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XXV Cycle

DEVELOPMENT OF A NEW PROCESS TO TREAT THE SOLID AND LIQUID BY-PRODUCTS OF OLIVE OIL INDUSTRY TO RECOVERY BIOPHENOLS AND TO REDUCE THE ENVIRONMENTAL CHARGE OF THE WASTES.

Ph.D. Dissertation

by

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0) PREFACE

L'olivicoltura rappresenta per tutta l'area mediterranea, e per tutta l'Italia in particolare, un settore produttivo di primaria importanza. Si tratta, tuttavia, di un settore gravato da una ingente generazione di sottoprodotti che comprendono in particolare tessuto vegetale (foglie e potature) e residui di produzione olearia quali, acque di vegetazione, sanse umide ed esauste. Questi ultimi sono caratterizzati da un forte carico inquinante, conseguentemente esiste la necessità impellente di soluzioni per la gestione di tali reflui, attraverso tecnologie che minimizzano il suo impatto ambientale e garantiscono un uso sostenibile delle risorse. L' acqua di vegetazione presenta una concentrazione fenolica variabile tra 3 e 10 g/L, che la rende recalcitrante alla degradazione microbica. D'altra parte, i composti fenolici, oltre ad essere responsabili dell'elevato carico inquinante delle acque di vegetazione, sono anche risorse preziose dal momento che possiedono una marcata attività antiossidante e numerose altre proprietà biologiche. Con l'obiettivo di risolvere i problemi legati allo smaltimento e simultaneamente recuperare e riutilizzare tutte le componenti delle acque di vegetazione ed in particolare l'idrossitirosolo, presso i laboratori del Centro Ricerche ENEA Casaccia di Roma è stato messo a punto in scala laboratorio un processo di frazionamento selettivo delle acque di vegetazione impiegando tecnologie di filtrazione tangenziale a membrana, permettendo il deposito di un brevetto a livello internazionale (WO2005123603).

Lo scopo del presente lavoro è quello di dimostrare la fattibilità in termini industriali (trasferimento tecnologico) sia per la messa a punto dei parametri di processo e sia di prodotto.

Parallelamente è stata studiata la fattibilità tecnica e pratica di un nuovo processo per il recupero dei biofenoli (in particolare oleuropeina) dalle foglie di ulivo attraverso l'impiego di tecnologie di membrane.

Il lavoro è stato svolto in collaborazione con la Società PhenoFarm S.r.l., sita nel Lazio.

Keywords: Olive mill wastewaters (OMW), Phenolic compounds, Hydroxytyrosol, Membrane Technologies.

1) STATE OF THE ART

1.1) Environmental Concerns of olive by products (Olive Mill Wastewaters OMW, Olive leaves)

Several million of cubic meters of Wastewater produced in three months by Olive Mill Wastewaters (OMW) in the Mediterranean countries as Spain, Italy and Greece, Morocco, etc., represent one of the greatest environmental problems of olive agro industry in the Mediterranean basin.
Table 1.1.1 reports an estimation of large volumes of OMW produced in European countries.

Countries	Ton/Year
Spain	8,0
Italy	9,0
Greece	2,0
Total	15-20

Table 1.1.1 Estimation of Olive Mill Wastewaters produced in Europe

Mediterranean countries are mostly affected by this serious environmental problem since they are responsible for 95 % of the worldwide olive-oil production.
These countries produce about 11 million of tons of olive are per year from which about 1.7 million tons of olive oil is extracted. The seasonal polluting load of olive oil production is reported to be equivalent to that of 22 million people per year (Aktas et al., 2001).
As a pollution sources, OMW were present already at the beginning of olive oil production, but their effect on the environment are at present are more noticeable, for the sensitivity of the public opinion to environmental concerns , but mainly for the increase of olive oil (and their by-product) across the centuries. The pollution charge of OMW is well represented by its chemical oxygen demand (COD) value that ranges between 80 e 160 g/L of O_2.
Where allowed by laws, OMW can be spreaded on the agricultural surfaces (ferti-irrigation) and in many countries this is the only way to dispose this waste. Conventional treatment methods as: biological and phisico-chemical depuration are un efficient for OMW.
Negative consequences on the soil properties are object of several studies (Fiorentino et al., 2003; Sierra et al., 2001).
Poliphenols of *Olea europaea* are present also in higher concentration in the OMW considered a very pollutant effluent of olive oil manufacturing.

