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# Subcritical extraction of flaxseed oil with *n*-propane: Composition and purity

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ABSTRACT

Flaxseed (*Linum usitatissimum* L.) oil was obtained via subcritical *n*-propane fluid extraction (SubFE) under different temperatures and pressures with an average yield of 28% and its composition, purity and oxidative stability were compared to oils obtained via conventional solvent extraction methods (SEMs). When the oxidative stability was measured by differential scanning calorimetry, the oil was found to be up to 5 times more resistant to lipid oxidation as compared to the SEM oils. Direct infusion electrospray ionization mass spectrometry (ESI-MS) analysis showed characteristic and similar TAG profiles for SubFE and SEMs oils but higher purity for the SubFE oil. The flaxseed oil content of β-tocopherol, campesterol, stigmasterol and sitosterol were quantified via GC–MS. SubFE showed to be a promising alternative to conventional SEM since SubFE provides an oil with higher purity and higher oxidation stability and with comparable levels of biologically active components.



## 1. Introduction

Flaxseed (*Linum usitatissimum* L.) is an oilseed crop that furnish an oil composed of 40–50% of α-linolenic acid (Pradhan, Meda, Rout, Naik, & Dalai, 2010) and rich in phytosterols, for example, β-sitosterol, campesterol and stigmasterol (Cert, Moreda, & Pérez-Camino, 2000; Moreau, Whitaker, & Hicks, 2002) and tocopherols. These bioactive components seem to act on many cellular functions in body immunity, preventing inflammation (Simopoulos, 2004), reducing the absorption of low density lipoprotein (LDL), decreasing cardiovascular diseases (Martins, Silva, Rita, Garbi, & Ito, 2004; Moreau et al., 2002), inhibiting the oxidation of cholesterol, decreasing the risk of other chronic diseases such as type 2 diabetes and cancer, and protecting against Alzheimer's disease (Köksal, Artik, Simsek, & Günes, 2006).

The commonly used processes of lipid extraction from oil seeds such as pressing and extraction with organic solvents (cold or hot) show a series of drawbacks including the use of time-consuming procedures, lower selectivity, solvent contamination and degradation of key bioactive components.

Subcritical fluid extraction (SubFE) for lipids (Nyam et al., 2010; Señoráns, Ibañez, Cavero, Tabera, & Reglero, 2000) seems therefore to provide the most attractive method with several advantages such as the use of a solvent with high density, diffusivity and low viscosity. The very mild temperature and pressure used for SubFE also reduces or eliminate degradation of the bioactive components resulting in a richer final product free of toxic solvent residues (Mariod, Matthaus, & Ismail, 2011; Passos, Silva, Silva, Coimbra, & Silva, 2010).

Pederssetti et al. (2011) and Corso et al. (2010), investigated the vegetable oil extraction using subcritical *n*-propane in the conditions of 30, 45 and 60 °C and 8, 10 and 12 MPa and supercritical $CO_2$ at 40, 50 and 60 °C and 20, 22.5 and 25 MPa. They obtained

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faster extraction with higher extraction yield using *n*-propane as compared with $CO_2$, with oils of similar oxidative stability and fatty acid profiles in both solvents.

Herein we present our evaluation of flaxseed oil extraction using subcritical n-propane under different temperature and pressure conditions. The composition of the oil obtained by SubFE was evaluated via GC–MS and ESI-MS analysis and compared to that of the flaxseed oils obtained via three conventional solvent extraction methods (SEMs).

## 2. Materials and methods

### 2.1. Sample preparation

Three packs of 500 g of 3 different lots of flaxseed (*L. usitatissimum* L.) samples were provided by Dubai Trade and Industry Food Production, Catuípe, RS, Brazil. The grains were previously dried in a ventilated oven at 40 °C for 48 h (Nova Ética, model 400/4ND, Brazil), ground in a Wiley mill (Tecnal, model TE 631/3, Brazil) to obtain a flour that was sieved, using the fraction that passed through a 14 mesh Tyler series sieve (WSTyler, USA). Later, the sample was thoroughly mixed and vacuum packed in polyethylene bags and frozen at −18 °C.

### 2.2. Proximate composition

Moisture, ash and crude protein content were determined according to AOAC (1998) and were expressed in percentage in wet basis (% $WB^{-1}$). The percentage of carbohydrate was estimated as the sum of moisture, ash, crude protein and overall lipid extracted by FLS method subtracted from 100%.

#### 2.2.1. Conventional method of lipid extraction

Total lipids were extracted according to Folch, Lees, and Sloane Stanley (1957) (FLS) with a mixture chloroform–methanol (2:1 v/v), Bligh and Dyer (1959) (BD) with a mixture chloroform–methanol–water (2:2:1.8 v/v/v) in two steps and crude fat by Soxhlet (1879) (SE) with a mixture of petroleum ether/ethyl ether (1:1 v/v). The results were expressed in dry basis percentage (% $DB^{-1}$).The term "lipids" will be used to generalize the terms "crude fat" and "total lipids".

