ (30 m × 0.25 mm ID; 0.1µm film thickness; Restek Corp., Bellefonte, PA, USA) was used. The temperature program was as follows: the column was held at 120°C for 30 s, then temperature increased 10°C/min up to 220°C, held there for 30 s, then increased at 6°C/min up to 350°C and held for 30 min. Total run time was 62.5 min. Helium (35 mL/min) was used as the carrier gas at 0.2 MPa. Injector and FID temperatures were 355ºC and 370ºC, respectively. Glyceryl trinanoate (100 µL; 1 mg/mL) was added as internal standard before the chomatograhic analysis and reference

butterfat BCR-519 of known composition was used for TAG determination and quantification.

2.4.3 Lipid classes composition by HPLC-Evaporative Light Scattering Detection (ELSD)

Separation of lipid classes present in the chia seed oil was accomplished in an HPLC system (model 1260; Agilent Technologies Inc. Palo Alto, CA, USA) coupled with an ELSD (SEDEX 85 model; Sedere SAS, Alfortville Cedex, France) as reported by Castro-Gomez et al. (2017). Prefiltered compressed air at 0.35 MPa and 60°C was used as the nebulizing gas and the gain was set at 3. Two Zorvax Rx-SIL columns (250 mm × 4.5 mm with 5-µm particle diameter; Agilent Technologies Inc.) in series and a precolumn of the same packing were used. Once the column was equilibrated at 40°C, 50 µL of sample (5 mg/mL in CH_2Cl_2) were injected. The solvent gradient was as detailed in Castro-Gómez et al., 2014). Both samples and standards were analyzed under the same conditions, using solvents freshly prepared. Assays were carried out in triplicate.

3. Results and discussion

3.1 Oil recovery

Figure 1 shows comparatively the oil recoveries achieved with the different methods assayed. By using PLE (Figure 1A) of untreated seeds, the total removal of the oil was practically accomplished (oil recovery of 99.7%) after only 10 min of extraction, using an agrochemical solvent (ethanol) at a relatively low temperature (60ºC). Similar results had been previously obtained by our group (Villanueva-Bermejo et al., 2019) when comparing the efficiency of PLE and SFE for oil extraction from two batches of Mexican chia seeds with different oil content.

As regards sequential SFE procedure (SFE+SFE), the mean oil recovery value obtained from the extractions carried out at 45 MPa, 40 ºC in the absence of co-solvent (first extraction step) was 86.6%, but it should be emphasized that the yield of extracted oil clearly enhanced when the second extraction step using EtOH as co-solvent was carried out, thus reaching values about 99% (Figure 1B-D). As can be observed, similar partial oil recoveries (around 12%) were obtained at the different EtOH concentrations. Therefore, the presence of EtOH positively affects the oil extraction process, by increasing the polarity of $SCCO_2$, however increasing EtOH concentrations did not improve the extraction yield at the operational conditions studied. As expected, the presence of co-solvent allowed the extraction of a higher assortment of lipid compounds including polar lipids (see Section 3.3). When sequential SFE+PLE procedure was performed (Figure 1E), the total oil recovery achieved was 96.5%. The oil recovery obtained specifically in the PLE step was 9.9%, which was slightly lower than that obtained in the second step of sequential SFE+SFE procedure (11.9-12.0%).

3.2 Fatty acid and TAG composition

The main fatty acids present in the oils extracted from chia seeds as well as the major TAG molecular species are shown in Table 1. As it can be seen, ALA was the major FA in all extracts, thus confirming that chia seed oil is the most important source of omega3 in nature (Dubois et al., 2007).

