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Qualitative phenolic profile (HPLC-DAD-MS) from olive oil mill waste waters at different states of storage and evaluation of hydrolysis process as a pretreatment to recover their antioxidants

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TITOLO
Profilo fenolico qualitativo (HPLC-DAD-MS) di acque reflue di vegetazione dell'industria olearia a diversi stadi di conservazione e valutazione dell'idrolisi quale pretrattamento per il recupero degli antiossidanti

KEY WORDS
Olive, olive oil mill waste waters, antioxidants, phenols

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Oliva, acque reflue, antiossidanti, fenoli

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Summary
Olive oil mill waste water (OOMWW) represents a waste that it is generated during the extraction process of olive oil from olives and it is an important environmental problem because of the huge volume generated in the mills. Nevertheless, it is highlighted in literature the high concentration of antioxidants, especially phenolic compounds, in the OOMWW. Principally, the qualitative phenolic profiles of OOMWW differ depending on the technological process of production of olive oil and the time of storage. The aim of this work was to compare qualitatively the phenolic profile of OOMWW by HPLC-DAD-MS. Glucosilated secoiridoids as oleuropein and ligstroside, their aglycon derivatives and simple phenols were the principal compounds found in these matrixes. Furthermore, different hydrolysis reactions have been studied for the phenolic fraction in the oleuropein standard as well as in the OOMWW samples. These steps have been evaluated as pretreatment for the selection and a possible subsequent recovery of most interesting phenolic compounds as hydroxytyrosol that has been proved to possess an important antioxidant activity

Riassunto
Le acque reflue di vegetazione (AV) dell'industria olearia rappresentano un rifiuto generato dal processo di estrazione dell'olio dalle olive e pertanto sono un problema ambientale per l'elevato volume generato presso gli impianti di produzione. Tuttavia, è nota dalla letteratura, l'elevata concentrazione nelle AV di composti antiossidanti a struttura fenolica. I profili fenolici qualitativi delle AV differiscono principalmente in funzione delle condizioni di produzione e delle modalità e tempi di conservazione. Questo lavoro ha l'obiettivo di confrontare qualitativamente i profili fenolici delle AV mediante analisi HPLC-DAD-MS. Nelle acque reflue di vegetazione appena prodotte sono state ritrovate principalmente secoiridoidi glicosilati (oleuropeina e ligstroside) nonché le loro forme derivate agliconiche e basse concentrazioni di fenoli semplici. Allo stesso tempo sullo standard puro di oleuropeina e sugli stessi campioni di AV sono state indagate diverse modalità di idrolisi della frazione fenolica. Tale operazione è stata valutata come pretrattamento per la selezione e l'eventuale successivo recupero dei composti fenolici di maggiore interesse come l'idrossitirosolo al quale è riconosciuta una elevata attività antiossidante.

Introduction

Olive oil mill waste water (OOMWW) represents the main environmental problem in the olive oil production process. 100 kg of olives are necessary to produce just 10-20 kg of olive oil, but it results in a huge amount of OOMWW (40-100 kg OOMWW) (1) that depends on the olive cultivar, ripening index, time of storage and quantity of water added during the olive oil extraction process.

The treatment of OOMWW is extremely difficult due to its large volume and the high concentration of organic matter (BOD varies between 15000 and 50000 mg/L and COD can reach values of around 250 g/L). Its principal components are polysaccharides, sugars, polyphenols, polyalcohols, proteins,organic acids, and oil (2). Moreover, OOMWW contains considerable amounts of suspended solids that may reach up to 190 g/L. Despite that, one of the major factors of the environmental problems caused by the OOMWW is the high concentration of polyphenols, because they present phytotoxicity (3, 4), toxicity against aquatic organisms (5), or suppression of soil microorganisms (6) and are difficult to decompose (7,8). Although polyphenols constitute an important environmental problem, their high antioxidant activity makes them interesting for the food, pharmaceutical and cosmetic industry. Because of that, to reutilize them, their extraction from OOMWW is a very important objective that also helps to diminish the volume of olive oil industry by-products.

