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Characterization and incorporation of extracts from olive leaves obtained through maceration and supercritical extraction in Canola oil: Oxidative stability evaluation

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ABSTRACT

Olive leaves (OL) are considered a potential source of bioactive compounds mainly due to its high content of phenolic compounds, widely known as natural antioxidants. The main objective of this study was to compare the performance of three OL extracts obtained by different extraction techniques in protecting canola oil against oxidative damage. The technologies evaluated were maceration with ethanol/water 75:25 (v/v), supercritical fluid extraction with CO_2 (SC–CO_2) and SC-CO_2 with 10% ethanol as modifier (SC–CO_2/EtOH). Each extract was analyzed as for total phenolic compounds (TPC), antioxidant activity (ABTS assay) and phenolic composition by reversed phase liquid chromatography-quadrupole-time of flight mass spectrometry. The oxidative stability of canola oil with or without the incorporation of 250 mg/kg of each extract was assessed during five weeks of storage at 60 °C. Peroxide, K_{232}, K_{270}, and Rancimat values, besides tocopherols content were determined. Macerated extract showed the highest TPC and antioxidant activity, but both SC-CO_2 extracts were more effective in preserving tocopherols. In addition, SC-CO_2 extracts delayed the oxidation progress as they lead to higher induction periods than control and macerated extracts, and a slower increase in peroxide values. Results obtained reinforce the use of supercritical fluid technology to obtain antioxidants compounds from natural sources.

1. Introduction

Canola oil is one of the most nutritive vegetable edible oils due to its high content of polyunsaturated fatty acids (PUFA) of about 35%, with a 2:1 ratio of monounsaturated to polyunsaturated fats, and also it is a rich source of vitamin E (Farahmandfar & Ramezanizadeh, 2018; Salami et al., 2020). However, also due to its fatty acid composition, with a high PUFA content, it is one of the most susceptible to suffer oxidation processes (Farahmandfar & Ramezanizadeh, 2018).

Natural antioxidants present in vegetable oils include: tocols, oryzanols and biophenols, although they are not necessarily found in all oils (Aguilar-Garcia et al., 2007). Synthetic antioxidants which are generally added include: butylhydroxyanisole (BHA), butylhydroxytoluene (BHT) and *tert*-butylhydroquinone (TBHQ). Although these types of antioxidants are attractive due to its low cost and because they have showed great effectiveness and stability, their use is limited as they may generate toxic effects (Xu et al., 2021). For instance, BHA and BHT have already been found to be responsible for adverse effects on the liver and for carcinogenesis in animal studies (Botterweck et al., 2000). The potential carcinogenic and teratogenic effects of synthetic antioxidants has increased the interest in searching for alternatives to the addition of this type of antioxidants and also in obtaining natural extracts with high antioxidant activity.

Although *Olea europaea* trees traditionally come from the Mediterranean areas, olive growing has been practiced in Uruguay for many years, and has been constantly increasing since the beginning of the twenty-first century (Silvera et al., 2012). Olive oil production, as well as table olives, generates great volume of solid residues among which are the leaves (Lama-Muñoz et al., 2020). Olive leaves (OL) result from tree pruning, that is necessary for the correct development and growing of the tree, likewise it allows obtaining higher productivity. Olive trees which are destined to olive oil production need to be pruned annually,

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while trees destined to produce table olives should be pruned biennially (Moya López & Mateo Quero, 2013). Therefore, OL constitute a residue during pruning and harvesting time reaching up to 10% of the total processed biomass (Lama-Muñoz et al., 2020). This residue can be revalorized, since it constitutes a potential source (and economical) of compounds with different type of bioactivity. For instance, the phenolic compounds, which present high free-radical scavenging capacity are able to play an important role in the protection against oxidative damage and cell ageing. Some studies have shown the great antioxidant activity that olive leaves have due to their high content of phenolic compounds, mainly Oleuropein and Hydroxytyrosol (Araújo et al., 2015; Galanakis & Kotsiou, 2017; Nunes et al., 2016). Taking advantage of this type of residue is interesting either in the pharmaceutical or food industry because it allows obtaining products with potential health benefits. Besides, revalorization of by-products that otherwise are actually discarded, also enables to minimize the negative effects on the environment and that are related to waste disposal (Dalla Rosa et al., 2019; Erbay & Icier, 2010).

As each vegetal matrix has its own characteristics, it is important to develop extraction processes in certain conditions for each raw material (Putnik et al., 2016). The success of the extraction depends on numerous factors. Among them, thermic stability of bioactive compounds which are intended to be recovered, the extraction technology being used, the type of solvent and time of the extraction process (Putnik et al., 2017; Roselló-Soto et al., 2015). The most reported extraction technique for polyphenols is the solid-liquid extraction through mechanic agitation with different organic solvents (Ameer et al., 2017; Tsimidou & Papoti, 2010). Several authors have used the following solvents in order to extract polyphenols: water, methanol, ethanol, acetone, ethyl acetate and their aqueous solutions (Contini et al., 2008; Dalla Rosa et al., 2019). However, this can imply negative effects upon performance, thermic degradation, and also consumption of great amounts of organic solvents (Putnik et al., 2018). Alternatively, it is important to obtain safe products for consumers, free of hazardous substances or negative effects which can risk their health and wellbeing. In this sense, both the extraction with pressurized liquids (PLE) and supercritical fluids (SFE) are the most used techniques based on the use of compressed fluids to obtain bioactive compounds (Herrero et al., 2013). These kind of processes are widely known as green technologies due to their low (or null) consumption of organic solvents. Moreover, there are successful experiences of their application to extract polyphenolic compounds in several vegetal matrixes which might be implemented at industrial scale (Ameer et al., 2017; Barba et al., 2016). However, few studies report the use of this kind of OL extracts as antioxidants in edible oils, focusing mostly on comparing them with conventional solvent extractions (Lafka et al., 2013). Due to low polarity of supercritical fluid extraction with CO_2 (SC–CO_2) alone, usually small amounts of a polar modifier are added in order to improve extract yield and selectivity of the process (Şahin & Bilgin, 2012).

In this context, the aim of this study was to compare the performance of three OL extracts obtained by different extraction techniques in protecting canola oil against oxidative damage The characterization of the extracts through reverse phase liquid chromatography-quadrupole-time of flight mass spectrometry (RP/HPLC-Q-TOF MS/MS) analysis was done. Moreover, the content of total phenols and the antioxidant activity (ABTS and ORAC-FL) was performed. Finally, the evaluation of the oxidative stability of Canola oil with and without the addition of extracts subjected to accelerated oxidation conditions (Schaal oven test) was carried out.

2. Materials and methods

2.1. Materials

The canola oil used for the experiments was a commercial sample without synthetic antioxidants added according to the information provided by the manufacturer.

The olive leaves used as plant material to obtain the extracts were collected from a local producer from Maldonado Department, Uruguay, and belong to the Arbequina cultivar (2019 harvest). Leaves were placed in trays at 20 °C protected from light and dried during 15 days. Subsequently, dried leaves were grinded using a Fritsch Pulverisette Model 14 mill, (1 mm particle size) and stored in low density polyethylene bags at −18 °C protected from light. Standards and reagents used for the analysis were supplied by Sigma-Aldrich (United States).

