Application of supercritical fluids for polyphenolic compounds extraction from olive pomace

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Abstract

Olive wastes generated during the olive oil processing produces a high impact on the environment. The compositions of these raw materials include antioxidants. These are products with relevant applications in the pharmaceutical and food industry. In this work, olive waste is use as a raw material for the development of extraction processes. It was demonstrated the effect of the use of supercritical fluid extraction (SFE) before the solvent extraction. For this, the SFE was carried out with variations in the process pressure (200, 250 and 300 bar). In addition, carbon dioxide was used as solvent and ethanol 60% as co-solvent. The best conditions for each one of the olive tree residues and the polyphenolic compounds present were determined. As a result, it was obtained that the highest antioxidant activity occurred when the process was carried out at 300 bar. Additionally, compounds with high antioxidant properties such as hydroxytyrosol, chlorogenic acid, caffeic acid and ferulic acid were identified.

Keywords: antioxidants, olive waste, supercritical fluid extraction

1. Introduction

The olive tree *(Olea europaea L.)* is a fruit tree grown in different parts of the world. Actually around 11 million hectares are cultivated (Ruiz et al., 2017). From this tree it is obtained the virgin olive oil, which is a product with high demand given its health benefits (Nocella et al. 2018). In addition, it is the second most important product of the agro-industrial sector in Europe.

Olive leaves, biomass form olive tree pruning and olive pomace are promising wastes for obtaining bioactive compounds and are all generated in huge amounts every year. For example, approximately 3 tons of biomass from pruning per hectare and per year are produced (Romero-García et al. 2014). From these residues, different studies have shown the presence of compounds with high antioxidant and anticancer capacity such as oleuroperin, luteolin, hydroxytyrosol, tyrosol and apigenin (Ahmad-Qasem et al. 2013, 2014; Čepo et al. 2017).

The objective of this study was to demonstrate the potential of olive biomass as raw materials for polyphenolic compounds extraction, using supercritical fluids as extraction technology and compare this with conventional extraction.

2. Methodology

2.1. Raw materials

The three raw materials (leaves, pruning biomass and pomace) were collected in the province of Jaén, Spain (37°23′00″N 5°59′00″O). Olive tree pruning biomass (OTPB) was obtained directly from the field by collecting the crushed material in an olive grove. The leaves were collected in a local olive mill after being separated from the olive fruit by means of a pneumatic separator. These OL were washed with water at room temperature to remove dirt. The OTPB and the OL were allowed to air drying and then crushed with a mesh size of 1 cm to homogenize them. The third material was the olive pomace (OP) that was received in the form of pellets from a local extraction company. All materials were stored at room temperature.

2.2. Reagents

The main reagents used in this work were ethanol 96% (Sigma-aldrich), acetone (Panreac), distilled water, hexane, sulfuric acid (97%) sodium hydroxide reactive grade, acetic acid 96% (MOL LABS) and sodium chlorite reagent grade. For the quantification of the polyphenolic compounds the following chemicals were used: gallic acid 96% (Sigma-Aldrich), anhydrous sodium carbonate (Panreac), Folin-Ciocalteu 1N (Sigma-Aldrich), 2,2-diphenyl-1-picrylhydrazyl (Sigma-Aldrich), methanol 99.8% (Panreac), HPLC grade water, mannitol, xylitol, glucose, chlorogenic acid, ferulic acid, vanillin, quercetin, vanillic acid and caffeic acid standards.

2.3. Characterization of olive waste

2.3.1. Characterization of solids

The characterization of the different olive biomasses was carried out following the procedures proposed by the National Renewable Energy Laboratory. (National Renewable Energy Laboratory, NREL). To complement this analysis the monomeric sugars content (after posthydrolysis) and mannitol were determined by HPLC. Additionally, the total phenols content in the aqueous extract produced in the quantification stage of extracts was obtained according to Folin-Ciocalteu method.