Many phenolic compounds have been identified in OOMWW, although the phenolic fraction is very different from that of olive fruit. OOMWW presents a high concentration of secoiridoids derivatives, such as hydroxytyrosol, and the dialdehydic form of decarboxymethyl oleuropein aglycone (1). The profile of OOMWW is characterized by a great complexity (9). A wide variety of methods have been suggested for the treatment of OOMWW, such as composting (10), anaerobic digestion (11), aerobic treatment (12), mixing with municipal wastewater (13), direct land application (irrigation) (14), chemical oxidation in combination with biological treatment (15) or adsorption (2), and even the utilization of fungi species (16). However, these methodologies had given rise to decomposition or destruction of the phenolic compounds contained on it and not to their exploitation.

In this work the phenolic profile of three different OOMWWs concerning their state of storage were studied by HPLC-DAD-MS (TOF, time of flight). After that, different hydrolysis reactions were studied using oleuropein as standard and better results were apply to the OOMWW phenolic samples.

The aim of this treatment was to evaluate the hydrolysis and to use it as a pretreatment for the selection and a possible subsequent recovery of most interesting phenolic compounds as hydroxytyrosol (HYTY) that has been proved to possess an important antioxidant activity.

Material and methods

Samples

Three different OOMWWs that vary on the state of storage and the ripeness of the olives were studied: OOMWW just produced from fresh olives (AV1), OOMWW just produced from very ripe olives (AV2) and OOMWW from very ripe olives and storage during three months (AV3).

Extraction of polar phenolic fraction

Phenolic compounds were extracted from OOMWW by a liquid-liquid extraction method. 10 mL of sample were centrifuged at 3500 rpm during 10 min. After that, they were washed twice with 15 mL of hexane in order to remove lipids. The extraction was carried out with 20 mL of ethyl

acetate for three times. Finally, the ethyl acetate fraction was evaporated under vacuum and then the dry residue was redissolved in 1 mL MeOH/H_2O (50/50).

Acid and basic hydrolysis

Four different kind of hydrolyses were studied: strong and weak acid hydrolyses, basic hydrolysis and enzymatic hydrolysis.

Three modalities of strong acid hydrolyses were performed: 0.5 M HCl/H_2SO4 2 h stirring at 80°C; 1 M HCl/H_2SO4 2 h stirring and 1 M HCl/H_2SO4 22 min ultrasounds.

The weak acid hydrolysis was carried out using a saturated solution of citric acid in MeOH/H_2O during 6 h at 80°C.

The basic hydrolysis was carried out in the following way: 2 M NaOH/KOH during 4h stirring, after that it was acidified with HCl until pH=1. Finally, the sample was extracted with diethyl ether/ethyl acetate.

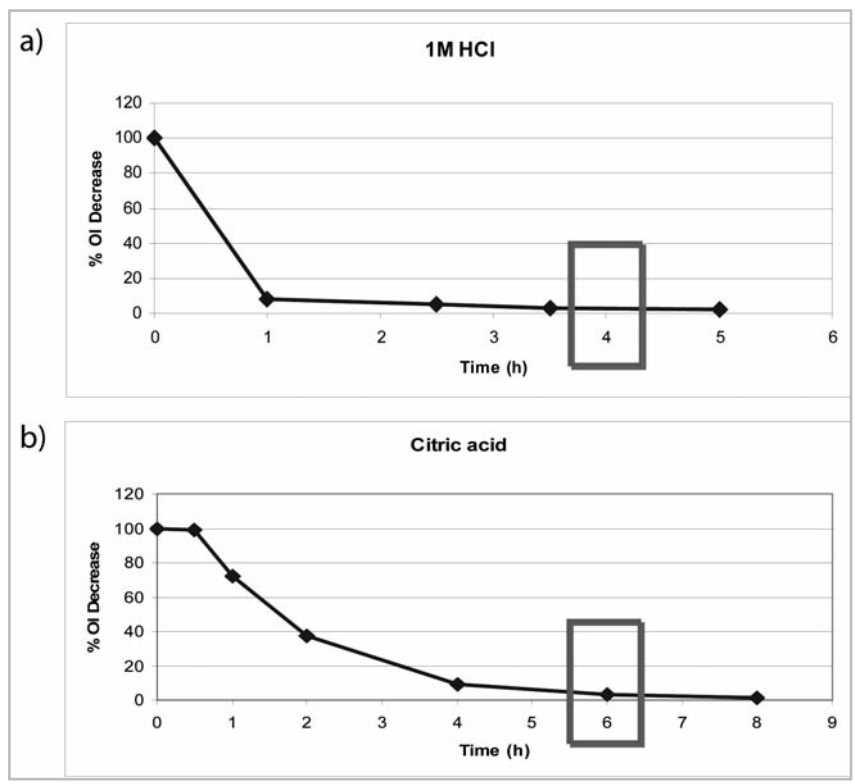
To perform the enzymatic hydrolysis 1 mg oleuropein (Ol) in sodium acetate buffer 0.1 M, pH=5.5 and 5 mg β-glucosidase (from almonds) ≥ 6 units/mg were heated at 37°C in a water bath during 1 h.