2.2. Gas chromatography determination of fatty acids in canola oil

Fatty acid profile of the oil was determined by gas chromatography, using nitrogen as the carrier gas. For that purpose, fat derivatization was performed according to IUPAC 2.301 (IUPAC, 1987). The equipment used was a Shimadzu Model GC-14B gas chromatographer equipped with a Supelco SP2560 column. Peak identification was accomplished through the analysis of authentic standards (F.A.M.E. Mix, C4–C24) supplied by Sigma–Aldrich (United States).

2.3. Tocopherols determination in canola oil

The analysis was performed by high-performance liquid chromatography (HPLC), using a Shimadzu Model 20 chromatographic system, equipped with a fluorescence detector (RF Model 20A XS) and a Macherey-Nagel C18 column (250 × 4.6 mm, 100 μm) at 40 °C. Briefly, 30 mg of oil were diluted in 1 mL of isopropanol (HPLC quality). A 50 μL aliquot was injected in the HPLC and the chromatogram was registered (λ_{ex} = 290 nm, λ_{em} = 330 nm), using acetonitrile (18%), methanol (13%), water with 0.5% of acetic acid (v/v) (69%) and isopropanol (0%) as the initial mobile phase, following the procedure described by Andrikopoulos et al. (1991). Tocopherols quantification was performed by using a calibration curve, in which α, β, γ and δ tocopherols were used as standards (Sigma Aldrich).

2.4. Supercritical CO_2 extraction

Supercritical extractions were performed in a laboratory scale system. For each experiment, the extraction cell (25 mL) was filled with dried and milled leaves. Flow rate was constant during the whole extraction procedure using an average CO_2 flow of 0.5 L/min (gas). The extraction conditions used were 30 MPa and 50 °C for pressure and temperature respectively, one without co-solvent (SC–CO_2) and the other one with the addition of 10% ethanol as co-solvent in relation to the CO_2 flow (SC–CO_2/EtOH). Ethanol was selected because of its low toxicity compared to other alcohols. Although the addition of a modifier to CO_2 was mandatory in order to extract polyphenols, we decided to compare both type of supercritical extractions since other compounds with antioxidant activity could also be extracted under SC-CO_2 conditions. Pressure conditions were chosen according to the results reported by Le Floch et al. (1998), where the amount of polyphenols increased with increasing the pressure until 33.4 MPa at 100 °C with ethanol as CO_2 modifier. However, in order to protect the thermolabile compounds temperature was set at 50 °C. The experimental extraction apparatus is described in several studies available in the literature (Abirached et al., 2020; Vieitez et al., 2018). Both extracts were stored at −18 °C until they were incorporated in the Canola oil. Experiments were done in triplicate.

2.5. Conventional solvent extraction (maceration)

Macerations were adapted and scaled from the method described by (Santos et al., 2012). Briefly, 30 mL of 75% ethanolic solution were added to 2 g of dried and ground olive leaves in a 50 mL Falcon tube. The mix was constantly stirred in the dark during 4 h, at room temperature. Then, it was centrifuged at 2146 g during 15 min and the supernatant

was separated. The residue was washed with 15 mL of the solvent used, and then stirred during 2 h. Again, the separation procedure was performed followed by the evaporation of the solvent under a nitrogen flow in a water bath at 40 °C. The extract obtained was stored at −18 °C and protected from light until it was incorporated in the Canola oil. Experiments were done in triplicate.

2.6. Characterization of the extracts

2.6.1. Total phenolic compounds (TPC)

Folin-Ciocalteau method was used to determine total phenol content of the extracts according to (Singleton et al., 1999) with modifications by (Fernández-Fernández et al., 2019). Briefly, 10 μL of standard solution or sample (extract dissolved in 6 mL/100 mL DMSO) were added to a 96-well microplate (Thermo Scientific), followed by 200 μL of Na_2CO_3 (20 mL/100 mL w/v). After 2 min, 50 μL of Folin reagent (1/5) were added to the mixture and the microplate was incubated in the dark during 30 min. Absorbance at 750 nm was measured using a Thermo-Scientific TM MULTISKAN GO microplate spectrophotometer (Waltham, Massachusetts, USA). A calibration curve was prepared using gallic acid as standard and results were expressed as gallic acid equivalents (mg GAE/g extract).

2.6.2. In vitro antioxidant activity

The antioxidant activity of the extracts was determined by the ABTS assay and the Oxygen Radical Absorbance Capacity (ORAC) method. The ABTS assay was performed according to (Re et al., 1999) modified by (Fernández-Fernández et al., 2019). Activated solution of ABTS•+ 7 mmol/L was added to a certain volume of phosphate buffer solution (5 mmol/L, pH 7,4) until an absorbance of 0,7 (750 nm) was obtained. Then, 10 μL of properly diluted extract (5.0 mg/mL for SFE and 0.5 mg/mL for macerated) or standard (0.25–1.5 mmol/L Trolox) were placed on the microplate and 190 μL of ABTS•+ working solution was added. After 10 min, the absorbance reading was performed at 750 nm using a MultiskanTM Go microplate spectrophotometer (Waltham, Massachusetts, USA). The ORAC-FL (Oxygen Radical Absorbance Capacity) test was performed according to (Ou et al., 2001) modified by (Dávalos et al., 2005). Fluorescence measurements of the final mixture (20 μL of sample or standard solution, 120 μL fluorescein 70 mmol/L and 60 μL AAPH 48 mM) were registered at excitation and emission wavelengths of 485 nm and 520 nm respectively on a Varioskan™ Lux multimodal plate (ThermoScientific, Massachusetts, USA) during 90 min at 37 °C. For both assays, a calibration curve with Trolox was used. The results were expressed as μmol Trolox eq. (TE)/g extract (dry matter).

2.7. Reversed phase liquid chromatography-quadrupole-time of flight mass spectrometry (RP/HPLC-Q-TOF MS/MS) analysis

Dried extracts (macerated, SC-CO_2 and SC-CO_2/EtOH) were dissolved in pure ethanol to a final concentration of 1 mg/mL, and 2 μL (in duplicates) were injected into a HPLC (model 1290) coupled to a quadrupole Q-TOF (6540 series) equipped with an Agilent Jet Stream thermal orthogonal ESI source, all from Agilent Technologies (Germany). MS control, data acquisition, and data analysis were carried out using the Agilent Mass Hunter Qualitative Analysis software (B.10.0). Compounds were separated using an Eclipe Plus C18 analytical column (100 × 2.1 mm, particle size 1.8 μm) with a C18 guard column (0.5 cm × 2.1 mm, particle size 1.8 μm), both from Agilent Technologies. LC-MS grade water and acetonitrile were used as mobile phase (A) and (B), respectively, and in both cases, 0.01% formic acid (v/v) was used as mobile phase modifier. The flow rate was set to 0.5 mL/min and the gradient started with 0% (B) at 0 min, 0–30% (B) in 7 min, 30–80% (B) in 2 min, 80–100% (B) in 2 min, 100% (B) in 2 min, and 3 min of post-time. The column temperature was held at 40 °C during the separation. The mass spectrometer was operated in negative mode using the following parameters: capillary voltage of 3000 V and with a m/z range from 25 to 1100. Nebulizer pressure was set at 40 psig and the drying gas flow rate was fixed to 8 L/min and 300 °C. The sheath gas flow was 11 L/min at 350 °C. 110 V was chosen for the fragmentor voltage, whereas the skimmer and octapole voltage were 45 V and 750 V, respectively. MS/MS analyses were performed employing the auto MS/MS mode using 5 precursor per cycle, dynamic exclusion after two spectra (released after 0.5 min), and collision energies of 20 and 40 V. For proper mass accuracy, spectra were corrected using ions m/z 119.0363 ($C_5H_4N_4$) and 966.0007 ($C_{18}H_{18}O_6N_3P_3F_{24}$ + formate), simultaneously pumped into the ionization source.