2.3.2. Sugars and other compounds

The solid samples were centrifuged and filtered through 0.45 µm membranes (Gelman Sciences, Inc., Michigan, USA). Subsequently these were analyzed by HPLC for quantitative carbohydrate analysis. The HPLC system (Waters, Milford, USA) was equipped with a refractive index detector (model 2414). A CARBOSep CHO-782 Pb (Transgenomic, Inc., Omaha, USA) carbohydrate analysis column operating at 70°C with ultrapure water as a mobile-phase (0.6 mL/min) was used for the monomeric sugars (arabinose, galactose, glucose, mannose and xylose) and mannitol determinations. Acetic acid content was analyzed by HPLC in a Hewlett-Packard 1100 system (Palo Alto, CA, USA) equipped with a refractive index detector. The separation was performed with a Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) operating at 65°C with 5 mM sulfuric acid as an eluent at a flow rate of 0.6 ml/min. Figure 1 shows the general diagram of the characterization of raw materials.

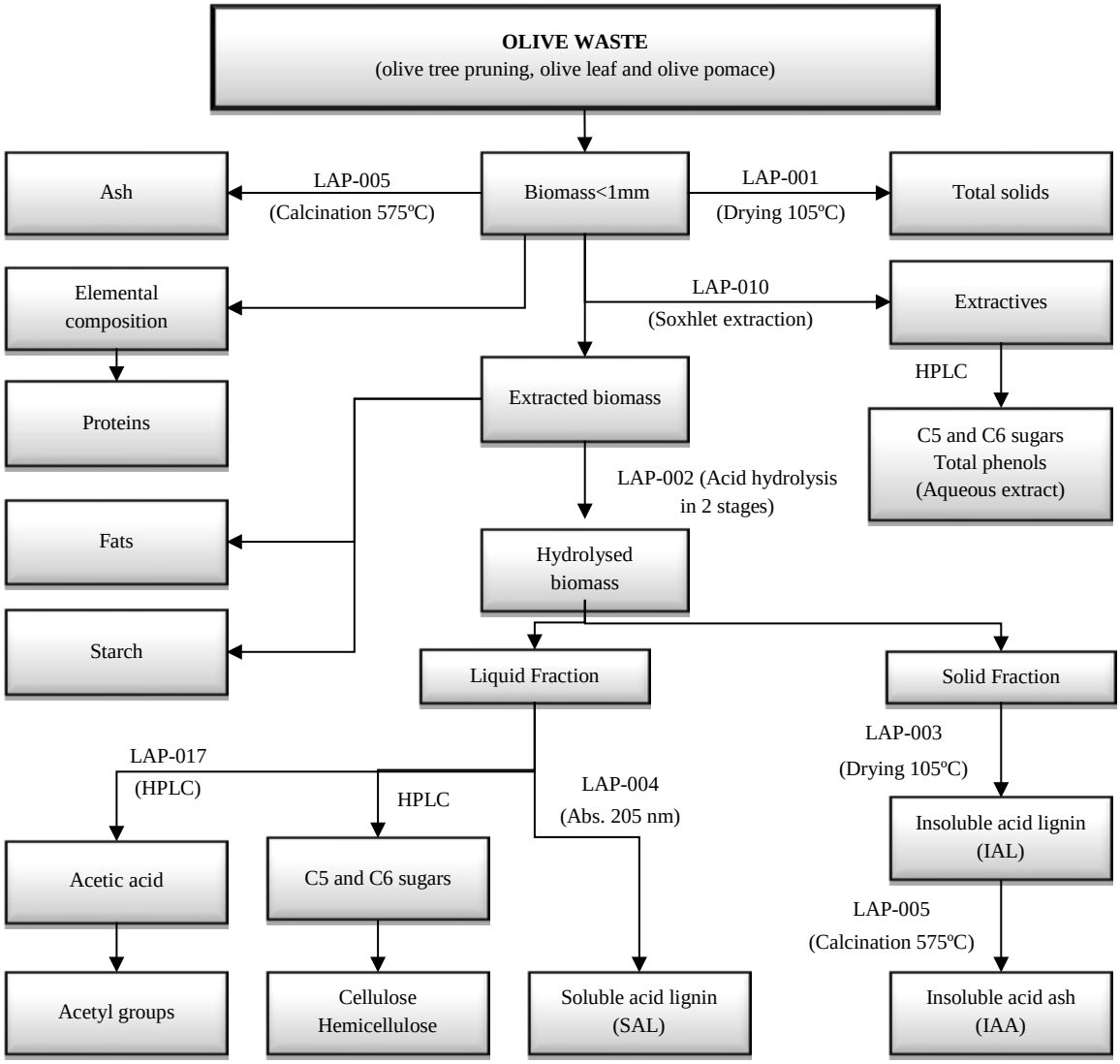

Figure 1. Scheme of the procedure for the characterization of olive residues.