Chemical composition of OMW, and consequently the value of COD depends on different parameters, first of all the process of olive crushing, traditional (two phases) or modern (three phases): one obtained from decanter, that produce tree phases: olive oil, olive mill wastewater and olive paste (semi-solid), and the traditional methods who produce only olive oil and a semi solid by-product named " *alperujo*" the two phase method is mostly applied in Spain were the main waste of olive oil industry is "alperujo".

The other parameters influencing OMW chemical composition are: cultivar, the geographic area of production, degree of olive ripening, olive harvesting procedure, etc.

Although the chemical composition of OMW vary as function of aforementioned parameters the chemical compounds are well known so the physic-chemical features of this matrix.

The representative and general chemical composition of OMW are reported in table 1.1.2.

Parameters	**OMW**
pH	4,5-6,0
Electric conductivity (mS/cm)	5-10
Minerals (g/L)	5-10
BOD_5 (g/L di O_2)	50-150
COD (g/L di O_2)	80-160
Total solids (%)	5,5-17,6
Organic substances (g/L)	30-160
Oil (g/L)	0,3-23
Sugars (g/L)	10-80
Nitrogen substances (g/L)	5-20
Total polyphenols (g/L)	3-11

Table 1.1.2 Physical-chemical properties of OMW

The OMW shows a brown-read colour, pH weakly acid, about 83,4 % of water, inorganic salts (1,8%), organic compounds (14,8%) and traces of olive oil (fats). (Cimato et al., 2001).

The COD charge is due to the high organic content related to molecules as sugars, organic acids, oils, proteins and polyphenolic compounds that are present in concentrations ranging between 3 to 11 g/L.

These chemicals represents the main obstacle to OMW disposal because they show antibacterial and phyto-toxic properties, they can inhibit both aerobic and anaerobic fermentation processes (Ranalli et

al., 2003; Rozzi et al., 1996), and consequently they require development of a specific treatment processes.

Treatment of OMW is extremely difficult not only for their chemical composition in terms of polysaccharides, sugars, polyalcohols, proteins, polyphenols, oil and a considerable amount of suspended solid that may reach 190 g/L (Zouari et al.,1998) but also and mainly for huge volumes produced each year by worldwide by olive oil industry.

Several treatments, based on chemical, physico-chemical, and biological processes, have been studied in order to reduce the pollution charge, but until now the practical results are very poor .

Most of processes designed to dispose OMW are focused to destroy polyphenols through oxidation path-way performed by air-oxygen, ozone etc insufflation. and very few of them are based on the application of microorganisms as selected yeasts able to metabolize poliphenols.

In fact, polyphenols are oxidizable substances that can determine acidification, saturation, waterproofing, of soils if they are spreaded after olive shredding and OMW production. If OMW are poured directly into water courses, they can induce anoxia til the detriment not only of fish species but also of those aerobic microorganisms, which require oxygen for living and to perform detoxyfication of waters (Ranalli et al., 1990).

Even the olive plant tissues (leaves and pruning) represent a disposal problem and also in this case oil mills must use appropriate measures of treatments in order to dispose of the aforesaid material.

Do not underestimate the disposal of olive leaves from pruning, consider that each plant annually produces 30-50 kg in leaves and 80-90% in the branches. Regarding instead the production of waste in the mills, the quantities of leaves and twigs that accompany the olives to the mill, and which must be separated, on average, amounted to 3-5% of the weight of pickled olives.

The disposal of plant material deriving from pruning operations is normally conducted in those countries who are involved in olive oil production, by burning of leaves and twigs directly on fields, this practice is actually permitted but new law disposition are ongoing with the aim of ruling this behaviour and put limits to the combustion of wooden that determine air pollution (smoke and combustion emissions) expecially for those civil settlements in the neighbors of olive cultivation.