For data processing, LC-MS raw data files were firstly converted to ABF format using Reifycs Abf Converter (accessible at: http://www.reifycs.com/AbfConverter/). Data processing was performed using MS-DIAL (v. 4.60) software for deconvolution, peak picking, alignment, and identification (Tsugawa et al., 2015). The following parameters were used: retention time begin, 0 min; retention time end, 14 min; mass range begin, 0 Da; mass range end, 1100 Da; MS1 tolerance, 0.01 Da; smoothing level, 3 scans; minimum peak width, 5 scans; minimum peak height, 1000 amplitude; mass slice width, 0.1 Da; sigma window value for deconvolution, 0.1; accurate mass tolerance for MSP library, 0.01 Da; identification score cut off for MSP library, 75%; retention time tolerance for alignment, 0.1 min; MS1 tolerance for alignment, 0.015 Da. Peak area calculation was performed by summing up the area of the different detected molecular species for each particular compound ([M-H]-, [2M-H]-, [M+Cl]-, [M+FA-H]- adducts), and total compound contribution was calculated as compound area/Total Ion Current area x 100. The MSP file used for annotation was generated by combining MS/MS spectra from NIST20 MS/MS database, the LipidBLAST mass spectral library (Kind et al., 2013), and the MassBank of NorthAmerica database (MoNA, available at https://mona.fiehnlab.ucdavis.edu/downloads). All metabolite were identified following the Metabolomics Standard Initiative (MSI) guidelines (Blaženović et al., 2019) as MSI level 2a (metabolites with precursor m/z and MS/MS spectral library matching). For compound classification, InChIKey identifiers were imported into the web-based ClassyFire application for batch conversion (https://cfb.fiehnlab.ucdavis.edu/) (Djoumbou Feunang et al., 2016).

2.8. Incorporation of the extracts obtained in Canola oil and experimental design

The amount of synthetic antioxidants allowed to be incorporated in vegetable oils is limited by the regulations of each country to values between 100 and 200 mg/kg depending on the compound to be used. However, for natural antioxidants there are no regulations. Therefore, a concentration of natural extract of 250 mg/kg was employed. A total of four systems were studied: Canola oil (control) and canola oil with the addition of 250 mg/kg of each extract (SC–CO_2, SC-CO_2/EtOH and macerated). The extracts were dissolved in ethanol at a final concentration of 25 mg/mL. To obtain the desired concentration of 250 mg/kg in the oils, aliquots containing 85 mg of the different extracts were incorporated into glass beaker with 340 g of canola oil. Then the oils were placed in ultrasonic bath at 40 °C for 10 min. Subsequently, all the oils with the different extracts were distributed in amber flasks. Oil storage experiments were conducted in an oven according to recommended practice AOCS Cg 5–97 (AOCS, 2017) (Schaal Oven test). Each sample was prepared and stored in 8 different amber flasks (one for each analysis time), closed, in an oven at 60 °C. During five weeks, the samples were analyzed on the following days of storage: initial time (0), 2, 7, 10, 14, 17, 22, 28 and 38.

2.9. Determination of oxidative stability of Canola oil

2.9.1. Peroxide value (PV)

Was determined by the standard method AOCS Cd 8–53 (AOCS, 1996) for all analysis times.

2.9.2. Spectrophotometric determination of oil deterioration

Conjugated dienes and conjugated trienes. Specific extinctions coefficients at 232 and 270 nm were determined using a ThermoScientific Genesys 10S UV–Vis spectrophotometer. Oil samples were diluted with cyclohexane to bring the absorbance within limits following the standard method of IUPAC method II. D. 23 (IUPAC, 1979). The analysis was performed on the following sampling times: initial (0), 1, 2, 4, 6 and 8.

2.9.3. Analysis of accelerated oxidative stability (Rancimat)

The efficiency of the different extracts in protecting Canola oil against an accelerated oxidation process was studied using 873 Biodiesel Rancimat (Metrohm, Switzerland). The procedure was performed according to the AOCS cd 12b-92 official technique (AOCS, 1997). The induction period (IP) of the oxidation process was determined at 100 °C, under air flow. The analysis was performed on the following sampling time: initial (0), 1, 2, 4, 6 and 8.

2.10. Statistical analysis

All the assays performed were done at least by triplicate. The data obtained for each assay were analyzed through variance analysis (ANOVA) and Tukey's test ($p<0.05$) to determine the existence of significant differences between the samples. Infostat software version 2020e was used.

3. Results and discussion

3.1. Weight yield and characterization of the extracts

Table 1 shows the weight yields of the extracts obtained by each of the different type of extractions. The highest performance was obtained by the ethanolic maceration (27.3%), followed by supercritical extraction using ethanol as co-solvent (SC–CO_2/EtoH) ($p < 0.05$). Şahin and Bilgin (2012) reported a similar extraction yield of 1.6% from olive tree leaves obtained by supercritical extraction at 30 MPa and 50 °C and 3.4% with ethanol as a co-solvent. Also, in accordance with the results reported by Vieitez et al. (2018).

The macerated extract demonstrated the higher results of TPC ($p < 0.05$) followed by the SC-CO_2/EtOH extract and lastly the SC-CO_2 extract (Table 1). This is consistent with the fact that phenols are polar compounds and are better extracted in similar solvents (Ameer et al., 2017; Vieitez et al., 2018). Specifically for olive leaves, previous studies have already reported that in case of supercritical extractions, it is essential to use a co-solvent if phenolic compounds are intended to be extracted (Xynos et al., 2012; Şahin & Bilgin, 2012). Regarding TPC content value in the extraction with ethanol maceration (2783 mg GAE/g dry olive leaves) is similar to that reported by Kiritsakis et al., 2010, where an ethanol solution of 60% was used and 2300–2400 mg of gallic acid per 100 g of dry leaves were obtained.

On the other hand, values reported by Jaski et al. (2018) related to total phenol content extracted through pressurized liquids was of 123 mg GAE/g extract, higher than the values obtained in the present study through supercritical extraction either using SC-CO_2 or SC–CO_2–EtOH (12.6 and 21.8 mg GAE/g extract respectively). This behavior is expected as ethanol was the pressurized liquid used.