As regards the oils extracted by SFE, all of them exhibited similar FA profile and no changes in composition were observed when co-solvent was used, independently of the proportion of EtOH added (Table 1). Likewise, FA profile obtained by the sequential SFE+PLE procedure was comparable to those obtained from the SFE+SFE procedure. However, the FA profile of sequential extractions (SFE+SFE and SFE+PLE) differed greatly from that found in oil obtained by using PLE (Table 1). ALA content in the PLE

extract reached 70%, but this value was around 59% in the case of the oils extracted by the other methods. A similar behavior was reported by Villanueva-Bermejo et al. (2019) when compared the efficiency of PLE and SFE for the extraction of chia seeds with oil concentrations different to those contained in the seeds of the present study. On the contrary, the concentration of LA, the second most abundant FA in all extracted oils, was ~30% higher in oils obtained from the sequential extractions than in PLE extract. Consequently, n-6/n-3 ratio achieved was more favorable for PLE (0.23 *vs.* 0.36-0.37). The n-6/n-3 ratio was in agreement with those reported in previous studies on chia oil (Dąbrowski, Konopka, Czaplicki & Tanska, 2017; de Mello et al., 2015; Zanqui et al., 2015). Similarly, due to the higher concentration of ALA and lower content of saturated FA (SFA), PLE extract exhibited a greater unsaturated-to-saturated fatty acid (UFA/SFA) ratio (10.70) compared to those obtained by sequential extractions (7.80-8.33). In general, the lipid profiles observed in this study were in agreement with those previously reported for chia seed oil obtained by using $SCCO_2$ (Ixtaina et al., 2011; Rocha Uribe et al., 2011; Dąbrowski et al., 2017) and PLE (Castejón et al., 2017; Villanueva-Bermejo et al., 2019).

As in the case of FA profile, the distribution of TAG molecular species depended mostly on the extraction method used and some differences were found between PLE and SFE extracts. Although CN54 was the predominant molecular specie of TAG in all oils analyzed (Table 1), its concentration in the PLE extract (56%) was considerably lower than that found in extracts from the sequential extractions. This effect could be explained considering the increasing TAG solubility in $SCCO_2$ as its unsaturation degree increases (Davarnejad, Kassim, Zainal, & Sata, 2008). Based on this, the extraction of CN54 with $SCCO_2$ could be positively affected by the high presence of unsaturated fatty acids (UFA), such as LA and ALA, esterified on their molecules

(Jokić et al., 2010). Similar results were previously reported by Villanueva-Bermejo et al. (2019) in extracts obtained by SFE from chia seeds with different oil contents as compared to those obtained by PLE using different solvents. With regard to the co-solvent effect, an increase in the concentration of EtOH had no effect on the TAG distribution in SFE oils. Similar results to those observed for SFE+SFE were found when the sequential SFE+PLE procedure was applied. In addition, a remarkable amount of the CN52 was also found in the chia oils, probably due to the high presence of TAGs containing palmitic acid esterified to the glycerol backbone. Co-elution of other lipid classes such as diacylglycerols (DAG), monoacylglycerols (MAG) and polar lipids could explain the presence of molecular species with ≤ CN50 in the isolated extracts (Castro-Gómez, Montero & Fontecha, 2017). Their concentration in SFE+SFE extracts was 2-fold lower than in the PLE extract. Similar distributions of TAG have been previously described for chia oil and twelve molecular species have been identified by using HPLC-APCI-MS (Ixtaina, Mattea et al., 2011) and RP-HPLC (Timilsena, Vongsvivut, Adhikari, & Adhikar, 2017), being trilinolenin (αLn-αLn-αLn) reported as the major TAG.

3.3. Lipid classes composition

Table 2 and Figure 2 show the distribution and profile of the lipid classes in the oil extracts obtained from chia seeds. As can be seen, their contents were markedly different depending on the extraction method used. TAG was the main lipid class in all extracts, but DAG, MAG, sterol esters (SE), sterols and free fatty acids (FFA) were also detected although in smaller amounts. With the exception of the SFE using neat CO_2 as solvent (first extraction step of sequential procedures), all methods tested allowed the extraction of the PL fraction.