Chromatographic procedure

Determination of the phenolic fraction was performed using an HPLC-DAD-MS(TOF) equipped with a reverse phase C18 LunaTM column according to Rotondi et al. (17). Phenolic compounds were tentatively identified based on their UV-vis and mass spectra obtained by HPLC-DAD-MS(TOF).

The HPLC system was coupled to a Bruker Daltonik microTOF mass spectrometer (Bruker Daltonik, Bremen, Germany) using an orthogonal electrospray interface (ESI) (model G1607A from Agilent Technologies, Palo Alto, CA, USA). Parameters for analysis were set using negative ion mode with spectra acquired over a mass range from m/z 50–1000. The optimum values of the ESI-MS parameters were: capillary voltage, +4.5 kV; drying gas temperature, 190°C; drying gas flow, 9 L/min; and nebulizing gas pressure, 2 bar. External calibration was performed using a sodium formiate solution injected at the beginning of the run and all the spectra were calibrated prior to the polyphenol identification.

Figura 1 - Decrease of oleuropein after hydrolysis Vs time of hydrolysis. a) hydrolysis with HCl, b) hydrolysis with citric acid.



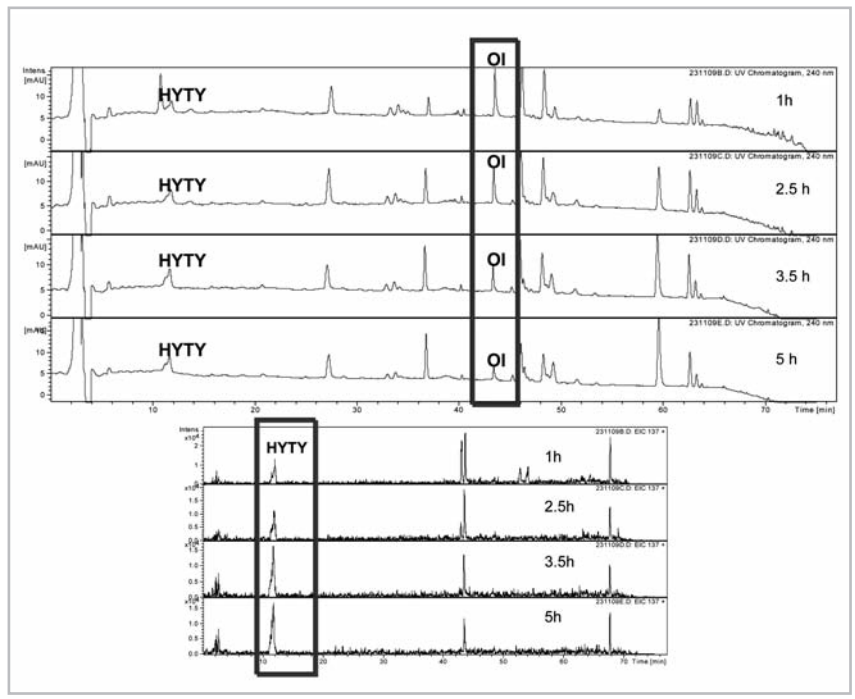
The accurate mass data for the molecular ions were processed using the software Data Analysis 3.4 (Bruker Daltonik), which provided a list of possible elemental formulas by using the Generate Molecular Formula TM editor.