According to (Japón-Luján et al., 2006), olive leaves constitute the part of the tree where the highest concentration of polyphenols is found. In addition, the high antioxidant power found in the extracts of olive leaves has been attributed to a synergistic effect between the different phenolic compounds present, compared to the same compounds separately (Vogel et al., 2015). The macerated extract showed the highest antioxidant activity ($p < 0.05$) either determined by the ABTS assay or ORAC assay (Table 1). This would indicate that, in the composition of the vegetal matrix used, there is a predominance of polar compounds (such as polyphenols) whose solubility in the selected solvent allows them to be extracted more easily. The results obtained for both of supercritical extracts are similar to the range reported by (Kamran et al., 2015), who obtained activity values between 100 and 550 μmol from TE/g extract (ABTS) by using different drying methods of olive leaves. On the other hand, results obtained for antioxidant activity of macerated extract is comparable with the reported by (Nicolí et al., 2019) who analyzed antioxidant activity determined by the ORAC method of macerated extracts from 15 different species of olive leaves, using the same type of solvent for the extraction, and the values obtained were between 810 and 4250 μmoles from TE/g.

Table 1
Weight yields (wt %), total phenolic compounds (mg GAE/g extract), and antioxidant activity of the different extracts.

Extract	SC-CO_2	SC-CO_2/EtoH	Maceration
Yield (wt %)	1.0 ± 0.1^{A}	1.9 ± 0.1^{B}	27.3 ± 0.2^{C}
TPC (mg GAE/g extract)	12.6 ± 0.3^{A}	26.1 ± 0.6^{B}	113.3 ± 3.2^{C}
Antioxidant activity (ABTS) (μmol of TE/g extract)	57.8 ± 1.9^{A}	163.3 ± 7.5^{B}	1424.0 ± 24.9^{C}
Antioxidant activity (ORAC) (μmol of TE/g extract)	225.1 ± 17.2^{A}	586.6 ± 13.7^{B}	2339.8 ± 30.4^{C}

In each row, those mean values that were significantly different from each other ($p < 0.05$) are shown with different capital letter.

3.2. Identified compounds obtained from different olive leaves extracts

Regarding the individual phenolic compounds identified by RP/HPLC-Q-TOF MS/MS, Table 2 summarizes the main polyphenols and their contribution to each of the extracts studied (qualitative analysis), ordered based on their retention time. As previously reported in the literature (Talhaoui et al., 2015), the most abundant compounds belong mainly to the family of simple phenols and phenolic acids derivatives (hydroxytyrosol, chlorogenic acid, *trans*-ferulic acid, *trans*-caffeic acid), flavonoids (quercetin-3-*O*-rutinoside, luteolin-4′-*O*-glucoside, luteolin 7-glucoside) and secoridoids (oleuropein, verbascoside). Other compounds such as lignans and triterpenoids were also present but with low peak intensities. High differences were found between the three extracts, not only referred to the total phenol content but also to their phenolic profile. As for the macerated extract, oleuropein and verbascoside, were the two compounds that presented higher contribution, followed by hydroxytyrosol, a group of four flavonoid glycosides (luteolin 7-glucoside, luteolin-4′-*O*-glucoside, quercetin-3-*O*-rutinoside and kaempferol-7-O-neohesperidoside), and *trans*-4-coumaric acid. In accordance with the results obtained for TPC analysis, this was the sample with higher amounts for most of the identified phenolic compounds, followed by SC-CO_2/EtOH and SC-CO_2 extracts respectively. In fact, from the 36 polyphenols reported, 34 were present in the macerated extract, while only 14 were identified in the SC-CO_2 extract and 30 in the SC-CO_2/EtOH. The most abundant phenolic compounds found in the SC-CO_2/EtOH extract were simple phenols and phenolic acids (*trans*-4-coumaric acid, hydroxytyrosol and *trans*-ferulic acid) followed by oleuropein, while the SC-CO_2 extract also showed predominance of this type of phenols except that oleuropein was not detected (Fig. 1). For instance, hydroxytyrosol and oleuropein (Fig. 1B and C) were demonstrated to possess high antioxidant activity responsible for scavenge free radicals. Free radical scavenging and antioxidant activity of phenolics compounds principally depends on the numbers and localization of hydrogen-donating hydroxyl groups on the aromatic ring of the phenolic molecules (Talhaoui et al., 2015). Besides, *trans*-Ferulic acid, which possess strong antioxidant activity and prevents lipid peroxidation (Rezaeiroshan et al., 2021), was present in higher amounts in SC-CO_2 and SC-CO_2/EtOH extracts.

Table 2
Tentatively identified compounds, compound class and total compound contribution (%) obtained from different olive leaves extracts (macerated, SC-CO_2 and SC-CO_2/EtOH) and analyzed by RP/HPLC-ESI (−) Q-TOF-MS/MS.

Tentative compound	Retention time (min)	Compound class	Total compound contribution (%)[a]		
			Macerated	SC-CO_2	SC-CO_2/EtOH
Chlorogenic acid	0.64	Phenolic acids	2.44	–	0.14
Succinic acid	0.74	Phenolic acids	0.56	0.58	1.10
Hydroxytyrosol	2.66	Simple phenols	**11.20**	**27.81**	**30.20**
Rosmarinic acid	3.24	Phenolic acids	0.51	–	–
Secologanoside	3.70	Secoiridoids	1.35		0.09
Vainillin isomer	3.75	Phenolic aldehyde	2.03	0.62	1.71
Methyl glucooleoside	3.81	Secoiridoids	1.10	–	–
Protocatechuic acid methyl ester	3.88	Phenolic acids	0.03	1.82	0.28
trans-Caffeic acid	3.96	Phenolic acids	1.96	–	3.85
4-Hydroxybenzaldehyde	3.97	Aldehyde	0.14	**12.74**	1.73
Pinoresinol	4.66	Lignans	0.02	1.65	0.09
trans-4-Coumaric acid	4.83	Phenolic acids	3.21	2.98	**37.51**
Luteolin-7,3′-di-O-glucoside	4.89	Flavonoids	1.39	–	–
trans-Ferulic acid	5,34	Phenolic acids	0.51	**24.60**	**10.06**
Eriodictyol-7-O-glucoside	5.51	Flavonoids	1.68	–	0.04
Quercetin-3-O-rutinoside	5,56	Flavonoids	**7.83**	–	–
Kaempferol-7-O-neohesperidoside	5,67	Flavonoids	**7.38**	–	–
Quercitin 3-glucoside	5,71	Flavonoids	0.54	–	0.02
Verbascoside	5.74	Secoiridoids	**16.23**	–	0.11
Luteolin 7-glucoside	5.80	Flavonoids	**10.38**	–	0.51
Coniferyl aldehyde	6,03	Methoxyphenols	0.03	**8.36**	0.27
Spherobioside	6,19	Flavonoids	2.35	–	–
Luteolin-4′-O-glucoside	6,39	Flavonoids	**8.33**	–	0.55
Apigenin-4′-glucoside	6.43	Flavonoids	0.41	–	0.02
Oleoside 11-methyl ester	6.84	Terpene glycosides	0.33	–	0.46
Oleuropein	7,20	Secoiridoids	**16.13**		**6.77**
Eriodictyol	7,34	Flavonoids	0.42		0.46
Methyl *p*-coumarate	7,67	Phenolic acids	–	2.47	0.08
Quercetin	7,70	Flavonoids	0.65	–	0.02
Luteolin	7,71	Flavonoids	0.18	–	0.03
Apigenin	8,36	Flavonoids	0.13	–	0.41
Chrysoeriol	8.48	Flavonoids	0.18	–	0.35
Ethyl *p*-coumarate	8,57	Phenolic acids	0.02	**9.06**	0.26
Euscaphic acid	9,44	Triterpenoids	0.28	0.44	0.99
3,19-Dihydroxyurs-12-ene-23,28-dioic acid	9,48	Triterpenoids	–	1.22	0.39
Pomolic acid	9,72	Triterpenoids	0.04	5.65	1.49