2.4. Extraction process

The antioxidant extractions from the olive pomace, olive tree pruning biomass and olive leaves were carried out through solvent extraction and SFE by triplicate. The solvent extraction (SE) was done with 60%ethanol with a solid liquid ratio 1:20 (w/v). The mixing process was developed at 300 rpm and the temperature of 25°C for 8 hours. Subsequently, the solutions were filtered under vacuum, obtaining the extracts from olive residues. The SFE was carried out at three pressure values (200, 250 and 300 bar). For this, carbon dioxide (CO_2) was used as a supercritical fluid. In addition, ethanol 60% was used as co-solvent (ratio 1:3 w/v solid-liquid) to increase the solubility with the compound of interest. This process was carried out at 50°C during 60 minutes. The extracts obtained from the two technologies were stored at -4°C in an amber bottle.

2.5. Total phenolic compounds

In the determination of total phenolic compounds (TPC), gallic acid (GA) was used as reference standard (Rover and Brown 2013). From each of the obtained extracts, 100 µL were mixed with 1600 µL of distilled water and mixed until homogenization. 100 µL of Folin-Ciocalteu reagent were added to this solution, mixing and letting equilibrate for 5 minutes. Then, 200 µL of Na_2CO_3 was added and left in a dark

room during 2 hours. The absorbance was determined at a wavelength of 765 nm in a UV-Vis spectrophotometer.

2.6. Antioxidant activity

The antioxidant activity of each of the extracts was obtained by the DPPH method. Trolox was used as the reference compound (Brand-Williams, Cuvelier, and Berset 1995). In this method it was necessary to carry out some dilutions of the extracts to obtain the inhibition percentage (Equation 1). Subsequently it was calculated the EC50 through Equations 2 and 3. To determine the absorbance, 150 µL of the diluted extract were mixed with 2,850 µL of DPPH solution, stirring and measuring at 515 nm in a spectrophotometer after 60 minutes. As a result, EC_{50} was obtained in micrograms of trolox per milliliter of extract.

$$\%Ihnibition = \left(1 - \frac{Abs_{final}}{Abs_{white}}\right) \times 100 \quad \text{Equation 1.}$$

$$EC_{50}\left(\frac{mL\ solution}{mL\ extract}\right) = \left(\frac{50 - Intercept}{slope}\right) \quad \text{Equation 2.}$$

$$EC_{50}\left(\frac{mg}{mL}\right) = \left(\frac{1}{IC_{50}\left(\frac{mLsolution}{mLetract}\right)}\right)\left(\frac{mass_{(mg)}}{Volumen_{(mL)}}\right)_{Trolox} \quad \text{Equation 3.}$$

2.7. Metabolites

The identification of chlorogenic acid, ferulic acid, hydroxytyrosol, vanillin, vanillinic acid, quercetin and caffeic acid was carried out in an HPLC system (LC-2010A HT) with UV-visible detector. A C18 column with dimensions of 150 mm x 4.6 mm and 5 µm particle diameter was used. The determination of chlorogenic acid was carried out according to the methodology proposed by Zhang et al. (Zhang et al. 2007). As a flow, 0.7 mL.min-1 and an elution gradient of acetic acid 0.5% v/v (A) and methanol (B) were used at a temperature of 25°C and a wavelength of 310 nm. For ferulic acid, the same elution gradient was used but this was determined at a wavelength of 280 nm (X. Li et al. 2007). In the case of hydroxytyrosol, acetic acid 2% v/v (A) and methanol (B) were used, measuring at 280 nm as reported by Smeriglio (Smeriglio 2015). For vanillin, a wavelength of 270 nm and acetic acid 0.01% v/v (A) were used (Li, Sun, and Zheng 2004). While quercetin, caffeic acid and vanillic acid were determined according to the methodology reported by Chen et al (Chen, Zuo, and Deng 2001). The acetic acid was 3% (A) and methanol (B) with a wavelength of 280 nm. The elution profiles for the identification of each of these compounds are shown in Table 1.