Results and discussion

Hydrolisis

After carrying out the different hydrolyses, optimum results were obtained with 1M HCl and saturated citric acid solution both in a reflux condenser at 80 ºC.
Both hydrolyses were proved at different times from 30 min until 8h. Optimum times of hydrolysis were individualized for each of them at 4h for HCl and 6h for citric acid (Figure 1).
Figure 2 shows how HCl hydrolysis affects the extract. It can be observed that oleuropein (Ol) decreases with time, while the concentration of HYTY increases. This fact is due to the fragmentation of Ol after the hydrolysis.
On the other hand, the citric acid hydrolysis also leads to the decrease of Ol. However, this kind of hydrolysis is softer than the one with HCl and the fragmentation gives caused to an intermediate fragment as oleuropein aglycone (Figure 3).

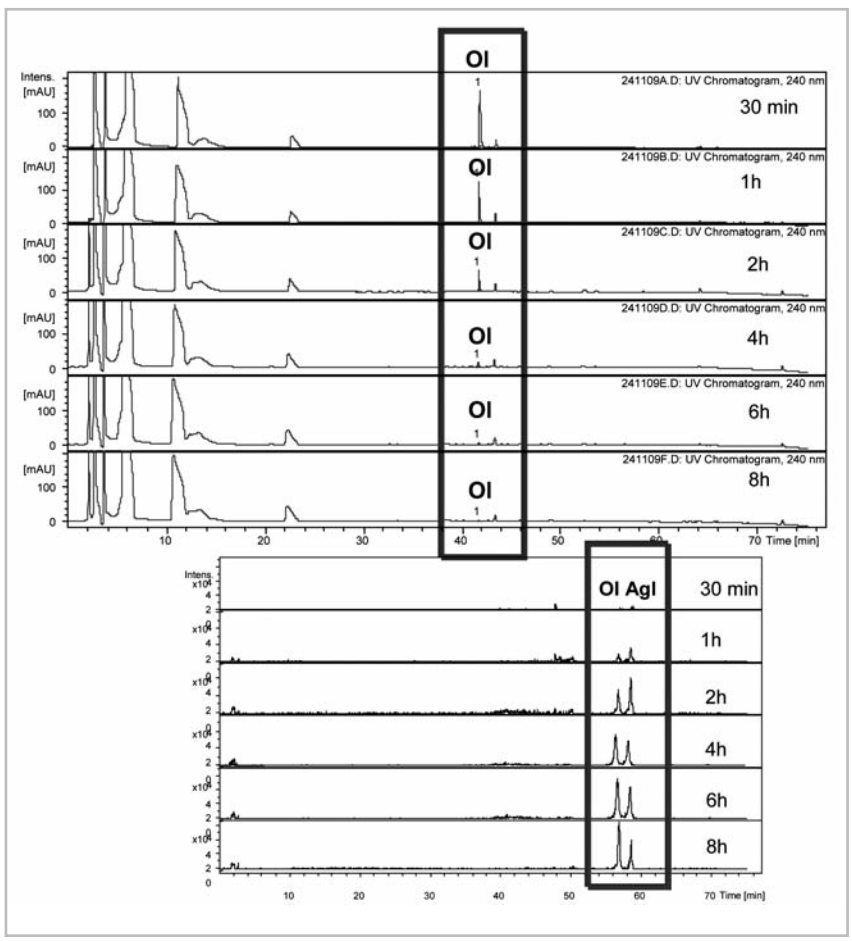
Figura 2 - Changes in the concentration of oleuropein and hydroxytyrosol during the HCl hydrolysis at different times.



Phenolic compounds in OOMWW before and after hydrolysis

OOMWWs have a high content of phenolic compounds that come from olives after olive oil production. These phenolics are principally secoiridoids as oleuropein, ligstroside and its derivatives. However, the enzymes that exist in olives, the milling of olives and the storage lead to a hydrolysis of phenolic compounds changing the initial composition of fresh OOMWWs.
AV1, AV2 and AV3 phenolic extracts from different OOMWWs have been characterized by HPLC-DAD-MS in order to see the differences among the phenolic profiles depending on the time of storage of the OOMWW and the freshness of the olives. Furthermore, the hydrolysis of the samples have been carried out as a pretreatment of the extracts to obtain simple phenols as hydroxytyrosol, compound that has been proved to possess a high antioxidant activity. This process would be a previous step to isolate these simple compounds to further use them as antioxidants in the food or pharmaceutical industry.