TAG concentration was higher in SFE+SFE and SFE+PLE extracts (97-95%) than in the PLE extract (78%). In the latter, DAG represents 21.4% of the total of lipid classes, which is a concentration 10-fold higher than that found in the samples obtained by sequential extractions. This may be related to residual lipase activity or to hydrolysis processes that take place during PLE extraction. Since there was no concomitant increase in MAG and FFA levels, it could be an unorthodox patatin-like TAG lipases (PTLs), similar to that described by Eastmond PJ. (2006) in sugar-dependent 1 (SDP1) *Arabidopsis thaliana* mutants. This enzyme exhibits strong specificity toward TAG and is significantly less active against DAG and inactive on MAG. Balvardi et al. (2015) did not observed this increase in DAG concentration when used PLE (EtOH, 150ºC, 20 min) for almond oil extraction. It is well known that some lipases have a high thermal stability (Barros, Fleuri & Macedo, 2010; Santos et al., 2013), remaining active even at the temperature used in our study (60ºC), while the enzyme deactivation would have occurred at 150ºC, the conditions used by Balvardi et al. (2015). On the other hand, it has been reported that $SCCO_2$ can suppress any lipolytic activity during the oil extraction by deactivating the lipases (King, Mohamed, Taylor, Mebrahtu & Paul, 2001), which would explain the lower concentration of DAG in oils obtained by the sequential extraction procedures.

In the case of the sequential extraction procedures, the removal of most part of oil during the first step favored the subsequent recovery of PLs from the chia cake during the second step. These results were consistent with the values concerning total and partial oil recoveries achieved (Figure 1). Even though the first extraction step was carried out at the same operational conditions (45 MPa, 40ºC, 0% ethanol), the application of PLE with EtOH on the chia cake (second extraction step of SFE+PLE procedure) brought about an enrichment on PL, probably as a consequence of the higher

polarity of EtOH compared to the CO_2-EtOH mixture (second extraction step of SFE+SFE procedure). It should be noted that the concentration of PL in the above mentioned PLE extracts (16.5%) was around 5-fold higher than those in the extracts obtained by SFE with the addition of co-solvent during the second extraction step (Table 2). On the other hand, increasing ethanol proportions did not lead to a variation in the profile of lipid classes nor substantially improve the PL extraction under the operational conditions studied. Recently, Fernández-López et al. (2018) have reported that chia cake might represent an important source of polyphenolic compounds and exhibits higher antioxidant activity than the original chia seeds. However, its potential as a PL source has not been thoroughly studied yet. It seems evident that the extraction of the polar fraction of the oil depends to a great extent on the extraction procedure used. This is a relevant aspect which must be taken into account when extraction and fractionation processes are designed.

The total PL content achieved under the different experimental conditions assayed in the present study ranged from 0.097% to 16.54% (Table 2). In all cases, these PL contents were much higher than those previously reported by Segura-Campos et al. (2014) (118 ppm = 0.0118%) and Ixtaina et al. (2011) (46 ppm= 0.0046%) for chia oil extracted with hexane by the Soxhlet method, which could be attributed to the low solubility of PL in hexane (Castro-Gómez, Rodriguez-Alcalá et al., 2014). Ixtaina et al. (2011) found a significantly higher PL content (225 ppm), when the oil was obtained by pressing. Our results clearly suggest a fractionation of PL, favored by the presence of EtOH as pressurized solvent (PLE) or as co-solvent in the $SCCO_2$ extraction.

3.4. Effect of extraction method on PL distribution

In contrast to what occurs in oils from soybean (Liu & Ma, 2011), rapeseed, (Woodfield et al., 2018 Ambrosewick-Walacik et al., 2015) flaxseed (Herchi et al., 2012) or