#### 2.2.2. Subcritical fluid extraction method

For lipid extraction with pressurized *n*-propane, 30.0 g of sample was filled into the extractor, on a laboratory scale, using pressurized *n*-propane solvent (White Martins, 99.5% purity) via a pump-type syringe with a temperature-controlled thermostatic bath at 10 °C, as described by De Souza et al. (2008).

Different temperatures and pressures were used as the two main factors for the $2^2$ factorial design (Table 1), with three replications of the central point. The answer was the final oil quantity (extraction yield). The extraction was carried out with 1 $cm^3$ $min^{-1}$ of *n*-propane flow, controlled by an expansion valve (Autoclave Engineers) maintained at 80 °C using a thermoregulator (Tholz, model CTM-04E). Lipids were collected in weighed glass vials and lipid content was determined gravimetrically in 5 periods of 5–60 min on an analytical balance (Marte, model AM 220, Brazil) and were expressed in dry basis percentage (% $DB^{-1}$).

### 2.3. Fatty acid composition

Fatty acid methyl esters (FAME) were prepared by the methylation of lipids, as described by Hartman and Lago (1973) and analyses were performed in duplicate. The FAME were separated by GC (Trace Ultra 3300 model – Thermo Scientific) equipped with a flame ionization detector (FID) and a cyanopropyl capillary column (100 m × 0.25 i.d., 0.25 μm film thickness, CP-7420 Varian, EUA) (Martin, Oliveira, Visentainer, Matsushita, & Souza, 2008) following the conditions used by Sargi et al. (2013). The peak areas were determined by the ChromQuest 5.0 software. For fatty acid identification, retention times were compared with those of standard methyl esters (Sigma–Aldrich Co., Brazil).

Quantification was performed against tricosanoic acid methyl ester as an internal standard (23:0) (Sigma–Aldrich Co., Brazil), as described by Joseph and Ackman (1992). Theoretical FID correction factor values (Visentainer, 2012).

**Table 1**
Factors and levels evaluated in the experimental design full $2^2$ for the subcritical fluid extraction, solvent and yield of the extraction of lipids by the method of Bligh & Dyer, Soxhlet, Folch, Less & Stanley and by subcritical fluid extraction.

| Analyses | Temperature (°C)* | Pressure (MPa)* | Percents (%) |
|---|---|---|---|
| Moisture | – | – | 6.50 ± 0.34 |
| Ash | – | – | 2.58 ± 0.07 |
| Crude protein | – | – | 20.37 ± 3.81 |
| Carbohydrates | – | – | 43.13 ± 4.36 |
| TL BD | – | – | 26.69[a] ± 0.16 |
| TL SE | – | – | 24.58[a] ± 2.94 |
| TL FLS | – | – | 27.42[a] ± 2.09 |
| TL A | 45 | 10 | 28.15**[a] |
| TL B | 30 | 8 | 27.08[a] |
| TL C | 30 | 12 | 26.93[a] |
| TL D | 60 | 8 | 28.62[a] |
| TL E | 60 | 12 | 28.78[a] |

TL: total lipids. BD: Bligh and Dyer, SE: Soxhlet, FLS: Folch, Less & Stanley. A, B, C, D and E: letters representing the testing of extraction with subcritical fluid. Averages of triplicates ± standard deviation absolute.

* Parameters used in subcritical fluid extraction.

** Average of triplicates of the center point. Values followed by different letters in the same column demonstrated significant difference by Tukey test ($p < 0.05$).

### 2.4. Phytosterols and tocopherols composition

Phytosterols and tocopherols were simultaneously evaluated by gas chromatograph coupled to a mass spectrometer (GC–MS) (Du & Ahn, 2002). The extracted oils were previously derivatized according to Beveridge, Li, and Drover (2002) using N-O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) (Sigma–Aldrich Co., Brazil) as the derivatizing agent. The analysis was performed in a gas chromatograph (Thermo–Finnigan, model Thermo Focus GC) equipped with a capillary column DB-5 (5% phenyl, 95% methylpolysiloxane) fused silica, 30 m, 0.25 mm id and 0.25 mm thick film stationary phase (J & W Scientific, Folson, CA) coupled to a mass spectrometer (Thermo–Finnigan, model DSQ II) equipped with an electron ionization (EI) source. The system of data acquisition was performed by Xcalibur software accompanying database of spectra contained in the NIST MS Search spectral library version 2.0. Flow rate of gas was 1.0 mL $min^{-1}$ for the carrier gas (He – 5.0). The injections were performed in triplicate; the injection volume was 2 μl and the sample splitting rate was 1:10. The temperature of the injector and detector was 280 °C. The initial temperature of the column was 200 °C for 8 min, programmed to increase to 235 °C by 3 °C $min^{-1}$, and then to 280 °C by 15 °C $min^{-1}$; the column remained at this temperature for 15 min. The temperature of the transfer line between GC and MS was 250 °C.