Figura 3 - Changes in the concentration of oleuropein and oleuropein aglycone during the citric acid hydrolysis at different times.



Tables 1, 2 and 3 show the phenolic profile in the different OOMWW studied and the changes that the hydrolyses produce on them.

AV1 (OOMWW just produced from fresh olives) is characterised for the presence of Ol and the absence of simple phenols. Nevertheless, after the hydrolysis Ol disappears and mainly, it can be seen the formation of HYTY and TY (Table 1).

The OOMWWs just produced from very ripe olives (AV2) do not have Ol, not even after production; while it can be noticed the presence of simple phenols as HYTY and also elenolic acid (EA) that come from the rupture of secoiridoids. This fact can be explained because of the ripeness of the olives. Olives are not fresh and the storage of them before milling causes the beginning of natural hydrolysis in the olives. Because of that, after hydrolysis the phenolic profile of AV2 is very similar to the one before hydrolysis (Table 2).

Finally, OOMWWs from very ripe olives and storage during a long time (AV3) as AV2 do not possess Ol; but they are characterised by the presence of simple phenols as HYTY and tyrosol (TY), and oxidised forms of secoiridoids as oxidised form of decarboxymethyl oleuropein aglycone (OxDOA), oxidised form of elenolic acid (OxEA) and decarboxymethyl oleuropein aglycone/oxidised form of decarboxymethyl ligstroside aglycone (DOA/OxDLA). As well as AV2, the acid hydrolysis do not affect particularly to the phenolic profile of AV3. This kind of OOMWW also comes from very ripe olives and besides it has been storage for a long time, which is the reason because it does not contain Ol but just oxidised form of secoiridoids and some simple phenols.

Conclusions

Different hydrolyses have been carried out and, after optimization, acid hydrolyses with HCl and citric acid show a good efficiency (>95%) after 4 and 6 h respectively.

Tabella 1 - Phenolic compounds found in OOMWW AV1 before and after hydrolysis.

Possible compound	Molecular formula	m/z calculated	AV1	AV1HCl	AV1CIT
Vanillin	C8H7O3	151.0401	X	-	-
Vanillic acid	C8H8O4	167.0328	X	X	X
Hydroxy DEA	C9H14O5	201.0788	X	X	X
HYTY	C8H10O3	153.0557	-	X	X
OxDEA	C9H12O5	199.0589	X	X	X
TY	C8H10O2	137.0608	-	X	-
Caffeic acid	C9H8O4	179.0331	X	X	X
DEA	C9H12O4	183.0663	X	X	X
Syringic acid	C9H10O5	197.045	-	X	X
p-coumaric acid	C9H8O3	163.0376	-	-	-
Syringaresinol	C22H26O8	417.1556	X	X	-
OxDOA	C17H20O7	335.1136	-	-	-
EA	C11H14O6	241.0722	X	X	X
Luteolin glucoside	C21H20O11	447.0941	-	-	-
p-coumaroyl-6'-secologanoside	C25H28O13	535.142	-	-	-
OxEA	C12H18O6	257.1019	-	-	-
10-H-Ol Agl	C19H22O9	393.1178	-	-	-
Oleuropein	C25H32O13	539.178	X	-	-
Ligstroside	C25H32O12	523.1833	-	-	-
Luteolin	C15H10O6	285.0407	X	-	-
DOA/OxDLA	C17H20O6	319.1187	-	-	-
Ac Pin	C22H24O8	415.139	X	-	-
Apigenin	C15H10O5	269.0432	X	-	-
DLA	C19H20O5	303.1238	-	-	-

Hydroxy DEA, hydroxy decarboxymethyl elenolic acid; HYTY, hydroxytyrosol; OxDEA, oxidised decarboxymethyl elenolic acid; TY, tyrosol; DEA, decarboxymethyl elenolic acid; OxDOA, oxidised decarboxymethyl oleuropein aglycone; EA, elenolic acid; OxEA, oxidised elenolic acid, DOA/OxDLA, decarboxymethyl oleuropein aglycone/oxidised decarboxymethyl ligstroside aglycone.