[a] Total compound contribution = compound area/Total Ion Current area * 100. Values showed in bold letters indicate the compounds with the higher contribution to each extract.

3.3. Incorporation of extracts in Canola oil

Table 3 shows the fatty acid composition of canola oil and its initial profile of tocopherols. Fatty acid profile is found within the expected values for a Canola oil (Orsavova et al., 2015) being oleic acid the highest (58.6%) followed by linoleic (23.8%) and linolenic acids (10.9%). Regarding the total tocopherol content, a value of 1683 mg/kg was obtained, being β and γ isomers the most abundant. The results related to the main tocopherols isomers are in agreement with those reported by Sayago et al. (2007). It has been shown that some tocopherols possess *in vitro* antioxidant activity that protects oils and foods from oxidation, while others protect human cells and tissues against free radicals *in vivo*. More specifically, α-tocopherol presents maximum activity *in vivo*, whereas β, γ and δ-tocopherols provide the higher protection to oils, and as a consequence, to the foods in which these oils are present (del Moral Navarrete, 2016), being γ and δ isomers the most stable against the oxidative process (Sayago et al., 2007).

In order to evaluate the stability of the different tocopherols in the analyzed oils, the loss percentage of each isomer respect to the initial values was calculated (Fig. 2). For all the systems studied, α tocopherol showed the higher loss percentages among all isomers. Additionally, when comparing as a function of storage time, the higher losses were found in the systems at the end of the study (38 days of storage) in most cases. This result was expected, as the systems were exposed to storage temperature during a longer period of time. It is worth noting that, the incorporation of the extracts to the oil resulted in the reduction of the loss percentages of total tocopherols. The lowest losses were found in the samples with the supercritical extracts added. Therefore, oil supplementation with olive leaf extract rich in polyphenols resulted in a more efficient protection of tocopherols against their decomposition, showing the mode of action of the different natural antioxidants on oil oxidative stability. This results were in agreement with that reported by Chiou et al., 2009, which studied the oxidative stability of different frying oils (sunflower oil, olive oil, and refined palm oil) supplemented with olive leaves extract obtained by methanol maceration. Thus, the use of agricultural by-products (olive leaves) and sustainable processes can allow solutions for preventing oils oxidation.

3.4. Oxidative stability of Canola oil

Fig. 3 shows the values of PV for each system in relation to the days of storage at 60 °C. In all cases, a continuous increase in PV with the increase of storage time was observed until a maximum (primary oxidation) followed by a decrease, indicating the stage where the rate of peroxide decomposition is higher than its rate of formation has been reached (start of the secondary oxidation). PV at initial time was 5.9 meq/Kg. From that point, the rate in which the initial increase occurs, as well as, the moment in which the maximum value is observed depends on the system being studied. Regarding the control system, it was observed that the value obtained for all sampling times (except for the fifth sampling time), presented significant differences ($p < 0.05$) with respect to the rest of the systems. As expected, the control system was the one that reached the highest level of peroxides (96.8 meq/kg) as it had no additional protection beyond its own antioxidants contained in