Quantitation were carried out in relation to the internal standard 5α-cholestane (Sigma–Aldrich Co., Brazil), according to Li, Beveridge, and Drover (2007).

### 2.5. Triacylglycerol composition

For the electrospray ionization mass spectrometry (ESI-MS) analysis, 1 μL of the oil was dissolved in 1.0 mL of HPLC-grade

methanol (Merck SA, Rio de Janeiro, Brazil) and injected into the ESI source of the HCT Ultra spectrometer (Bruker – Bremen, Germany) with an auxiliary syringe pump (Harvard Apparatus, Holliston, MA) by a flow of 400 μL $h^{-1}$. The ion trap analyzer was operated in the ultra scan mode with a range of $m/z$ 100–1200. Spectra were acquired under the following conditions: capillary and skimmer of −3000 V and 40 V, respectively, source temperature of 300 °C. The mass spectra were processed using the ESI Compass 1.3 for HCT/esquire software.

### 2.6. Differential scanning calorimeter (DSC)

The oxidative stability of the oils extracted by different extraction methods was evaluated by the midpoint, according to Tan, Che Man, Selamat, and Yusoff (2002). An amount of 12.0 ± 0.5 mg of oil was placed in platinum capsules and introduced into the differential scanning calorimeter (DSC) (Netzsch, model STA 6000 Perkin-Elmer) to be analyzed at four different temperatures: 110, 120, 130 and 140 °C. While the temperature was being increased, the sample was kept in contact with an inert atmosphere of nitrogen (White Martins S.A., 99.9% purity)with a flow of 50 $cm^3$ $min^{-1}$, contacting with a flow of 50 $cm^3$ $min^{-1}$ of oxygen (White Martins S.A., 99.9% purity) in the set temperature.

### 2.7. Statistical and principal components analysis (PCA)

Proximate composition, phytosterols and tocopherols analyses were performed in triplicate and fatty acid analysis was done in quadruplicate. Means and standard deviations of the analytical error propagation were calculated. The results were submitted to variance analysis (ANOVA) and mean values were compared by Tukey's test, using the Statistica software (StatSoft, 2007), version 8.0. The Principal Component Analyses (PCA) was performed with the Statistica software, version 8.0.

## 3. Results and discussion

### 3.1. Proximate composition

The moisture (6.5%), ash (2.6%) crude protein (20.4%), total lipids (27.4%) and carbohydrates contents (43.1%) in flaxseed measured. The values of crude protein and ash were similar to those measured by Khan, Sharif, Sarwar, and Ameen (2010), who studied some varieties of flaxseed and obtained crude protein between 22.37–27.24% and ash 3.18–4.35%, whereas lipid contents were lower (35.03–41.23%). The chemical composition were also similar to the TACO (Brazilian Food Composition Table, UNICAMP, 2006) for flaxseed with values of 6.7% of moisture, 3.7% of ash, 14.1% of crude protein and 32.3% of carbohydrates. Variations in proximate composition may result from several factors such as edaphoclimatic, genetic and aging factors, and the conditions of extraction. As Table 1 shows, variations in SubFE conditions and the three different SEM methods led to quite similar extraction yields.

### 3.2. Fatty acid quantification

Table 2 shows the results for FA composition of the SubFE oil. Palmitic (16:0), stearic (18:0), oleic (18:1 $n-9$), linoleic (18:2 $n-6$) and α-linolenic (18:3 $n-3$) were the main FA present in the extracted oils (Table 2), and similar results have been reported by Pradhan et al. (2010) and Bozan and Temelli (2002). The most abundant FA was α-linolenic (18:3 $n-3$), which values ranging from 458.1 to 485.7 mg of FA $g^{-1}$. Note that linoleic and α-linolenic FA are essential in the human diet since they are used in the synthesis of longer chain fatty acids such as docosahexanoic and docosapentanoic acid. They also prevent inflammation and act in immunity (Simopoulos, 2004).

There was no significant difference among the two extraction methods in regard to the FA quantitation values. Foods placed in the healthy group are also characterized by the FA ratio of the $n-3$ family of the $n-6$ family ($n-3/n-6$). The flaxseed oils showed $n-3/n-6$ values of 3.95 in 45 °C and 10 MPa (test A) and 3.37 in 30 °C and 8 MPa (test B), with a mean of 3.79. According to the (WHO (1995)), the daily intake of $n-3/n-6$ between 1:5 and 1:10 are excellent values in regard to the prevention of cardiovascular diseases, and action in combating hypertension and chronic diseases. All flaxseed oils obtained by the two different extraction methods and conditions presented $n-3/n-6$ ratio excellent values, according to WHO, and it may cooperate to healthy eating.

For the sum of saturated fatty acids (SFA) and polyunsaturated fatty acids (PUFA), the quantity of SFA oil was higher in the SE and the lowest was for the oil obtained using 45 °C and 10 MPa (Test A), whereas the amount of PUFA was higher in this test. Therefore SE extraction seems to have caused PUFA oxidation to some extent, which is avoided by SubFE. PUFA, due to their highly unsaturated fatty acids structures, are more susceptible to oxidation as compared to SFA (Marques, Valente, & Rosa, 2009).