Actually for these matrices the energetic exploitation (co-generation from wood and plant material) is the most followed path, however since olive leaves contains important polyphenols (Oleuropein, luteolin-7-glucoside, verbascoside) (Silva, et al.,2006), it can be suggested the adoption of different ways of valorisation for this matrices aiming to recovery valuable compounds before burning wooden parts to obtain energy.

1.2) The Phenolic fraction of OMW and olive leaves: properties chemical and functional characterization.

1.2.1) Polyphenolic compounds

The terms "phenols", "polyphenols" or "biophenols" refer to any chemical species bearing one or more aromatic ring substituted with one or more hydroxyl groups. This includes priority phenols that are important synthetic chemicals.

In the olive literature, the term "biophenol" has gained widespread usage, this term is sufficient to distinguish between industrial phenols and those of plant origin.

The term covers those phenolic compounds that have been of interest in the chemistry of olive oil as well as those of more recent pharmaceutical interest. It also covers compounds that have been traditionally known as polyphenols, such as the flavonoids whose presence in olive may be important. Within this communication the term "polyphenols" or "phenolic compounds" identify the Biophenols of the olive pulp.

1.2.2) Polyphenol chemical classes and occurrence in olives and olive oil

Phenolic compounds are plant secondary metabolites, which play important roles in disease resistance protections against pests and species dissemination (Servili and Montedoro et al., 2002; Antolovich et al., 2000). The interest on phenolic compounds has raised attention for its study in olive fruits and other parts of the olive tree. Virgin olive is an important dietary oil, rich in natural antioxidants. Olives contain high concentrations of phenolic compounds ranging between 1-3% of the fresh pulp weight. The main classes of phenolic compounds present in olives are phenolic acids, phenolic alcohols, flavonoids and secoridoids. Hydroxytyrosol and tyrosol are the most abundant phenolic alcohols in olives. The flavonoids include flavonol glycosides such as luteolin-7-glucoside and rutin well as anthocyanins. The phenolic content and the specific composition of these phenols in whole olives depend on the altitude where the olive trees are grown, the harvesting time and the processing conditions. Similarly, the levels of phenolics in olive oil depend upon several factors (cultivar, climate, ripeness of olives, preparation and storage of the oil. (Monti et al., 2001). These phenolics are responsible for the stability of the oil from oxidation and for the organolectic properties (Galli-Visioli et al., 2001).

While phenolic acids, phenolic alcohols and flavonoids occur in many fruits and vegetables belonging to various botanical families, secoiridoids are present exclusively in plants belonging to the Oleaceae family which includes Olea europaea L. (Robards et al., 1999; Servili-Montedoro et al., 2002; Servili

et al., 2004; De la Torre-Carbot et al., 2005). There are more than 100 different phenolic molecules reported in olive products (fruit, oil , leaves, and by-products).

Secoiridoids are compounds that are usually glycosidically bound and produced from the secondary metabolism of terpenes. They are characterised by the presence of elenolic acid in its glucosidic or aglyconic form, in their molecular structure. In particular, they are formed from a phenyl ethyl alcohol (hydroxytyrosol and tyrosol), elenolic acid and, eventually, a glucosidic residue. Oleuropein is an ester of hydroxytyrosol (3,4-DHPEA) and the elenolic acid (EA) glucoside (oleosidic skeleton common to the secoiridoid glucosides of Oleaceae) (Montedoro-Servili et al., 1992; Montedoro-Servili et al., 1993). While oleuropein is present in high amounts in unprocessed olive fruit, hydroxytyrosol is more abundant in the processed fruit and virgin olive oil (VOO). This compound amounts up to 14% of the dry weight in unripe olives (Tan et al., 2003).