sunflower (Guiotto et al., 2013; Ayerdi-Gotor & Rhazi, 2017), there is no available data in the literature concerning the composition of the PL fraction in chia seed oil. As can be observed in Figure 3, the distribution of the different components of the PL fraction and their concentration are clearly dependent on the extraction conditions used. In SFE obtained in presence of co-solvent (Figure **3A-C**), only two PL, phosphatidylcholine (PC) and phoshatidylethanolamine (PE), were found. PC was the main compound in the extracts obtained at the different ethanol concentrations, but a reduction in its concentration was observed when the amount co-solvent increased (79% *vs* 71%). PC plays a key role in lipid metabolism in plants, where in addition to its structural function in the oleosome membrane, is actively involved in the synthesis of TAG enriched in PUFA. Besides acting as a substrate on which desaturation occurs, PC acts as acyl donor in a unique reaction where PUFA attached on its structure are transferred to DAG to produce TAG (Bates et al., 2009; Castro-Gómez et al., 2015). This mechanism for PUFA enrichment becomes even more relevant in the case of seeds like chia which accumulates high amounts of ALA (Sreedhar et al., 2015).

With respect to the PLE extract (Figure **3D**), in addition to PC and PE, a substantial amount (16%) of phosphatidylinositol (PI) was also detected. The enzymes involved in PI metabolism have been identified by Sreedhar et al. (2015) during characterization of the developing chia seeds transcriptome. However, the greatest variety of compounds in the PL fraction was observed in the extract obtained by re-extraction of the chia seed cake with PLE after the SFE process (Figure **3E**). Moreover, the relative amounts of those PL were markedly different than that observed in the other extracts. Thus, PC, PE and PI were the mayor PL attaining concentrations of 43.6%, 20.5% and 34.5%, respectively. Phosphatidylserine (PS) was also detected although in a much smaller amount (1%). Small traces of sphingolipids (SLs) were even detected in this extract,

which likely corresponded to glycosylinositol phosphoryl ceramides, the most abundant in plants (Buré, Cacas, Mongrand & Schmitter, 2014). It should be noted that the presence of phosphatidic acid (PA) was only found in this extract and its concentration reached a high value (11.6%). PA is generated as a result of phospholipase D activity (PLD), which cleaves PL at the terminal phosphodiester bond. Given that this enzyme exhibits high thermal stability, withstanding at least 60ºC for 30 min (Chen, Snyder, Greer & Weselake et al., 2011) even in a "non water" environment, it could be possible that it remained active during extraction. At this respect, Ambrosewick-Walacik et al. (2015) emphasized the importance of controlling the extraction parameters as a means of limiting the presence of active PLD. Our results suggest that PLD activity could be affected by the extraction time, since when one–step extraction (PLE, 10 min) was used, PA was not detected (Figure **3D**).

4. Conclusions

The fractionation of PL from chia seeds has been carried out for the first time. The majority of TAG were extracted in the first extraction step by using neat $SCCO_2$.The second extraction step using ethanol allowed the extraction of polar lipid fraction, specially PLs. Therefore, two different fractions were obtained: an oil rich in PUFA, and an ethanol extract containing a high concentration of PLs and still considerable amounts of omega-3. The highest amount of PLs (16.54 %) was obtained in the PLE extract from the sequential SFE+PLE procedure. Moreover, up to 5 different individual species of PL (PC, PE, PI, PS, PA and even SLs traces) were observed in this extract, unlike the rest of extraction procedures studied. This may have important technological and nutritional properties. Moreover, the wide variety of individual species of PL identified in the PLE oil makes it interesting as potential ingredient for multiple purposes in both pharmaceutical and food industries. Nevertheless, it should be stated

the importance of controlling the extraction parameters to limit the presence of active PLD and thus, prevent PA formation.