### 3.3. Phytosterols and tocopherols quantitation

β-Tocopherol was the only tocopherol obtained. For the phytosterols class, the ones obtained were: campesterol, stigmasterol and sitosterol in all flaxseed oils. Table 3 shows the results for quantitation of β-tocopherol, and for three major phytosterols (campesterol, stigmasterol and sitosterol). Significant variations in the amount of all of the bioactive components were noted. For campesterol and sitosterol, the highest amounts were found for the SE oil, which likely results from the higher temperature used for SE. High temperatures likely assist in the extraction of campesterol and sitosterol, which are also less susceptible to thermal degradation.

Ciftci, Przybylski, and Rudzinska (2012), using the methodology proposed by Folch et al. (1957) for lipid extraction, have reported the same sterols in flaxseed oil with extraction yields of 145.0, 23.8 and 58.4 mg 100 $g^{-1}$ of sitosterol, stigmasterol and campesterol, respectively.

Accordingly, Bozan and Temelli (2002) have also reported on the levels of tocopherols in flaxseed oil extracted by either supercritical $CO_2$ or Soxhlet and found that the sum of β and γ-tocopherol extracted by Soxhlet (73.9 mg 100 $g^{-1}$ of flaxseed oil) was also higher than that in the oil extracted by supercritical $CO_2$ (53.7 mg 100 $g^{-1}$ of flaxseed oil).

### 3.4. Triacylglycerol composition

TAG profiles of the flaxseed oils where then rapidly and efficiently compared via direct infusion ESI(+)-MS. Mass spectra of Fig. 1 show the ions distribution in flaxseed oils by different extractions methods. Representative mass spectra of different conditions of SubFE extraction are available in the Supplementary data (Fig. 3). The TAG molecules were detected in these spectra mainly in three different cationic ion forms: as their protonated $[M+H]^+$, sodiated $[M+Na]^+$ or ammoniated $[M+NH_4]^+$ molecules.

Flaxseed oils obtained by SEM and SubFE extractions were therefore directly analyzed by ESI(+)-MS and unique TAG profiles were obtained (Fig. 1). Note in particular the unique feature of the ESI(+)-MS profile of flaxseed oil with a major cluster of ions around $mV/z$ 877, a second minor cluster around $m/z$ 851 and two small clusters of ions around $m/z$ 577 and 599. This profile is quite unique and contrasting to those for other more common vegetable oils which normally display a richer set of TAG ions (Simas et al., 2012).

**Table 2**
Quantification of fatty acids (mg $g^{-1}$ of oil), summations and $n-3/n-6$ ratio of flaxseed oil (*Linum usitatissimum* L.) extracted by the method of Bligh & Dyer, Soxhlet, Folch, Less & Stanley and subcritical fluid using propane.