AV1, OOMWW just produced from fresh olives; AV1HCl, OOMWW hydrolysed with HCl; AV1CIT, OOMWW hydrolysed with citric acid.

Tabella 2 -Phenolic compounds found in OOMWW AV2 before and after hydrolysis

Possible compound	Molecular formula	m/z calculated	AV2	AV2HCl	AV2CIT
Vanillin	C8H7O3	151.0401	X	-	-
Vanillic acid	C8H8O4	167.0328	X	X	X
Hydroxy DEA	C9H14O5	201.0788	X	X	X
HYTY	C8H10O3	153.0557	X	X	X
OxDEA	C9H12O5	199.0589	X	X	X
TY	C8H10O2	137.0608	-	X	-
Caffeic acid	C9H8O4	179.0331	X	X	X
DEA	C9H12O4	183.0663	X	X	X
Syringic acid	C9H10O5	197.045	-	X	-
p-coumaric acid	C9H8O3	163.0376	X	X	X
Syringaresinol	C22H26O8	417.1556	-	-	-
OxDOA	C17H20O7	335.1136	-	-	-
EA	C11H14O6	241.0722	X	X	X
OxEA	C12H18O6	257.1019	-	-	-
Luteolin glucoside	C21H20O11	447.0941	X	-	X
p-coumaroyl-6'-secologanoside	C25H28O13	535.142	X	X	X
10-H-Ol Agl	C19H22O9	393.1178	X	-	-
Oleuropein	C25H32O13	539.178	-	-	-
Ligstroside	C25H32O12	523.1833	X	-	-
Luteolin	C15H10O6	285.0407	X	X	X
DOA/OxDLA	C17H20O6	319.1187	-	-	-
Ac Pin	C22H24O8	415.139	X	-	X
Apigenin	C15H10O5	269.0432	X	-	X
DLA	C19H20O5	303.1238	-	-	-

Abbreviations see Table 1.

AV2, OOMWW just produced from very ripe olives; AV2HCl, OOMWW hydrolysed with HCl; AV2CIT, OOMWW hydrolysed with citric acid.

Tabella 2 - Phenolic compounds found in OOMWW AV3 before and after hydrolysis

Possible compound	Molecular formula	m/z calculated	AV3	AV3HCl	AV3CIT
Vanillin	C8H7O3	151.0401	X	-	-
Vanillic acid	C8H8O4	167.0328	X	X	-
Hydroxy DEA	C9H14O5	201.0788	X	X	-
HYTY	C8H10O3	153.0557	X	X	X
OxDEA	C9H12O5	199.0589	-	-	-
TY	C8H10O2	137.0608	X	X	X
Caffeic acid	C9H8O4	179.0331	-	X	-
DEA	C9H12O4	183.0663	X	X	X
Syringic acid	C9H10O5	197.045	X	X	X
p-coumaric acid	C9H8O3	163.0376	X	X	X
Syringaresinol	C22H26O8	417.1556	-	-	-
OxDOA	C17H20O7	335.1136	X	-	-
EA	C11H14O6	241.0722	-	-	-
Luteolin glucoside	C21H20O11	447.0941	-	-	-
p-coumaroyl-6'-secologanoside	C25H28O13	535.142	-	-	-
OxEA	C12H18O6	257.1019	X	X	-
10-H-Ol Agl	C19H22O9	393.1178	-	-	-
Oleuropein	C25H32O13	539.178	-	-	-
Ligstroside	C25H32O12	523.1833	-	-	-
Luteolin	C15H10O6	285.0407	-	X	-
DOA/OxDLA	C17H20O6	319.1187	X	X	-
Ac Pin	C22H24O8	415.139	-	-	-
Apigenin	C15H10O5	269.0432	-	-	-
DLA	C19H20O5	303.1238	X	X	-

Abbreviations see Table 1.

AV3, OOMWW from very ripe olives and storage during three months; AV3HCl, OOMWW hydrolysed with HCl; AV3CIT, OOMWW hydrolysed with citric acid.

The state of ripeness of the olives and the time of storage of OOMWW influence the phenolic profile, specially affects to secoiridoids. O1 disappears if OOMWW are not produced from fresh olives.