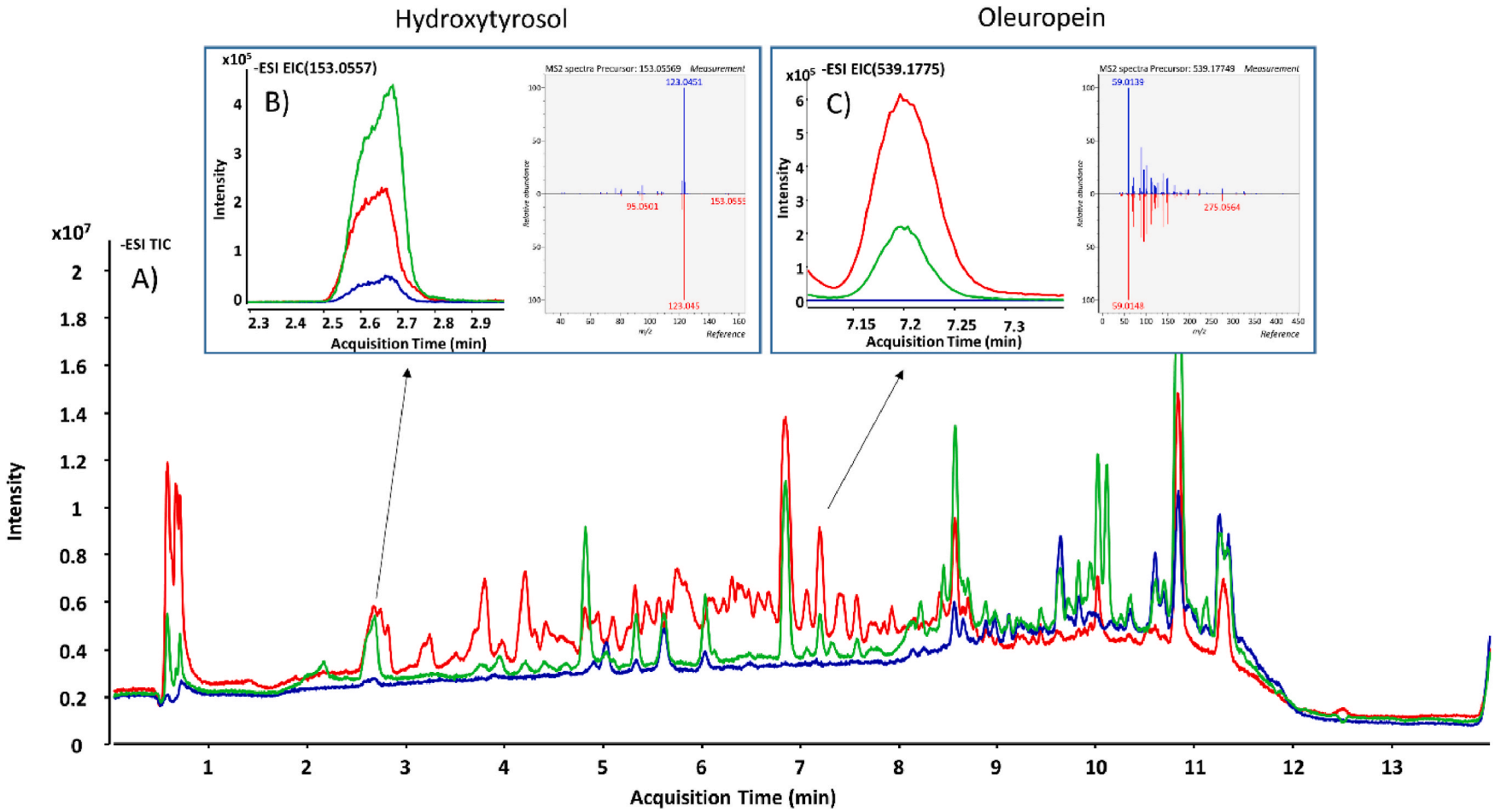

Fig. 1. Total ion chromatogram obtained by RP/HPLC-ESI (−) Q-TOF from macerated (red), SC-CO_2 (blue) and SC-CO_2/EtOH (green) olive leaves extracts (A). Extracted ion chromatogram and MS/MS fragmentation analyses of hydroxytyrosol (B) and oleuropein (C). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Fatty acid (%) and tocopherol composition (mg/kg) of Canola oil.

	Canola oil
C 16:0 (wt %)	4.1 ± 0.2
C18:0 (wt %)	1.5 ± 0.1
C 18:1 (wt %)	58.6 ± 0.7
C 18:2 (wt %)	23.8 ± 0.4
C18:3 (wt %)	10.9 ± 0.5
C 20:0 (wt %)	0.6 ± 0.1
α tocopherol (mg/kg)	228.1 ± 0.4
β+γ tocopherols (mg/kg)	1386.0 ± 0.7
δ tocopherol (mg/kg)	69.0 ± 0.4

the oil (tocopherols). This maximum was registered at 22 days of storage and then a decrease in the PV was observed, in accordance with the results reported by (Baik et al., 2004). For the oil systems with the extract added the maximum PV obtained were 68.9 meq/kg (17 days), 69.5 meq/kg (17 days) and 71.7 meq/kg (28 days) for the macerated extracts, SC–CO_2–EtOH and SC-CO_2 respectively. Therefore, during the first 17 days of storage (fifth sampling time) in which all systems are on the stage where primary oxidation is predominant, it is observed that the system with the SC-CO_2 extract added had the lowest PV. This means that this extract was the most effective to delay the oxidation process during the first stages, as the peroxide content was kept at the lowest values during more time before the triggering of an accelerated increase. Moreover, it was observed that the control system was the one with the higher PV followed by the system with the addition of the macerated extract. Once the secondary oxidation is triggered, it can be observed that the final PV in the control system is lower than in the rest of the systems. This is the result of a higher decomposition rate of the hydroperoxides (Domínguez et al., 2019). When comparing the three extracts incorporated in the Canola oil, results showed that the addition of the macerated extract resulted in the lowest level of protection against oxidation. This behavior differs from the expected, according to the results found in the characterization of the extracts where the macerated extract had shown the highest antioxidant activity, as well as, TPC content (Table 1). However, there are some compounds present in higher amounts in SC-CO_2/EtOH and SC-CO_2 extracts, like the *trans*-Ferulic acid, with high antioxidant activity to prevent the lipid peroxidation (Rezaeiroshan et al., 2021), methyl 4-hydroxycinnamate (Venkateswarlu et al., 2006), and ethyl *p*-coumarate (Neudörffer et al., 2004). Another possible explanation could be differences in affinity existing between the extract and the oil to which it is incorporated. Since SC-CO_2 is a non-polar solvent, this implies that the extract obtained has mostly hydrophobic character and that confers a higher capacity of dissolution when incorporated into an oily matrix (Vieitez et al., 2018). The polar paradox suggests that polar antioxidants are more active in bulk lipids than nonpolar antioxidants. However, recent results in bulk oils, demonstrate that the principal site of oxidation is not the air-oil interface, but association colloids formed with traces of water and surface active molecules such as phospholipids, free fatty acids, and hydroperoxides (Laguerre et al., 2015). Therefore, in bulk oil, some antioxidants, like phenolic compounds, do not exhibit behavior in accordance with the polar paradox. Thus, the effectiveness of polar and nonpolar antioxidants in edible oils not only depends on the type and concentration, also depends in the presence of minor components in bulk oil systems (Mishra et al., 2021). Consequently, even though it is important to increase the extract yield as occurred in the maceration with ethanol, this is not necessarily accompanied by a higher antioxidant activity for protecting edible oils. The use of polar solvents improves the extraction of polyphenols since they are polar compounds but at the same time they may present solubility issues in oils (non-polar).