| FA | A | B | C | D | E | SE | FLS | BD |
|---|---|---|---|---|---|---|---|---|
| 16:0 | $60.38^{b} \pm 0.4$ | $60.88^{ab} \pm 0.3$ | $61.05^{ab} \pm 0.3$ | $63.74^{a} \pm 3.0$ | $61.80^{ab} \pm 1.3$ | $62.30^{ab} \pm 0.2$ | $62.20^{ab} \pm 0.3$ | $62.62^{ab} \pm 0.1$ |
| 16:1*n*−9 | $1.09^{abc} \pm 0.1$ | $1.04^{bc} \pm 0.1$ | $1.00^{c} \pm 0.1$ | $1.17^{a} \pm 0.1$ | $1.06^{bc} \pm 0.1$ | $1.13^{ab} \pm 0.1$ | $1.03^{bc} \pm 0.1$ | $1.06^{bc} \pm 0.1$ |
| 18:0 | $59.11^{cd} \pm 1.2$ | $60.71^{bc} \pm 0.2$ | $61.15^{b} \pm 0.8$ | $60.65^{bc} \pm 0.4$ | $61.76^{ab} \pm 0.2$ | $63.29^{a} \pm 1.0$ | $60.95^{bc} \pm 0.5$ | $58.18^{d} \pm 0.1$ |
| 18:1*n*−9 c | $215.91^{b} \pm 2.7$ | $219.76^{ab} \pm 0.8$ | $220.39^{a} \pm 0.9$ | $220.53^{a} \pm 1.1$ | $220.69^{a} \pm 1.7$ | $220.02^{ab} \pm 2.3$ | $219.03^{ab} \pm 0.8$ | $216.78^{ab} \pm 0.4$ |
| 18:1*n*−7 | $7.76^{abc} \pm 0.1$ | $7.48^{bc} \pm 0.3$ | $7.34^{c} \pm 0.2$ | $8.08^{a} \pm 0.3$ | $7.93^{ac} \pm 0.1$ | $7.69^{abc} \pm 0.2$ | $7.77^{abc} \pm 0.1$ | $7.99^{a} \pm 0.1$ |
| 18:2*n*−6 | $123.03^{ab} \pm 0.4$ | $123.25^{ab} \pm 0.9$ | $123.32^{ab} \pm 0.4$ | $135.94^{a} \pm 13.7$ | $127.05^{ab} \pm 3.6$ | $121.91^{b} \pm 0.2$ | $124.22^{ab} \pm 0.3$ | $124.97^{ab} \pm 0.3$ |
| 18:3*n*−3 | $485.73^{a} \pm 5.2$ | $478.12^{a} \pm 1.4$ | $478.25^{a} \pm 1.6$ | $458.11^{b} \pm 18.2$ | $469.21^{ab} \pm 6.8$ | $475.08^{ab} \pm 3.2$ | $473.76^{ab} \pm 1.7$ | $482.63^{a} \pm 0.1$ |
| 24:0 | $1.07^{abc} \pm 0.1$ | $1.02^{bcd} \pm 0.1$ | $0.96^{cd} \pm 0.1$ | $1.06^{abc} \pm 0.1$ | $1.10^{ab} \pm 0.1$ | $1.15^{a} \pm 0.1$ | $1.01^{bcd} \pm 0.1$ | $0.89^{d} \pm 0.1$ |
| SFA | $120.56^{d} \pm 1.3$ | $122.61^{bcd} \pm 0.3$ | $123.17^{abcd} \pm 0.9$ | $125.45^{ab} \pm 3.0$ | $124.66^{abc} \pm 1.4$ | $126.74^{a} \pm 1.1$ | $124.16^{abcd} \pm 0.6$ | $121.69^{cd} \pm 0.2$ |
| MUFA | $224.76^{b} \pm 2.7$ | $228.28^{ab} \pm 0.8$ | $228.73^{ab} \pm 0.9$ | $229.78^{a} \pm 1.1$ | $229.68^{a} \pm 1.7$ | $228.85^{ab} \pm 2.3$ | $227.82^{ab} \pm 0.8$ | $225.83^{ab} \pm 0.4$ |
| PUFA | $608.76^{a} \pm 5.2$ | $601.36^{ab} \pm 1.6$ | $601.57^{ab} \pm 1.6$ | $594.05^{b} \pm 22.8$ | $596.26^{b} \pm 7.6$ | $597.00^{b} \pm 3.2$ | $597.99^{b} \pm 1.7$ | $607.60^{a} \pm 0.3$ |
| *n*−3 | $485.73^{a} \pm 5.2$ | $478.12^{a} \pm 1.4$ | $478.25^{a} \pm 1.6$ | $458.11^{b} \pm 18.2$ | $469.21^{ab} \pm 6.8$ | $475.08^{ab} \pm 3.2$ | $473.76^{ab} \pm 1.7$ | $482.63^{a} \pm 0.1$ |
| *n*−6 | $123.03^{ab} \pm 0.4$ | $123.25^{ab} \pm 0.9$ | $123.32^{ab} \pm 0.4$ | $135.94^{a} \pm 13.7$ | $127.05^{ab} \pm 3.6$ | $121.91^{b} \pm 0.2$ | $124.22^{ab} \pm 0.3$ | $124.97^{ab} \pm 0.3$ |
| *n*−3/*n*−6 | $3.95^{a} \pm 0.1$ | $3.88^{ab} \pm 0.1$ | $3.88^{ab} \pm 0.1$ | $3.37^{b} \pm 0.1$ | $3.69^{ab} \pm 0.1$ | $3.90^{ab} \pm 0.1$ | $3.81^{ab} \pm 0.1$ | $3.86^{ab} \pm 0.1$ |

BD: Bligh and Dyer, SE: Soxhlet, FLS: Folch, Less & Stanley. Mean values ± standard deviation; means followed by different letters in the same row demonstrated significant difference by Tukey test ($p < 0.05$). A, B, C, D and E: letters representing the testing of extraction with subcritical fluid. MUFA = total monounsaturated fatty acids; PUFA = total polyunsaturated fatty acids; *n*−6 = total *n*−6 fatty acids; *n*−3 = total *n*−3 fatty acids; (detection limit = 0.015 mg $g^{-1}$).