Table 1. Elution profiles of polyphenolic compounds.

Time (min)	(A) Acetic acid	(B) Methanol
Elution profile of chlorogenic acid		
0	10	90
4	10	90
15	30	70
25	30	70
Elution profile of ferulic acid		
0	20	80
4	45	55
9	45	55
12	80	20
25	80	20
Elution profile of vanillin		
0	60	40
5	60	40
7	50	50
14	100	0
18	100	0
19	60	40
Elution profile of hydroxytyrosol		

0	5	95
10	35	75
13	5	95
15	5	95
Elution profile of quercetin, caffeic acid and vanillinic acid		
0	0	100
10	10	90
40	70	30
44	0	100
47	0	100

3. Results and discussion

3.1. Characterization of olive waste

Table 2 shows the composition of the three biomasses used in this work. It should be noted that in the three cases the largest fraction are the extractives with values between 28.6 and 49.7% (OTPB<OL<OP). These values are in the range reported by other authors (Cara et al. 2008, Romero-García et al. 2016, Manzanares et al. 2017). These values are much higher than those in other biomass in the olive grove, such as olive stone, which shows values of 5.5-8.9% (Lama-Muñoz et al. 2014). On the other hand these results were also superior to other biomass such as rapeseed straw 13.1-14.6% (López-Linares et al. 2014), sunflower stalks 15.9-21.6% (Díaz et al. 2011) or sugarcane bagasse 5.6-5.75% (Mesa et al. 2010).

Regarding the fraction of structural carbohydrates (cellulose + hemicellulose), it was found between 20.5 and 36.1% (OP <OL <OTPB) (Romero-García et al. 2014). In order to valorize this carbohydrate fraction, the previous removal of the extractives has shown a very substantial improvement in the overall recovery of sugars (cellulosic + hemicellulosic). This improvement may be due to the avoidance of "lignin-like" compounds formation during pretreatment, which improves the subsequent stage of enzymatic hydrolysis (Ballesteros et al., 2011). In addition, these extracts show an important content of phenols between 2.92 and 6.14% (OP <OL <OTPB). There compounds are toxic for the microorganisms responsible for the fermentation of sugars (Jönsson et al., 2013).

To remove part of the extractives (phenolics) can have a double benefit. One is the reduction of the toxicity of liquor improving biotransformation. Additionally the recovering of bioactive compounds with antioxidant capacity and high added value, that can make more viable a potential biorefinery. Bioactive compounds with antioxidant capacity and a high added value such as oleuropein, hydroxytyrosol, tyrosol, among others, are present in the different biomasses of the olive grove (Romero-García et al. 2014; Ruiz et al. 2017).

Finally, regarding the lignin content, the great difference between OL and, EOP and OTPB should be highlighted, 35.7% at 20.9% and 17.7% respectively. The value found of lignin in OL is quite similar to that reported by García-Maraver (39.6%) (Garcia-Maraver et al. 2013). The transformation of this lignin in bioproducts with high added value would also allow the advancement of biorefinery from the biomasses studied (Fernández-Rodríguez et al. 2017). In summary the composition of these olive waste raw materials with a large fraction of extractives makes them different from the rest. An initial stage of extraction is essential in order to remove the phenolic compounds. From this, it is needed to improve later stages of the process, in addition to obtaining compounds with high added value.

Table 2. Composition of extracted olive pomace (OP), olive tree pruning (OTPB) and olive leaves (OL). Results are expressed as g/100 g raw material oven dry weight.