Secoiridoids of (VOO) in aglyconic forms arise from glycosides in olive fruits by hydrolysis of endogenous β-glucosidases during crushing and malaxation. These newly formed substances, having amphiphilic characteristics, are partitioned between the oily layer and the OMW, and are more concentrated in the latter fraction because of their polar functional groups. During storage of VOO hydrolytic mechanisms that lead to release of simple phenols, such as hydroxytyrosol and tyrosol, from complex phenols as secoiridoids may be involved (Gutierrez Gonzales-Quijano et al., 1977; Gutfinger et al., 1981; Tsimidou et al., 1998).

1.2.3) Polyphenols in vegetation water (Olive mill wastewaters - OMW) and Olive leaves

The polyphenolic fraction of olive oil comprises only 2% of the total phenolic content of the olive fruits, with the remaining 98% being in olive mill water (OMW) (Rodis-Karathanos et al., 2002).

It is noteworthy that during the olive milling process, for olive oil production, the olive paste is continuously hosed with lukewarm water during the milling, a process that is called malaxation (Aktas-Imre et al., 2001). The OMW is produced in extremely large quantities (~800,000 tons/year in Italy) and, despite the fact that it contains a considerable amount of phenols (more than 1% w/v), is currently disposed of. More than a decade ago, Visioli et al. demonstrated that OMW extracts have powerful (in the ppm range) in vitro antioxidant activity (Aktas-Imre et al., 2001; Longhi-Vodopivec et al., 2001); In animal experiments (Visioli-Galli et al., 2000; Visioli-Caruso et al., 2001) and a couple of human studies (Visioli-Galli et al., 2003; Leger-Carbonneau et al., 2005) confirmed that waste waters are a source of bioactive phenols with a wide array of biological activities and dietary supplements derived from olive mill waste water are already available in the market. The latest of such

studies showed that OMWW increases glutathione levels in healthy volunteers (Visioli-Wolfram et al., 2009) (see below in addendum "Biological activities of olive polyphenols").

There are differences in levels and type of phenolic compounds in *olea europaea L*. leaves, fruits and seeds. Traditionally, olive tree leaves have been used as a folk remedy for combating fevers and other diseases, such as malaria (Benavente-Garsia et al., 2000). Oleuropein a typical secoiridoid of the olive tree, has hypocholesterolemic and hypoglycaemic activities (Romani et al., 1999) and is a potent antioxidant with anti-inflammatory properties (Benavente-Garsia et al., 2000).

Chemically, olive leaves are characterized by the presence of oleuropein (that can reach up to 6-9% of dry matter) and other related secoiridoids (Bruneton-Iridoides et al., 1993), but flavonoids, triterpenes and other classes of compounds have also been isolated (Van Hellemont et al., 1986). Although much of the observed pharmacological activity has been attributed to the secoiridoids, authors also reached the conclusion that other compounds must be implicated in the referred activity (Bruneton-Iridoides et al., 1993). Flavonoids, for their many described properties, namely anti-oxidant, anti-hypertension, anti-inflammatory, anti-allergic, anti-carcinogenic, hypoglycemic and hypocholesterolemic, anti-bacterial and anti-fungal (Harborne-Williams et al., 2000; Havsteen et al., 2002), may also be responsible for a part of the pharmacological actions of olive leaves or, at least, for reinforcing synergistically those actions. A recent report (Silva et al., 2006) demonstrates the presence of polyphenols: Verbascoside, luteolin-7-glucoside and oleuropein abundant in leaves. The high antioxidant activities suggesting the possible application of olive leaves as sources of natural antioxidants.

1.2.4) Active olive polyphenolic components

Hydroxytyrosol

Hydroxytyrosol, also known as 3,4-dihydroxytyrosol or 3,4-dihydroxyphenylethanol (CAS No.: 10597-60-1); is the major component of the phenolic fraction of olive extract and olive oil (Figure 1.2.4.1); hydroxytyrosol is present in olive oil either as simple phenol or esterified with elenolic acid to form oleuropein aglycone. Hydroxytyrosol, is the most investigated molecule among olive polyphenols, and it represent the biochemical target in the majority of bioavalability studies performed on humans and animal systems (see below in following sections) .