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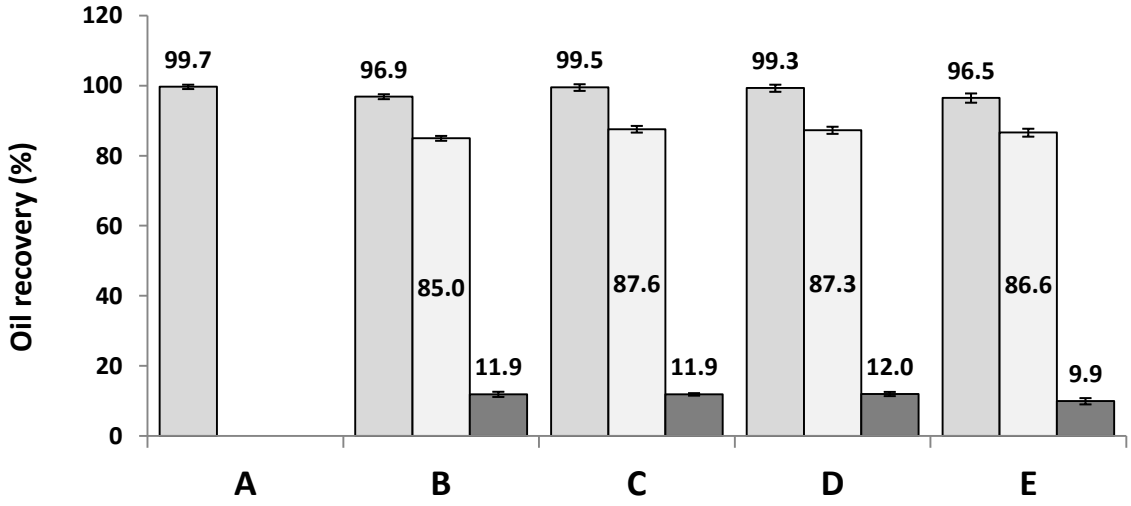
Figure Captions

Figure 1. Oil recoveries (mass of oil extracted / mass of oil in seeds x 100) obtained under the different experimental conditions tested. PLE with EtOH, 60ºC,10 min (**A**); SFE + SFE: 45 MPa, 40ºC and 40 g/min CO_2 flow rate, 120 min, no co-solvent (Step 1), followed by 60 min extraction with EtOH as co-solvent (Step 2) at 15.5% (**B**), 25.3% (**C**) and 36.9% (w/w) (**D**); SFE+PLE: 45 MPa, 40ºC and 40 g/min CO_2 flow rate, 120 min, no co-solvent (Step 1), followed by PLE (EtOH, 60ºC, 10 min) (Step 2).(**E**). Total oil recovery (■). Partial recoveries corresponding to the first (■) and second extraction steps (■). Mass of oil in seeds = 33.8% (data provided by the supplier).

Figure 2. Comparison of lipid classes profiles, obtained by HPLC-ELSD of the oils extracted from chia seeds. Experimental conditions: (**A**) PLE with EtOH, 60ºC. (**B**) SFE at 45 MPa, 40ºC without co-solvent (first extraction step), (**C**) SFE at 45 MPa, 40ºC and 36.9% of ethanol (second extraction step), and (**D**) PLE with EtOH, 60ºC (second extraction step). SE: sterols esters; TAG: triacylglycerols; DAG: diacylglycerols; MAG: monoacylglycerols; ST: sterols; FFA: free fatty acids; PL: phospholipids; PE: phosphatidylethanolamine; PI: phosphatidylinositol; PS: phosphatidylserine; PC: phosphatidylcholine; PA: phosphatidic acid; SLs: sphingolipids.

Figure 3. Distribution of individual species of phospholipids (PL) in the oils extracted from chia seeds under different experimental conditions. SFE (45 MPa, 40ºC) with EtOH as co-solvent (second extraction step) at 15.5% (**A**), 25.3% (**B**) and 36.9% (**C**) (w/w); PLE with EtOH, 60ºC, (**D**); PLE with EtOH, 60ºC (second extraction step) (**E**). PE: phosphatidylethanolamine; PI: phosphatidylinositol; PS: phosphatidylserine; PC: phosphatidylcholine; PA: phosphatidic acid; SLs: sphingolipids.

Figure 1.


Oil recovery (%)
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86.6
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A
B
C
D
E

Figure 2.