	OP			**OTPB**			**OL**		
Total solid (%)	91.43	±	0.15	93.41	±	0.01	94.16	±	0.09
Composition (% dry matter)									
Extractives	49.71	±	0.61	28.62	±	1.33	37.93	±	0.97

Water-extract	45.78	±	0.45	23.49	±	1.39	25.29	±	0.92
Glucose	7.63	±	0.26	7.27	±	0.11	4.19	±	0.18
Xylose	0.45	±	0.09	nd	±	0.00	nd	±	0.05
Galactose	1.37	±	0.04	0.73	±	0.06	0.67	±	0.05
Arabinose	1.67	±	0.06	0.28	±	0.25	1.38	±	0.16
Mannose	0.89	±	0.01	1.13	±	0.05	0.00	±	0.00
Mannitol	5.03	±	0.15	3.00	±	0.05	0.22	±	0.10
Total phenols*	6.14	±	0.14	2.92	±	0.01	4.25	±	0.08
Ethanol-extract	3.93	±	0.22	5.13	±	0.24	12.64	±	0.23
Cellulose	9.78	±	0.34	21.58	±	0.18	13.89	±	0.26
Hemicellulose	10.71	±	0.20	14.47	±	0.20	7.88	±	0.20
Xylose	9.90	±	0.27	10.18	±	0.02	5.05	±	0.15
Galactose	0.98	±	0.03	2.23	±	0.04	1.31	±	0.04
Arabinose	0.95	±	0.01	3.22	±	0.15	2.55	±	0.08
Mannose	0.25	±	0.04	0.64	±	0.09	0.00	±	0.06
Lignin	20.90	±	0.08	17.72	±	0.39	35.72	±	0.24
Acid-soluble lignin	1.91	±	0.01	2.33	±	0.07	2.67	±	0.04
Acid-insoluble lignin	18.99	±	0.07	15.39	±	0.39	33.05	±	0.23
Acetyl groups	1.15	±	0.06	0.90	±	0.06	1.84	±	0.06
Ash	8.70	±	0.19	3.85	±	0.55	8.22	±	0.05

*expressed as gallic acid equivalent (GAE)

3.2. Total phenolic compounds (TPC)

A high content of TPC was found mainly in olive pomace as seen in Table 3. However, this difference was not significant compared to the olive leaf. Moreover, from the use of olive pomace, the highest concentration of TPC was obtained with SFE at 300 bar (14.01 ± 0.03 mg GAE/g). While using the conventional technology (solvent extraction) a concentration of up to 12.89 ± 0.22 mg GAE/g was obtained. Additionally, by using a pelletized olive pomace, higher TPC values could be found in this work in comparison to those found in literature. Čepo et al. (2017) reported concentrations between 2.2 - 3 mg GAE/g through solvent extraction, while Goldsmith obtained higher values of TPC (22.01 mg GAE/g) (Goldsmith et al. 2018). On the other hand, Chanioti and Tzia (2017) for olive pomace oil reported a TPC between 0.165 - 0.262 mg GAE/g. Alburquerque (2004) determined concentrations between 6.2 - 23.9 mg GAE/g for the alperujo, the residue of the olive tree coming from the second phase of decantation, while the olive pomace is obtained in the third phase through hot water.

For olive tree pruning biomass the best values were obtained with solvent extraction followed by the SFE at 300 bar with 11.54 ± 0.20 mg GAE/g and 10.39 ± 0.18 mg GAE/g, respectively. These values were higher than those determined by Conde et al. (2009), 1.89 mg GEA/g. For the olive leaf a similar behavior was observed with the best result for TPC at 300 bars (13.12 ± 0.26 mg GAE/g). The TPC value obtained in this study in the olive leaf extract was lower than that reported by some studies. This may be due to the use of other technologies, longer extraction time and origin of the raw material. Al-Rimawi et al. (2014) through maceration with water at 40°C obtained a TPC between 18.63 - 48.30 mg GAE/g. Hussam et al. (2013) obtained up to 66 mg GAE/g with solvent extraction, while Ahmad et al. (2014) reported values of 25-67 mg GAE/g and Ibbay et al. (2014) from 21.56 to 47.58 mg GAE/g. Using olive cake there were found values of up to 42.26 mg GAE/g with methanol during 24 hours (Uribe et al., 2014).

Table 3. TPC of olive waste extract.