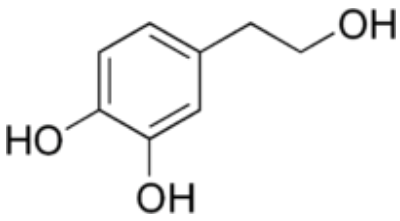

Figure 1.2.4.1 Chemical structure of hydroxytyrosol

Oleuropein

Oleuropein is a phenolic secoiridoid glycoside found in the bark, leaves and fruit of the olive tree, as well as in some other genera of the Oleaceae. The most abundant phenolic substance in the drupe is oleuropein, a bitter glycoside that constitutes up to 14% of the fruit's dry weight. Oleuropein (CAS No.: 32619- 42-4) has the chemical formula C25H32O13 and a molecular weight of 541 Da. (Figure 1.2.4.2).

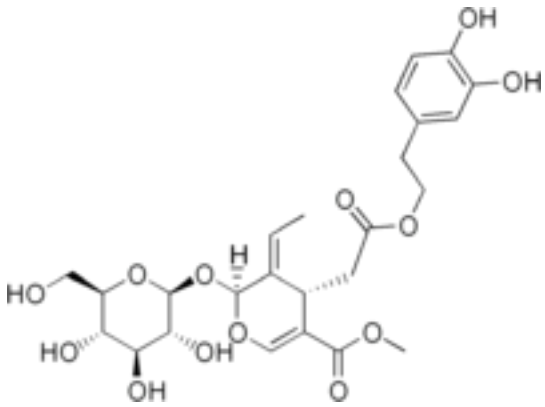

Figure 1.2.4.2 Chemical structure of oleuropein

Tyrosol

Tyrosol (CAS No.: 501-94-0), a minor component of OPE, has a faint sweet fruity-floral odor and a sweet but very weak taste. Tyrosol has the chemical formula C8H10O2 (Figure 1.2.4.3) and a molecular weight of 138 Da. (Soni-Burdock et al. 2006)

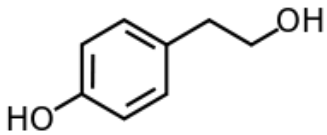

Figure 1.2.4.3 Chemical structure of tyrosol

Oleocanthal

Oleocanthal (CAS No: 289030-99-5) (Figure 1.2.4.4) is a natural organic compound isolated from extra virgin olive oil. It is responsible for the slightly peppery "bite" of extra virgin olive oil. Oleocanthal is a tyrosol ester and its chemical structure is related to oleuropein that is also found in olive oil. Oleocanthal has been found to be have anti-inflammatory and antioxidant properties. 50g of olive oil per day is thought to have the same effect as 1/10 of the adult ibuprofen dose (Beauchamp-Keast et al. 2005).

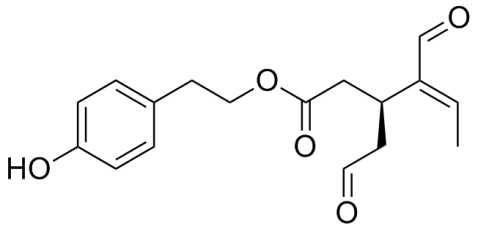

Figure 1.2.4.4 Chemical structure of oleocanthal

1.2.5) Biological activities of olive polyphenols

Recently the new studies concerning the more interesting biological properties of the polyphenols present in the OMW have driven the attention to the recovery and reuse its, rather tan destroy.

Although polyphenols can be considered an environmental problem, they are a very important category of antioxidant, phytochemicals that are useful for the pharmaceutical and cosmetic industry. The concentration of polyphenols in olive oil ranges from 100 to 800 mg/kg of oil, depending on the olive variety, fruit ripening, extraction system, etc. This amount of antioxidants in the olive oil is only 1-2 % of the available pool of antioxidants present in the olive fruit, the rest is lost either in the wastewater (approximately 50%) and in the pomace (about 50 %) (Rodis et al., 2002).