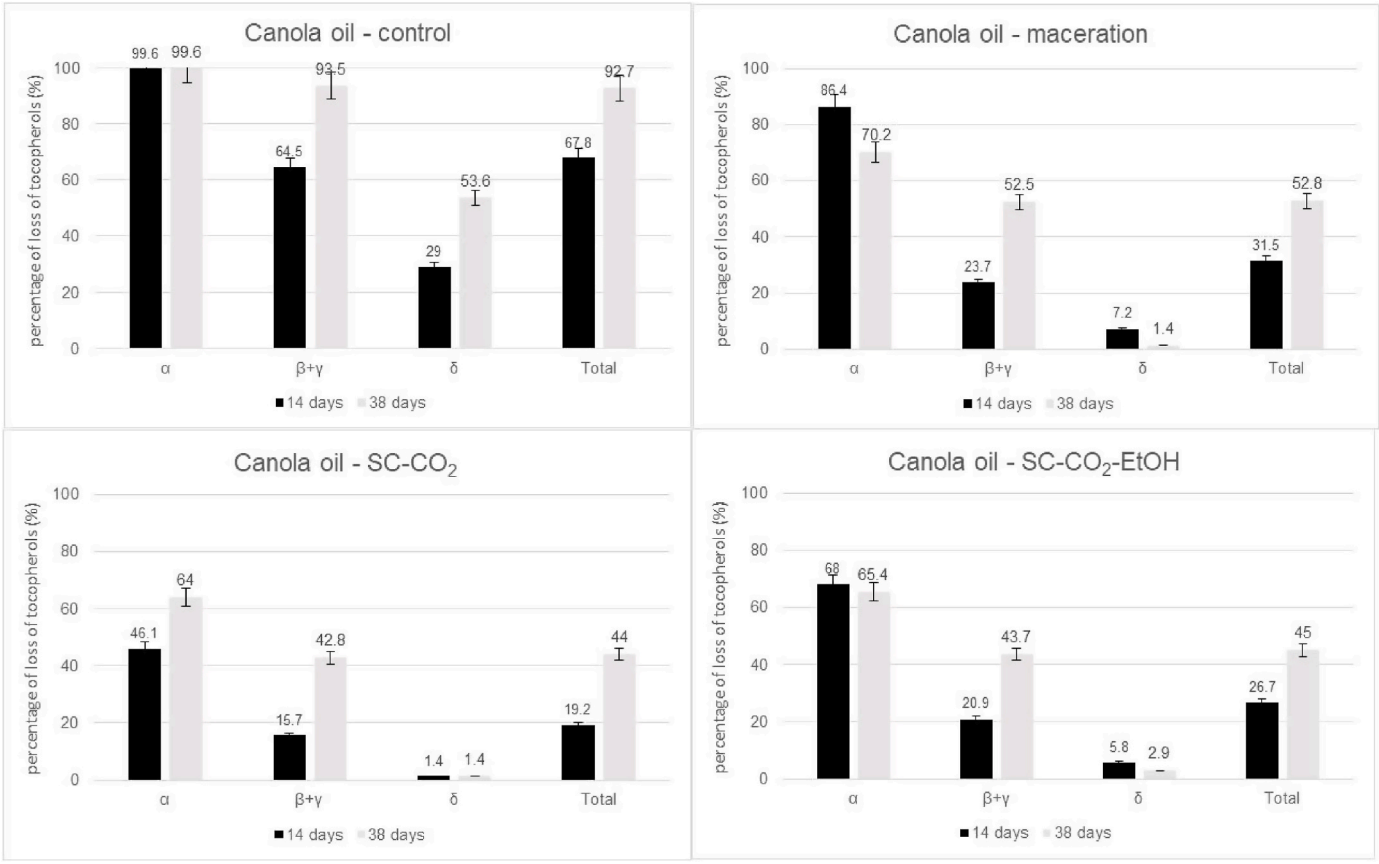

Fig. 2. Loss percentage of the different tocopherols at 14 and 38 days respect to the initial value in Canola oil: (a) control, (b) macerated extract, (c) SC-CO_2 extract and (d) SC–CO_2–EtOH extract.

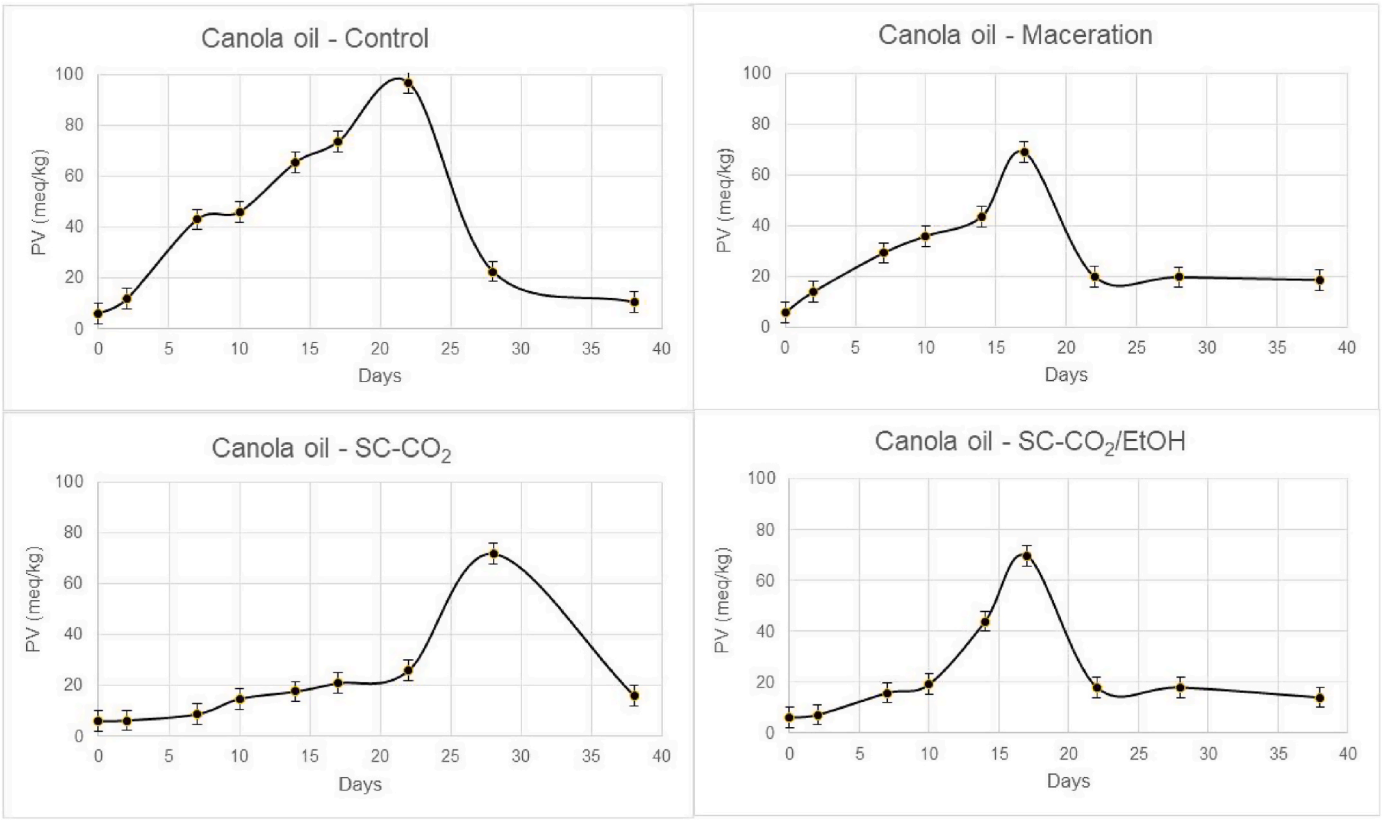

Fig. 3. Evolution of peroxide value during the storage in Canola oil: (a) control, (b) macerated extract, (c) SC-CO_2 extract and (d) SC–CO_2–EtOH extract.

Thus, not only total phenol content is important but also the identity of individual compounds as it is linked to their activity. Hence, the extracts with a higher TPC not necessarily have more antioxidant power. The use of more polar solvents could extract other non-active compounds from the OL matrix. As a consequence, the concentration of the compounds of interest could be reduced in those extracts and, therefore, the extracts could show lower antioxidant power (Chen et al., 1992). According to this author, when water content in aqueous-ethanol solutions is high, a decrease in the extraction of antioxidant compounds is expected. Thus, a decrease in the protection power is expected if they are incorporated into an oil. In conclusion, the extraction yield increases with increasing polarity of the solvents used in the maceration process. However, not all the compounds extracted are active, which is evidenced in its antioxidant activity in the protection of Canola oil. Taking this into account, it is important to properly select the type of food to which the extract is incorporated to, because depending on the matrix used different results could be obtained.