Technology	Olive pomace (mg GAE/g)	Olive tree pruning biomass (mg GAE/g)	Olive leaf (mg GAE/g)
SE	12.89 ± 0.22	11.54 ± 0.20	11.28 ± 0.14

SFE-200 bar	9.18 ± 0.17	7.94 ± 0.13	5.83 ± 0.10
SFE-250 bar	12.35 ± 0.25	8.66 ± 0.19	9.76 ± 0.16
SFE-300 bar	14.01 ± 0.31	10.39 ± 0.18	13.12 ± 0.26

3.3. Antioxidants activity

The analysis of the antioxidant activity presented the highest EC_{50} for the olive leaves (274.91 - 382.43 μg/mL), while olive tree pruning biomass had the lowest value (8.38 - 13.77 μg/mL) as shown in Table 4. The use of olive pomace for the extraction of its compounds presented the highest antioxidant activity by SFE-300 bar (85.33 ± 7.04 μg/mL). With this raw material, supercritical fluid extraction obtained 23.33% antioxidant activity higher than through solvent extraction. This EC_{50} value was lower than that reported by Vitali (750 μg/mL) (Čepo et al. 2017) and Goldsmith up to 263 μg/mL for the olive pomace (Goldsmith et al. 2018). On the other hand, this work presented results similar to those found by Alexandra et al when using alperujo (28.79 - 41.56 μg/mL) (Alexandra and Gameiro 2016).

In the case of olive tree pruning, the best values were obtained with SFE-200 and SFE 250 bars with 13.77 ± 0.72 μg/mL and 12.8 ± 0.42 μg/mL, respectively. These antioxidant activity values were higher than those reported by Zbid et al,. (2009), 6.8 μg/mL through solvent extraction. For the olive leaves, Taamalli et al. (2012) determined an antioxidant activity of 550.5 - 796.1 μg/mL and 284.9 - 633.5 μg/mL with SE and SFE, respectively, while in this work the best values were obtained with SE (382.43 ± 10.47 μg/mL) and SFE-300 bar (365.18 ± 8.99 μg/mL), in the range reported by Taamalli et al.

Table 4. Antioxidant activity of olive waste extract.

Technology	Olive pomace EC_{50} (μg/mL)	Olive tree pruning biomass EC_{50} (μg/mL)	Olive leaf EC_{50} (μg/mL)
SE	69.19 ± 5.20	11.68 ± 0.83	382.43 ± 10.47
SFE-200 bar	46.20 ± 3.58	13.77 ± 0.72	274.91 ± 9.18
SFE-250 bar	64.72 ± 6.41	12.84 ± 0.42	321.25 ± 11.35
SFE-300 bar	85.33 ± 7.04	8.38 ± 0.54	365.18 ± 8.99

3.4. Identification through HPLC

The results of the quantification of polyphenolic compounds present in the olive pomace, olive tree pruning biomass and olive leaf extracts are shown in Table 5. Among the compounds identified in these extracts hydroxytyrosol, chlorogenic acid, quercetin, vanillic acid, ferulic acid, caffeic acid and vanillin (only present in olive tree pruning extracts) were present. In the case of hydroxytyrosol, it showed the highest concentration through SFE-300 bar from the olive leaf (1.35 ± 0.05 mg/g) and olive pomace (1.25 ± 0.01 mg/g). While for chlorogenic acid, it was obtained the highest concentration with SE from olive pomace (0.31 ± 0.02 mg/g) and olive leaf (0.09 ± 0.004 mg/g). But in the case of OTPB as a raw material up to 0.24 ± 0.04 mg/g with SFE-300 bar were obtained. The quantification of vanillin gave concentrations of up to 0.79 ± 0.04 mg/g with SE from olive tree pruning. Quercetin and caffeic acid showed the highest concentrations in the extracts of olive pomace with up to 0.09 mg/g. Fot the quercetin the SE contributed to get the best results, while for the caffeic acid the SFE was the best. Moreover, ferulic acid was the second compound followed by hydroxytyrosol with greater presence in the extracts analyzed. This compound presented the greatest presence from OP (0.52 - 0.99 mg/g) and the best results with SFE-200 bar (0.99 ± 0.01 mg/g).