**Table 3**
Quantification of tocopherols and phytosterols (mg 100 $g^{-1}$) in flaxseed oil (*Linum usitatissimum* L.) extracted by the methods of Bligh & Dyer, Soxhlet, Folch, Less & Stanley and subcritical fluid using propane.

| Method | β-Tocopherol | Campesterol | Stigmasterol | Sitosterol |
|---|---|---|---|---|
| A | $33.84^{a} \pm 2.2$ | $42.81^{b} \pm 2.7$ | $12.39^{a} \pm 1.1$ | $75.67^{c} \pm 1.9$ |
| B | $29.43^{bcd} \pm 0.6$ | $41.04^{b} \pm 2.8$ | $12.25^{a} \pm 1.7$ | $72.36^{c} \pm 1.9$ |
| C | $32.38^{abc} \pm 0.5$ | $38.92^{b} \pm 1.1$ | $11.92^{a} \pm 1.3$ | $77.93^{bc} \pm 8.3$ |
| D | $33.76^{ab} \pm 1.1$ | $43.07^{b} \pm 2.7$ | $13.92^{a} \pm 2.8$ | $89.99^{b} \pm 2.3$ |
| E | $32.42^{abc} \pm 1.0$ | $39.07^{b} \pm 1.7$ | $12.69^{a} \pm 1.4$ | $81.47^{bc} \pm 5.3$ |
| SE | $25.23^{d} \pm 1.9$ | $60.08^{a} \pm 4.1$ | $4.63^{b} \pm 1.9$ | $147.11^{a} \pm 6.5$ |
| FLS | $28.44^{cd} \pm 2.2$ | $41.37^{b} \pm 1.7$ | $9.74^{a} \pm 1.4$ | $77.74^{bc} \pm 3.6$ |
| BD | $25.28^{d} \pm 1.6$ | $44.88^{b} \pm 3.8$ | $4.31^{b} \pm 0.6$ | $79.30^{bc} \pm 6.8$ |

Mean values ± standard deviation; means followed by different letters in the same row demonstrated significant difference by Tukey test ($p < 0.05$). BD: Bligh and Dyer, SE: Soxhlet, FLS: Folch, Less & Stanley. A, B, C, D and E: letters representing the testing of extraction with subcritical fluid.

Major ions in the spectra of Fig. 1 are assigned, according to the FA composition of flaxseed oil summarized in Table 2 (α-linolenic (18:3*n*−3, Ln), linoleic (18:2*n*−6, L), oleic (18:1*n*−9, O), palmitic (16:0, P) and stearic (18:0, S)), as follow: *m*/*z* 851 ($[TAG+NH_4]^+$, OPP), *m*/*z* 855 ($[TAG+H]^+$, LLP), *m*/*z* 873 ($[TAG+H]^+$, LnLnLn), *m*/*z* 875 ($[TAG+H]^+$, LnLnL), *m*/*z* 877 ($[TAG+H]^+$, LnLnO and LnLL), *m*/*z* 879 ($[TAG+H]^+$, LnLO and LLL), *m*/*z* 881 ($[TAG+H]^+$, LLO; $[TAG+Na]^+$, OOP), *m*/*z* 883 ($[TAG+Na]^+$, PSO) and *m*/*z* 885 ($[TAG+H]^+$, OOO).

Note that the SubFE oils produced nearly identical spectra (Fig. 1) which indicate very similar and characteristic TAG compositions, and no traces of oxidation, which is characterized by sodium adducts with +16 and +36 *m*/*z* shifts (Simas et al., 2012). Flaxseed oils obtained by the SEM methods of Soxhlet, Folch and Bligh & Dyer extractions showed additional ions particularly in the *m*/*z* 890 above (Fig. 1), which were attributed mainly to degradation products and/or oil components. This ions relative abundance were low in Flaxseed oils and their presence is expected in a crude oil with high unsaturation degree (Chasquibol et al., 2014).

For instance, the ESI(+)-MS of the flaxseed oil obtained by Soxhlet extraction (Fig. 1, SE) displayed a large set of unique ions mainly of *m*/*z* 485, 529, 551, 573, 595, 617, 639, 661, 683 and 705, which were attributed mainly to diacylglycerols (DAG) due to TAG hydrolysis. Facilitated hydrolysis and therefore the presence of DAG is also common for crude oils with high unsaturation degrees. For the Folch extraction (Fig. 1, FLS), the ESI(+)-MS showed another unique set of ions mainly of *m*/*z* 496, 522, 760, 784, 921, 977 and 1040, whereas the profile for the Bligh & Dyer oil (Fig. 1, BD) displayed the unique set of ions of *m*/*z* 667, 740, 814, 963 and 1040. These differences in the ESI(+)-MS profiles of the SubFE (Fig. 1, A) flaxseed oils when compared to the SEM oils show that the SubFE flaxseed oil displays fortunately similar TAG qualitative profiles, with therefore no extraction discrimination, but higher purity.

ESI-MS (Cabral et al., 2012), as well as other related techniques such as EASI-MS (Simas et al., 2010), offer rapid and efficient protocols for vegetable oil quality control and typification, with the detection of characteristic and representative TAG profiles. In addition, these spectra also reveal oxidation level (Simas et al., 2012), FA composition and unsaturation levels.

### 3.5. Differential scanning calorimetry (DSC)

Table 4 shows the induction time, which determines the point where the oils begin to be oxidized, the logarithmic equations and the values of their regression coefficients ($R^2$) obtained for different temperatures in the DSC analysis. The highest values of the induction time for the SubFE oil indicate higher resistance to oxidation compared to all other SEM oils, showing the effectiveness of the SubFE method. This result also confirms that SubFE extraction more efficiently preserves the natural antioxidants present in the oils. This preservation has been also noted when comparing seed oil extractions with supercritical $CO_2$ and subcritical *n*-propane with conventional extraction with hexane (Corso et al., 2010; Pederssetti et al., 2011).