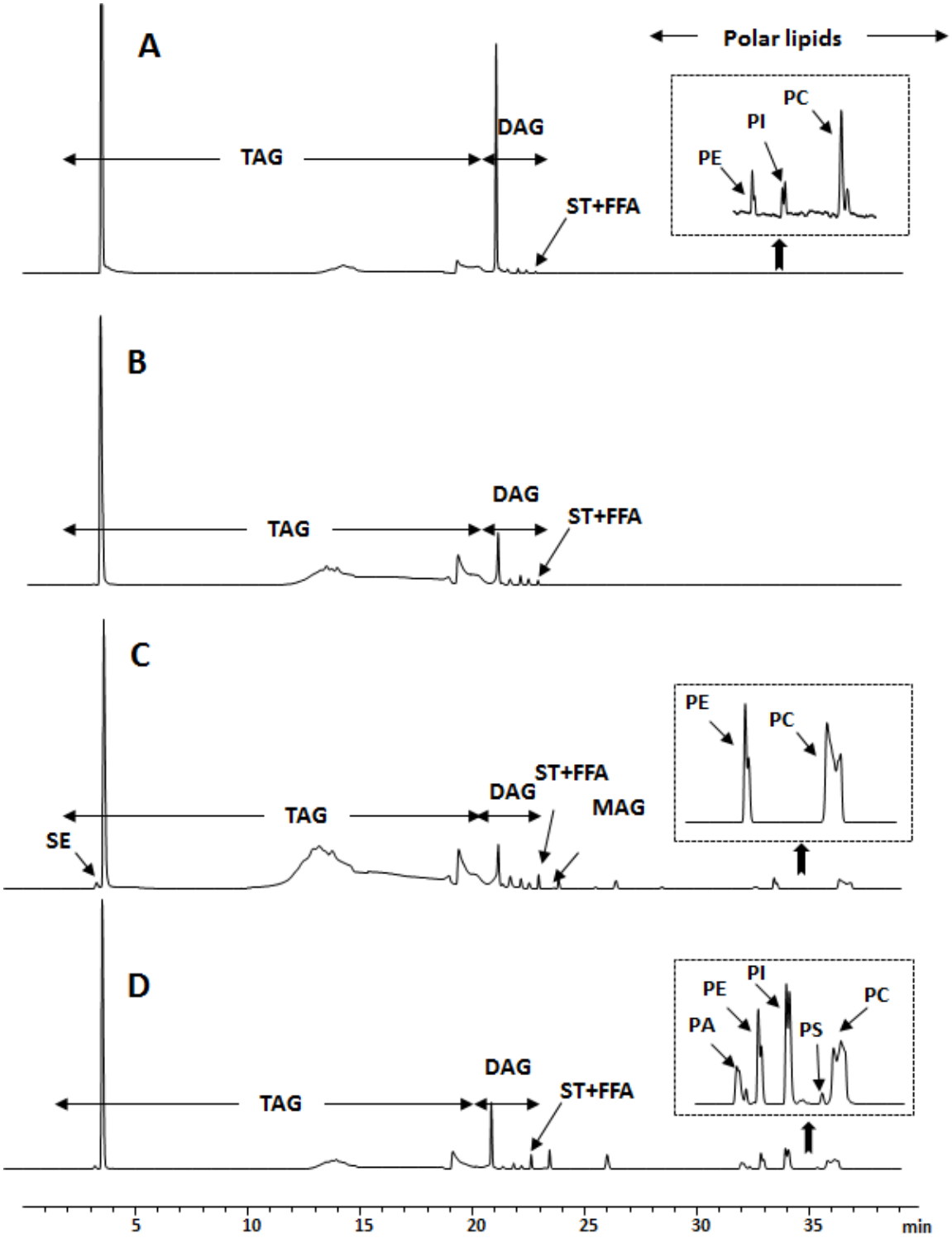
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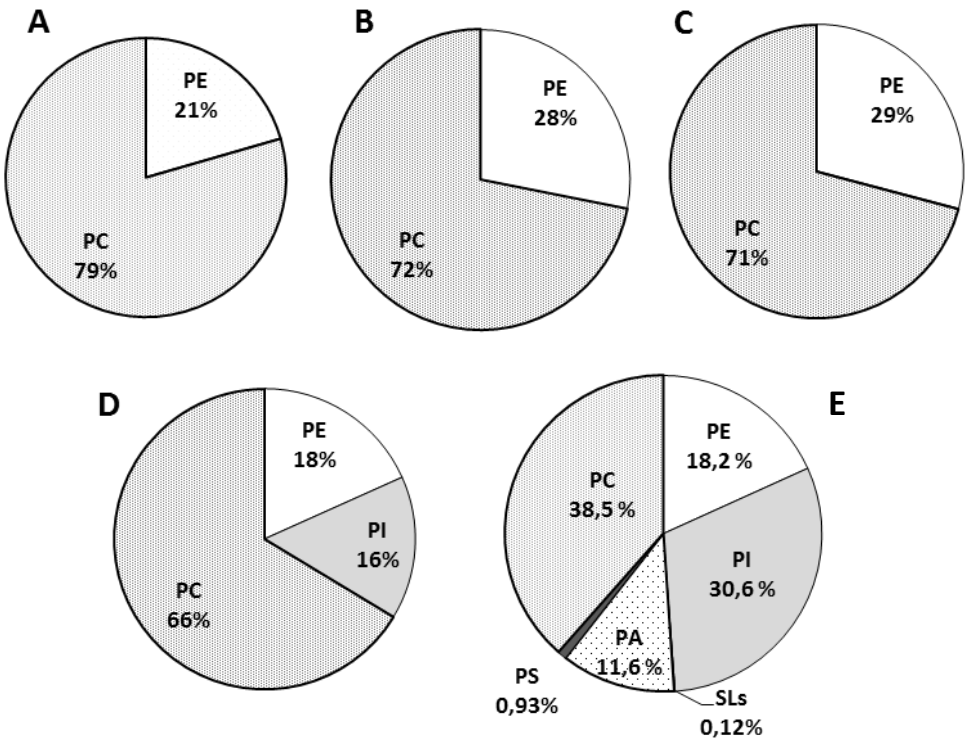
Figure 3.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SLs
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Table