These concentrations of polyphenolic compounds obtained showed values in the range of those reported in other investigations. Alexandra et al. reported hydroxytyrosol content (0.1 - 0.9 mg/g) (Alexandra and Gameiro 2016). While other studies determined in the olive pomace a hydroxytyrosol concentration of 0.08 mg/g (Čepo et al. 2017). In OTPB Conde et al. (2009) obtained concentrations of 1.2 - 1.9 mg/g for vanillin, 1.3 - 1.9 mg/g for vanillic acid and 25.4 - 49.3 mg/g for hydroxytyrosol. For the olive leaves, Jemai et al. (2008) reported the presence of hydroxytyrosol. Hussam et al. (2013) and Taamalli et al. (2012) determined the presence of luteolin, caffeoyl and oleuropein, quinic acid and apigenin.

Table 5. Polyphenolic compounds present in olive waste extracts.

Raw material	Technology	Hydroxytyrosol (mg/g)	Chlorogenic acid (mg/g)	Ferulic acid (mg/g)	Vanillin (mg/g)	Quercetin (mg/g)	Vanillic acid (mg/g)	Caffeic acid (mg/g)
Olive pomace	SE	1.02 ± 0.008	0.31 ± 0.02	0.76 ± 0.008	NR	0.06 ± 0.002	0.16 ± 0.01	0.09 ± 0.001
	SFE-200 bar	0.91 ± 0.005	0.13 ± 0.005	0.99 ± 0.01	NR	0.09 ± 0.005	0.07 ± 0.004	0.02 ± 0.001
	SFE-250 bar	0.95 ± 0.007	0.11 ± 0.008	0.71 ± 0.003	NR	0.05 ± 0.003	0.11 ± 0.01	0.04± 0.002
	SFE-300 bar	1.25 ± 0.01	0.08 ± 0.003	0.52 ± 0.005	NR	0.04 ± 0.02	0.13 ± 0.008	0.05 ± 0.002
Olive tree pruning biomass	SE	0.12 ± 0.02	0.06 ± 0.005	0.11 ± 0.01	0.79 ± 0.04	0.01 ± 0.002	0.04 ± 0.004	0.02 ± 0.003
	SFE-200 bar	0.03 ± 0.00	NR	0.19 ± 0.03	0.40 ± 0.04	0.02 ± 0.001	0.03 ± 0.002	0.02 ± 0.002
	SFE-250 bar	0.07 ± 0.01	0.09 ± 0.002	0.15 ± 0.01	0.45 0.03	0.03 ± 0.002	0.01 ± 0.001	NR
	SFE-300 bar	0.18 ± 0.01	0.24 ± 0.04	0.04 ± 0.01	0.60 0.07	0.03 ± 0.002	0.01 ± 0.002	NR
Olive leaf	SE	0.97 ± 0.04	0.09 ± 0.004	0.22 ± 0.02	NR	0.01 ± 0.001	0.05 ± 0.002	0.03 ± 0.001
	SFE-200 bar	0.42 ± 0.02	NR	0.10 ± 0.2	NR	0.02 ± 0.002	0.02 ± 0.003	0.01 ± 0.02
	SFE-250 bar	0.73 ± 0.00	0.01 ± 0.002	0.11 ± 0.02	NR	0.04 ± 0.003	0.02 ± 0.001	0.01 ± 0.01
	SFE-300 bar	1.35 ± 0.05	NR	0.13 0.02	NR	0.07 ± 0.005	0.01±0.002	NR

4. Conclusions

The use of olive residues presents a high economic and environmental benefits. This work demonstrated that these raw materials present high potential for obtaining antioxidants. These residues are not used totally today. From the extraction of these residues, it was possible to identify the presence of a great variety of polyphenolic compounds. Within these, hydroxytyrosol, chlorogenic acid and caffeic acid were identified, which have a high antioxidant, anticancer, antidiabetic capacity, among others. Additionally, the effect of the supercritical fluid extraction was observed against conventional extraction (solvent extraction). In the SFE case there was a higher concentration of total polyphenolic compounds and higher antioxidant activity. On the other hand, a higher performance at high pressures (300 bar) was observed. In addition, this work demonstrated that the implementation of non-conventional technologies such as the SFE is a promising alternative for the future applications at the industrial level due to the fact that SFE is a technology that requires less time and quantity of solvent for its development.

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