The flaxseed oil extracted by the BD method was oxidized instantly upon contact with oxygen flow at 140 °C. The Induction times and oxidation temperature showed a linear behavior with satisfactory $R^2$ values in all samples.

### 3.6. Principal components analysis

Variation ($p > 0.05$) in the quantification of sterols and tocopherols was observed in the SubFE oils extracted by using different temperatures and pressures as well as for the three SEM methods. PCA analysis was performed to try to find correlations for the amount of key components in relation to the extraction method (Fig. 2A).

The explained variance (%) for each principal component (PC) indicated the optimal number of principal components (Fig. 2A). PCA clearly separates two groups represented by ellipses. The variances explained by PC1 and PC2 were 78.32% and 19.1%,

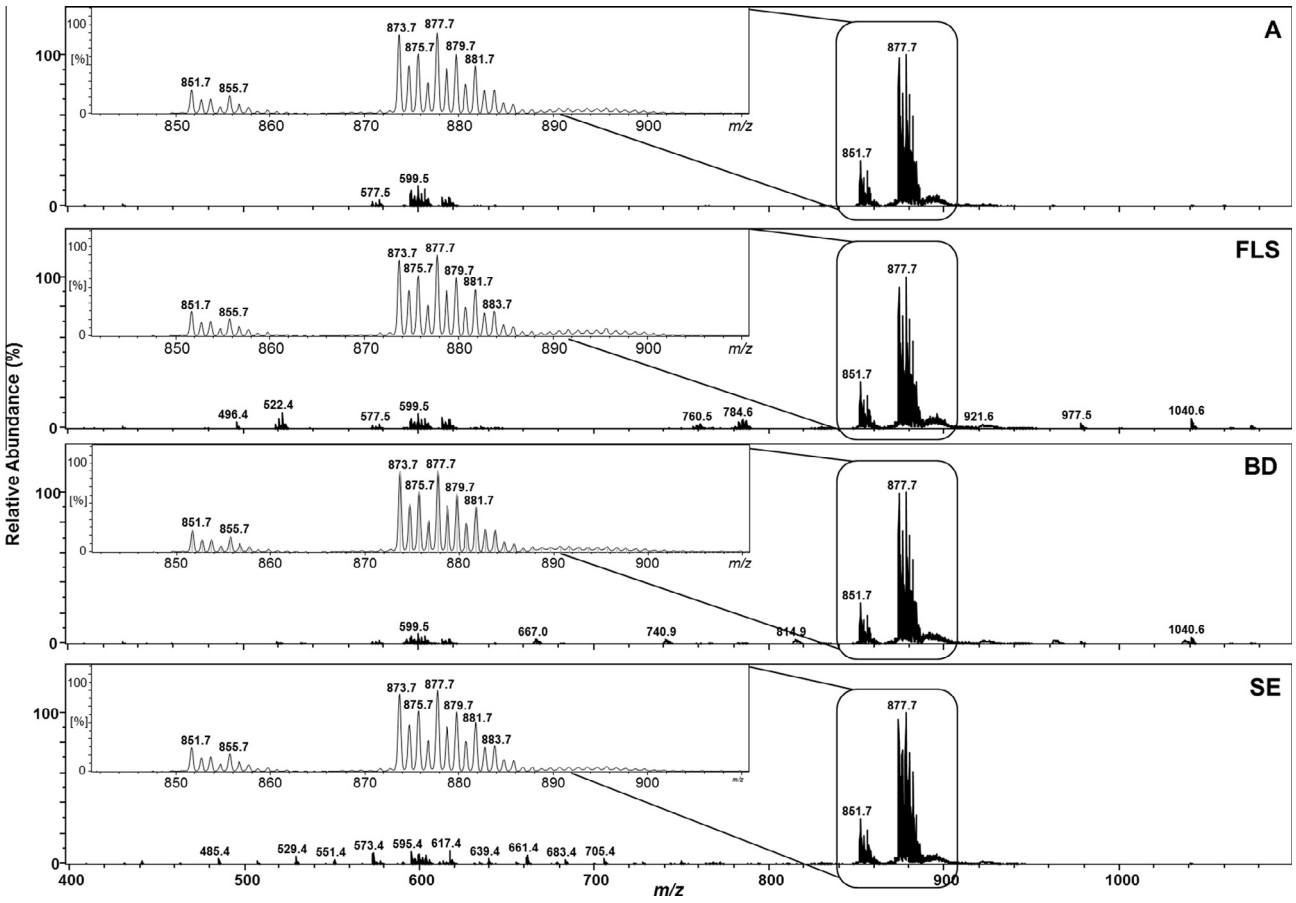


**Fig. 1.** ESI(+)-MS finger printings of methanol solutions of flaxseed oil obtained by follow methods: (A) subcritical fluid extraction with propane, (FLS) Folch, (BD) Bligh & Dyer and (SE) Soxhlet.