In the OMW are identified more than 30 biophenols compounds, the majority of them exhibit antioxidant activity, cardioprotective action and cancer-preventing activities in human, as reviewed by Obied et al. (Obied et al., 2005; Visioli et al., 1999).

Biophenol	Bioactivity	Remarks
hydroxytyrosol	antioxidant	Isolated from OMWW, antioxidant in rat plasma Antioxidant in rat liver Protects human erythrocytes against oxidative damage
	cardioprotective and antiatherogenic	Multiple effects Scavenges and reduces superoxide anion production in human promonocyte cells
	chemopreventive	Inhibition of peroxynitrite-dependent DNA damage Induces cytochrome C-dependent apoptosis Inhibition of the proliferation of tumor cells
	antimicrobial	Human pathogens Agricultural pathogenic bacteria
	Anti-inflammatory	Prostaglandin sparing and antioxidant activity also were detected Inhibition of leukocytes leukotriene B_4
	Skin bleaching	Topical and bath preparation
oleuropein	antioxidant	Olive cake extract In vivo and in vitro activity Radical scavenging activity within biomembrane
	Antiatherogenic and cardioprotective	Inhibition of LDL oxidation and platelets aggregation Fatty acid composition of rat heart Enhances nitric oxide production
	Hypoglycemic Antihypertensive Antimicrobial and antiviral	In rats (normal and diabetic) Vasodilator Antibacterial Antimycoplasmal Antifungal effects Anti-HIV activity of olive leaf extract
	Anti-inflammatory	Inhibition of 5-lipoxygenase
	cytostatic	Against McCoy cells
	molluscicidal	South American snail Biomphalaria glabratus
	Endocrinal activity	Thyroid stimulation; modulation of hypolipidemic-hypoglycemic activity
	Enzyme modulation	Activates pepsin and inhibits trypsin, lipase, glycerol, dehydrogenase, glycerol-3-phosphate dehydrogenase, and glycerokinase
tyrosol	antioxidant	DPPH scavenging Protects against oxidized LDL Reduces DNA oxidation at high concentrations
	Anti-inflammatory	Inhibition of 5-lipoxygenase (less active than HT)
	antiatherogenic	In humans
	cardioactive	Antiarrhythmic and cardioprotective
Caffeic acid	antioxidant	Tert-butyl hydroperoxide induced oxidative stress Reactive species of oxygen and nitrogen
	chemoprotective	Inhibits DNA oxidation (less active than hydroxytyrosol but more efficient than tyrosol) in prostate cells
	antiatherogenic	Inhibition of LDL oxidation Pro-oxidant activity on LDL
	antimicrobial	Antibacterial and antifungal
	Anti-inflammatory	Inhibition of 5-lipoxygenase (more active than tyrosol and less active than hydroxytyrosol and oleuropein)
	Antidepressive-like activity	Unknown mechanism
Vanillic acid	antioxidant	Alkylperoxyl radical-scavenging
	Antimicrobial activity	Antibacterial and antifungal
	antioxidant	Ballota nigra extract
	chemoprevention	Reverse malignant phenotypic characteristics
	cardioactive	Chronotropic, inotropic, and coronary vasodilatator mediated through cAMP

verbascoside	antihypertensive	Angiotensin converting enzyme (ACE) inhibitor
	Anti-inflammatory	Multiple mechanisms
	antiatherogenic	Plasma lipid peroxidation and erythrocyte membrane fluidity
	sedative	Ballota nigra extract
Elenolic acid	antimicrobial	Antibacterial
	antiviral	Calcium elenolate
p-coumaric acid	antioxidant	Protection of rat heart from oxidative stress of doxorubicin Hypochlorite scavenging activity
	antimicrobial	Antibacterial and antifungal
	chemoprevention	Antileukemic activity
catechol	phytotoxic	Toxic to tornado plants
	antimicrobial	Against plant pathogens
	carcinogenic	Activity in rat stomach differs among strains
	Antioxidant and anticancer	Contrasting effects
rutin	antioxidant	Hepatoprptective Hemoglobin oxidation
	antiatherogenic	Less active than quercetin
	Anti-inflammatory	Only in chronic inflammation
	chemopreventive	Blocking agent for heterocyclic amine-induced rat liver carcinogenicity

Table 1.2.5.1 Major Biophenols in OMW and Their Reported Activities (reviewed by Obied et al.)