Furthermore, the storage for several months of OOMWW causes the formation of oxidised forms of secoiridoids.

Because of that, it will be very important to assess the state of the olives and the time of storage of OOMWW in order to use them to optimize a methodology to separate the phenolic compounds present in these olives by-products.

Finally, the performances of the phenolic fraction isolated will have to be studied in order to use them as food antioxidants.

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References

1. De Marco E, Savarese M, Paduano A, Sacchi A. Characterization and fractionation of phenolic compounds entracte from olive oil mill wastewaters. Food Chem 2007; 104: 858-67.
2. Zouari N. Decolorization of olive oil mill effluent by physical and chemical treatment prior to anaerobic digestion. J Chem Technol Biotechnol 1998; 73: 297-303.
3. Aliotta G, Fiorentino A, Oliva A, Temussi F. Olive oil mill wastewater: Isolation of polyphenols and their phytotoxicity in vitro. Allelopathy J 2002; 9: 9-17.
4. Capasso R, Cristinzio G, Evidente A, Scognamiglio F. Isolation, spectroscopy and selective phytotoxic effects of polyphenols from vegetable waste waters. Phytochem 1992; 31: 4125-28.
5. Fiorentino A, Gentili A, Isidori M, et al. Environmental effects caused by olive mill wastewaters: Toxicity comparison of low-molecularweight phenol components. J Agric Food Chem 2003; 51: 1005-9.
6. Kotsou M, Mari I, Lasaridi K, Chatzipavlidis I, Balis C, Kyriacou A. The effect of olive oil mill wastewater (OMW) on soil microbial communities and suppressiveness against Rhizoctonia solani. Appl Soil Ecol 2004; 26: 113-21.
7. Davies LC, Vilhena AM, Novais JM, Martins-Dias S. Olive mill wastewater characteristics: Modelling and statistical analysis. Grasas Aceites 2004; 55: 233-41.
8. Obied HK, Allen MS, Bedgood DR, Prenzler PD, Robards K, Stockmann R. Bioactivity and analysis of biophenols recovered from olive mill waste. J Agric Food Chem 2005; 53: 823-37.
9. Bianco A, Buiarelli F, Cartoni G, Coccioli F, Jasionowska R, Margherita P. Analysis by liquid chromatography tandem mass spectrometry of biophenolic compounds in olives and vegetation waters, Part I. J Sep Sci 2003; 26: 409-16.
10. Papadimitriou EK, Chatjipavlidis I, Balis C. Application of composting to olive mill wastewater treatment. EnViron Technol 1997; 18: 101-7.
11. Sabbah I, Yazbak A, Haj J, Saliba A, Basheer S. Biomass selection for optimal anaerobic treatment of olive mill wastewater. EnViron Technol 2005; 26: 47-54.
12. Hoyos SEG, Nieto LM, Rubio FC, Cormenzana AR. Kinetics of aerobic treatment of olive-mill wastewater (OMW) with Aspergillus terreus. Process Biochem 2002; 37: 1169-76.
13. Croce F, Poulsom S, Hendricks D. Combined treatment of olive mill effluent and municipal waste-water in a small tourist community. Water Sci Technol 1994; 29: 105-10.
14. Lopez R, Martinez-Bordiu A, de Lome ED, Cabrera F, Sanchez MC. Soil properties after application of olive oil mill wastewater. Fresenius EnViron Bull 1996; 5: 49-54.
15. Fiorentino A, Gentili A, Isidori M, Lavorgna M, Parrella A, Temussi F. Olive oil mill wastewater treatment using a chemical and biological approach. J Agric Food Chem 2004; 52: 5151-54.
16. Aggelis G, Iconomou D, Christou M, et al. Phenolic removal in a model olive oil mill wastewater using Pleurotus ostreatus in bioreactor cultures and biological evaluation of the process. Water Res 2003; 37: 3897-904.
17. Rotondi A, Bendini A, Cerretani L, Mari M, Lercker G, Gallina Toschi T. Effect of olive ripening degree on the oxidative stability and organoleptic properties of cv. Nostrana di Brisighella extra virgin olive oil. J Agric Food Chem 2004, 52: 3649-54.