**Table 4**
Time of oxidative induction obtained by differential scanning calorimetry (DSC) and its logarithmic regression equation among $T_0$ and the temperatures of the isotherms for flaxseed oils (*Linum usitatissimum* L.) extracted by the methods of Bligh & Dyer, Soxhlet, Folch, Less & Stanley and subcritical fluid using propane.

| Temperature (°C) | 110 | 120 | 130 | 140 | Regression equation | Coefficient of determination ($R^2$) |
|---|---|---|---|---|---|---|
| Extraction | DSC $T_0$ (min) | | | | | |
| A | 89.5 | 44.8 | 25.5 | 15.1 | $T = 458.25 - 38.85 \log_{10} T_0$ | 0.9936 |
| FLS | 30.2 | 12.8 | 8.1 | 5.1 | $T = 438.76 - 38.65 \log_{10} T_0$ | 0.9588 |
| BD | 33.2 | 11.2 | 4.9 | 0.5 | $T = 419.15 - 23.92 \log_{10} T_0$ | 0.9877 |
| SE | 53.9 | 18.5 | 14.9 | 8.3 | $T = 444.77 - 36.63 \log_{10} T_0$ | 0.8909 |

A: conditions of temperature (°C) e pressure (MPa), respectively: 45 e 10; BD: Bligh e Dyer; SE: Soxhlet, FLS: Folch, Less & Stanley.

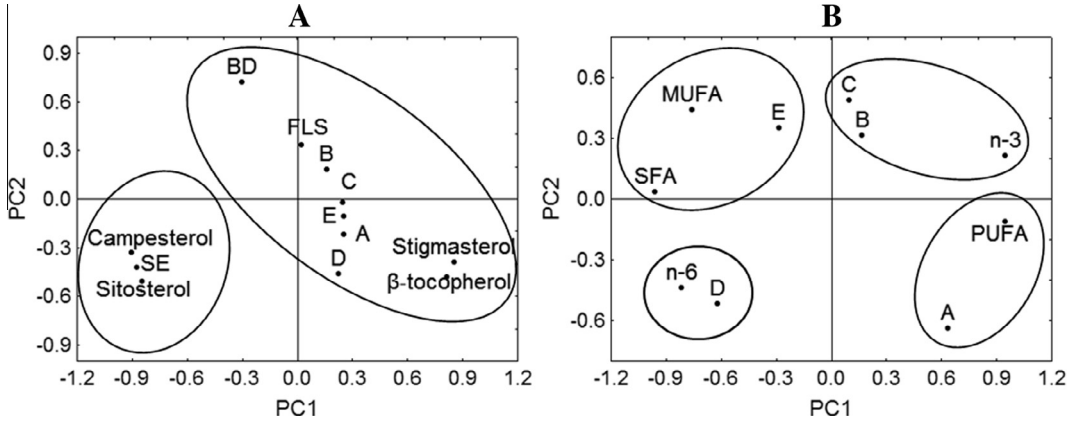


**Fig. 2.** PCA of sterols and tocopherols, PCA of sums de FA for flaxseed Oil. A, B, C, D and E: letters representing the testing of extraction with subcritical fluid. MUFA = total monounsaturated fatty acids; PUFA = total polyunsaturated fatty acids; $n-3$ = total $n-3$ fatty acids.

respectively, totalizing as much as 97.42%. The SE extraction method was characterized by the highest concentrations of campesterol and sitosterol, whereas the other methods were characterized by their levels of β-tocopherol and stigmasterol.

The extraction of higher quantities of sterols by the SE method likely results from the high temperature used, due therefore to increased solubility, whereas reduced degradation were due to high thermal stability of these molecules (SE extraction was likely aided also by the presence of the natural oxidant vitamin E in the flaxseed oils.

A PCA was also performed to evaluate the influence of conditions used in the SubFE via the sums of FA (Fig. 2B). This PCA shows that the extraction conditions significantly influences the amount of FA since groups were clearly separated. The explained variance in Fig. 2B was 89.57% for PC1 and 10.08% for PC2, totaling as much as 99.65%. Conditions B and C were more efficient in relation to the $n-3$ PUFA extraction. As Fig. 2B shows, there is a similarity for $n-6$ and PUFA, according to PC2, and between $n-3$ and PUFA, according to PC1.

## 4. Conclusion

Subcritical *n*-propane at 45 °C and 10 MPa applied to flaxseed oil extraction has been shown to be an effective alternative method when compared to conventional methods. SubFE offers a relatively fast procedure and an oil that is free of toxic residual solvents. The oil obtained by SubFE has also higher quality as measured by the amounts of bioactive compounds ($n-3$, tocopherol and phytosterols), greater oxidative stability and higher purity compared with oils obtained by SEM.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.05.033.

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