Table 4 shows the values of the spectrophotometric constants K232 and K270 obtained. This methodology allows detecting the presence of primary oxidation compounds such as conjugated dienes and hydroperoxides of linoleic acid, as well as secondary oxidation compounds such as conjugated trienes, ethyl diketones and carbonyl compounds (Malvis et al., 2019). PV and conjugated dienes by means of the specific extinction at 232 nm are alternatively used for the evaluation of primary oxidation compounds in oils and fats oxidation studies (Malvis et al., 2019; Marmesat et al., 2009). As Table 4 shows, the specific extinction coefficient values obtained (K_{232}) followed the expected tendency in accordance with the results found with the PV analysis. Our results indicate that all systems studied had an increase of the specific extinction coefficient at 232 nm followed by a decrease, accompanied by a more pronounced increase of the specific extinction coefficient at 270 nm, indicating the presence of secondary oxidation compounds.

Additionally, the induction period (IP) of Canola oils by the Rancimat method was determined (Table 5). In this regard, the three systems with the addition of the extracts obtained in Canola oil presented longer IP ($p<0.05$) with respect to the control system, except for the initial and final time in the case of the macerated extract. This agrees with the results reported by (Volpini-Klein et al., 2020). At the same storage time, the same tendency found with the PV analysis was obtained. The highest PV corresponded to the control system followed by the system with the addition of the macerated extract, in accordance with the IP obtained, as these same systems had the lowest times. The same pattern was observed

Table 4
Values of the extinction coefficient found for canola oil at different times of analysis.

Days	K, 232 nm				K, 270 nm			
	SC-CO_2	SC-CO_2/EtOH	Macerated	Control	SC-CO_2	SC-CO_2/EtOH	Macerated	Control
0	4.40 ± 0.02^{A}	4.40 ± 0.02^{A}	4.40 ± 0.02^{A}	4.40 ± 0.02^{A}	0.75 ± 0.02^{a}	0.75 ± 0.02^{a}	0.75 ± 0.02^{a}	0.75 ± 0.02^{a}
2	5.75 ± 0.15^{B}	5.25 ± 0.05^{A}	5.31 ± 0.12^{A}	6.01 ± 0.09^{B}	1.00 ± 0.05^{b}	0.85 ± 0.01^{a}	0.82 ± 0.04^{a}	0.96 ± 0.07^{b}
7	6.50 ± 0.04^{A}	7.48 ± 0.15^{B}	8.06 ± 0.09^{C}	8.72 ± 0.21^{D}	1.18 ± 0.04^{a}	1.01 ± 0.11^{a}	1.11 ± 0.07^{a}	2.75 ± 0.12^{b}
14	6.21 ± 0.03^{A}	9.38 ± 0.07^{C}	8.82 ± 0.09^{B}	10.54 ± 0.08^{D}	0.90 ± 0.02^{a}	1.96 ± 0.09^{b}	0.92 ± 0.07^{a}	3.09 ± 0.03^{c}
22	8.25 ± 0.05^{B}	6.44 ± 0.05^{A}	6.42 ± 0.03^{A}	12.77 ± 0.23^{C}	2.34 ± 0.05^{a}	2.38 ± 0.03^{a}	2.74 ± 0.05^{b}	3.39 ± 0.015^{c}
38	6.24 ± 0.08^{A}	6.01 ± 0.24^{A}	6.24 ± 0.06^{A}	8.75 ± 0.18^{B}	2.87 ± 0.11^{a}	$2.97 \pm 0.07^{a.b}$	3.10 ± 0.04^{b}	3.51 ± 0.05^{c}

Different letters yield significant differences among samples for each of the times studied (Tukey, $p < 0.05$). Capital letter for absorbance values at 232 nm and lowercase letter for absorbance values at 270 nm.

Table 5
Values of induction period obtained for canola oil at different times of analysis.

Days	Induction Period (h)			
	SC-CO_2	SC-CO_2/EtOH	Macerated	Control
0	6.25 ± 0.05^{B}	6.21 ± 0.02^{B}	5.34 ± 0.07^{A}	5.55 ± 0.05^{A}
2	5.53 ± 0.03^{D}	5.32 ± 0.05^{C}	5.08 ± 0.04^{B}	4.77 ± 0.07^{A}
7	3.81 ± 0.05^{D}	3.27 ± 0.05^{C}	2.77 ± 0.05^{B}	2.44 ± 0.06^{A}
14	3.77 ± 0.11^{D}	2.93 ± 0.06^{C}	2.42 ± 0.06^{B}	1.33 ± 0.02^{A}
22	1.40 ± 0.02^{D}	1.32 ± 0.05^{C}	1.04 ± 0.03^{B}	0.84 ± 0.03^{A}
38	0.89 ± 0.02^{B}	0.84 ± 0.04^{B}	0.54 ± 0.02^{A}	0.50 ± 0.07^{A}

In each row, those mean values that were significantly different from each other ($p < 0.05$) are shown with different capital letter.

with the systems which had the lowest PV: SC-CO_2 followed by SC–CO_2–EtOH, accordingly had the higher IP observed. In all cases, as expected, by increasing the storage days, a decrease in the IP values was observed. This showed that the oil becomes more susceptible to oxidation as storage time at 60 °C increases, since the antioxidant compounds present are being consumed. As a consequence, there is a lower concentration of molecules available to neutralize the damage caused by free radicals. Jimenez et al. (2011) studied the oxidative stability of different oils with the addition of olive leaves extracts that were obtained by supercritical and solvent extraction. The extract obtained by hydroalcoholic maceration (ethanol–water 1:1) showed the highest phenol content (7.7 mg CAE/mL), whereas the extract obtained by SC-CO_2, presented the lowest (2.2 mg CAE/mL). Besides, they showed that the addition of SFE-CO_2 extract increases the IP at 110 °C with respect to control and the macerated extract in the oil systems under study.

4. Conclusions

The addition of olive leaf extracts obtained by three different techniques to canola oil allowed its shelf life extension by delaying the oxidation process. Compared to the control oil, lower PV were observed for oils with 250 mg/kg of the three different extracts studied. Regarding the extraction method, it was observed that both SFE extracts (SC–CO_2 or SC–CO_2–EtOH) offered higher protection to Canola oil than the macerated extract. Hence, it can be concluded that although maceration provided the highest extraction yield, TPC and antioxidant activity, supercritical extracts were more effective to delay oxidation. In case of the development of a large-scale process at an industrial level, a cost-benefit evaluation should be done for the three alternatives as a way to be able to discern which of them would be the most convenient. Therefore, in terms of process feasibility, the selectivity extraction of the SC-CO_2 towards the compounds of interest, the possibility of reutilization of the solvent associated to better solvent separation with less energy consumption should be taking into account. However, in a large scale environment the extraction of natural antioxidant cannot be considered as an individual and isolated activity, instead, the biorefinery concept must be better associated to the processing of this residue.

CRediT authorship contribution statement

Cecilia Dauber: Data curation, Writing – original draft, Formal analysis, Investigation. **Tatiana Carreras:** Visualization, Investigation. **Laura González:** Methodology, Investigation. **Adriana Gámbaro:** Resources, Supervision. **Alberto Valdés:** Investigation, Software. **Elena Ibañez:** Supervision. **Ignacio Vieitez:** Conceptualization, Project administration, Funding acquisition, Writing – review & editing, Validation, Supervision.

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