Table 1. Composition of fatty acid and molecular species of TAG (%) in oils extracted from chia seeds using both SFE and PLE under different experimental conditions. *Data shown mean values of the final oil extract obtained from two extraction steps. CN: Carbon number.

FA (g/100 g fat)	PLE[1]	Sequential extraction procedures*				
		SFE[2] (without co-solvent)	SFE[3] (15.5% EtOH)	SFE[3] (25.3% EtOH)	SFE[3] (36.9% EtOH)	PLE[4]
C16:0	5.89	6.94	7.24	7.24	7.25	7.48
C18:0	2.66	3.77	3.80	3.86	3.84	3.89
C18:1c9	5.47	7.31	7.40	7.33	7.32	7.30
C18:2 c9.c12	15,89	21,31	21,67	21,65	21.76	21.99
αC18:3 n3	70.09	59.89	59.06	59.16	59.05	58.53
SFA	8.54	10.72	11.05	11.10	11.09	11.37
MUFA	5.47	8.08	8.23	8.09	8.10	8.11
PUFA	85.99	81.20	80.73	80.80	80.81	80.52
UFA	91.46	89.28	88.95	88.90	88.91	88.63
n6/n3	0.23	0.36	0.37	0.37	0.37	0.38
UFA/SFA	10.70	8.33	8.05	8.01	8.02	7.80
TAG molecular species (g/100g fat)						
<CN50	4.96	2.16	2.68	2.18	2.42	3.98
CN50	4.57	2.89	3.45	3.02	2.93	3.08
CN52	34.24	22.81	23.97	22.96	22.80	22.78
CN54	55.95	72.08	69.89	71.84	71.84	70.15

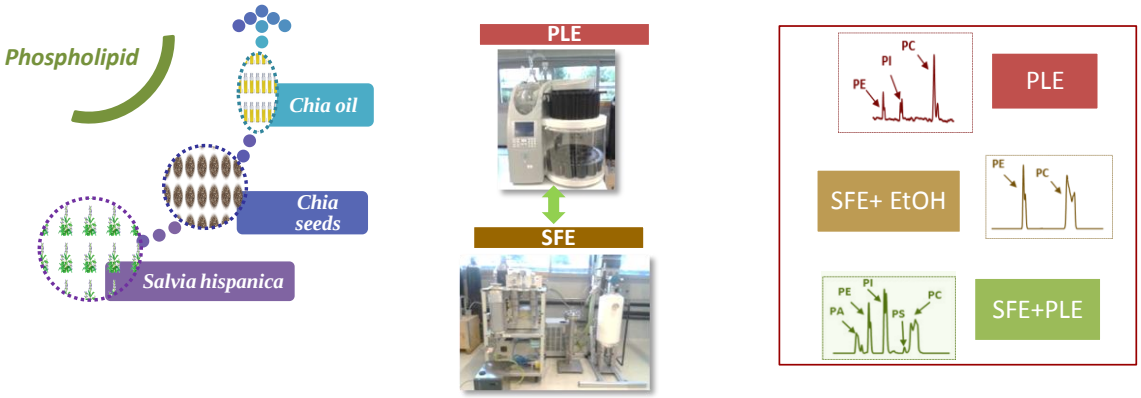
[1] 60ºC, EtOH (food grade),10 min.
[2] First extraction step: 45 MPa, 40ºC, 40 g/min CO_2 flow rate, 120 min, no co-solvent.
[3] Sequential SFE+SFE procedure (first + second extraction step). Second extraction step: 45 MPa, 40ºC, 40 g/min CO_2 flow rate, 60 min, co-solvent addition).
[4] Sequential SFE+PLE procedure (first + second extraction step). Second extraction step: 60ºC, EtOH (food grade), 10 min.

Table 2. Lipid classes composition of the oil extracted from chia seed using both SFE and PLE under different experimental conditions.

Lipid classes* **(g/100g fat)**	PLE[1]	Sequential extraction procedures[2,3]								
		Step 1	Step 2				Total (Step1 + Step2)			
		SFE	EtOH (15.5%)	EtOH (25.3%)	EtOH (36.9%)	PLE	EtOH (15.5%)	EtOH (25.3%)	EtOH (36.9%)	PLE
SE	0.08	0,08	0,47	0,45	0,44	0,50	0,13	0,12	0,12	0,12
TAG	78.27	97,78	94,81	94,23	94,59	72,41	97,55	97,32	97,32	95,28
DAG	21.39	1,86	1,34	1,38	1,70	7,38	1,69	1,85	1,90	2,41
ST + FFA	0.12	0,28	0,51	0,48	0,45	1,27	0,29	0,30	0,32	0,38
MAG	0.04		0,45	0,46	0,44	1,90	0,05	0,05	0,05	0,20
ΣPL	0.098		2,41	2,99	2,39	16.54	0,29	0,35	0,29	1,61

[1] Pressurized liquid extraction (PLE): 60ºC, EtOH (food grade), 10 min.
[2] SFE + SFE: 45 MPa, 40ºC and 40 g/min CO_2 flow rate, 120 min, no co-solvent (Step 1), followed by 60 min extraction with co-solvent addition (Step 2).
[3] SFE + PLE: 45 MPa, 40ºC and 40 g/min CO_2 flow rate, 120 min, no co-solvent (Step 1), followed by PLE (60ºC, EtOH (food grade), 10 min) (Step 2).
* SE: sterols esters; TAG: triacylglycerols; DAG: diacylglycerols; MAG: monoacylglycerols; ST: sterols; FFA: free fatty acids; PL: phospholipids.

***Graphical Abstract**



***Conflict of Interest form**

Statement of author contributions

The authors have all contributed to this manuscript and approve of this submission.

Statement of related content

There is not a manuscript of related content under consideration for publication elsewhere, nor has one been published nor made available elsewhere.

Statement of disclosures of conflict of interest

The authors declare no conflict of interest.