1.2.6) Antioxidant activity in humans

The antioxidant properties of the olive polyphenols have been extensively studied and their effects are described in several reviews (Covas et al., 2007; Visioli-Poli et al., 2002; Manna-Della Ragione et al., 1999; Fito et al., 2007). Olive polyphenols are pontent radical scavengers and they inhibit the oxidation of lipids and of low-density lipoprotein (LDL) particle; their antioxidative properties have been demonstrated both in vivo and in vitro. Hydroxytyrosol , an ortho-diphenol, is considered to be a potent antioxidant due to its two adjacent hydroxyl groups. Hydroxytyrosol inhibits copper induced LDL oxidation (Visioli-Bellomo et al., 1995) while a mono-phenol such as tyrosol, has little antioxidant activity and does not protect LDL from chemically induced oxidation. Therefore, the olive oil polyphenols with a cathecol moiety , such hydroxytyrosol or its derivatives are considered the major antioxidant in olive products, several investigations and studies support the antioxidant properties of hydroxytyrosol and its derivatives (Fito-Covas et al., 2000; Schaffer-Podstawa et al.,2007; Weinbrenner-Fito et al., 2004; Corona-Tzounis et al., 2006). Rietjens showed that hydroxytyrosol efficiently protects vascular tissue against oxidative stress (Rietjens-Bast et al., 2007). Hydroxytyrosol was also shown to reduce oxidative damage in intestinal epithelial cells (Manna-D'Angelo et al., 2002) hepatocytes (Goya-Mateos et al., 2007), and human erythrocytes (Manna-Galletti et al., 1999). In addition hydroxytyrosol was able to inhibit COX-2 and iNOS gene expression, in LPS-stimulated j774 murine macrophages (Maiuri et al., 2005).

Hydroxytyrosol is an efficient radical scavenger (superoxide anion, hydroxyl radial, peroxynitrite) and has metal chelating capacities; it efficiently protects against LDL oxidation in vitro at relatively low concentrations. The data show that hydroxytyrosol is a potent inhibitor oh lipid peroxidation wihich is considered to be one of the main mechanisms of tissue damage by free radicals. Finally, the antioxidant properties of olive polyphenols were also demostrated in vivo in animal models (Visioli-Galli et al., 2000; Deiana-Rosa et al., 2007). Thus, olive polyphenols and particularly hydroxytyrosol clearly can reduce oxidative damage in vitro and in vivo and protect cells from oxidative damage.

1.2.7) Markers of oxidation in humans

Oxidized low density lypoprotein (oxLDL) are modified LDL particles exhibiting proatherogenic, proinflammatory, and highly immunogenic activities.

They play a key role in development atherosclerosis, coronary heart disease (CHD) (70-72). High concentrations of oxLDL are predictive of future CHD also in apparently health subjects and are related to metabolyc syndromes. Plasma oxLDL levels were measured in 6 randomized, placebo-controlled, cross-over studies (Marrugat-Covas et al., 2004; Gimeno-De la Torre-Carbot et al., 2007; Covas-Nyyssonen et al., 2006; Salvini-Sera et al., 2006; Covas-de la Torre et al., 2006; Fito-Cladellas et al., 2008; Weinbrenner-Fito et al., 2004). Several studies investigated the effects of olive polyphenols on postprandial oxidative stress, which is linked with postprandial lipemia and hyperglycemia. These studies consistently showed that plasma levels of oxLDL level was observed in the groups with a higher intake of olive polyphenols. Moreover, in 5 of the 6 studies a significant decrease was observe ind oxLDL levels between the low- and the high olive polyphenols groups. The Eurolive study (Covas-Nyyssonen et al., 2006), performed in 200 healthy subjects from five European countries is the largest clinical study showing that olive polyphenols decreased biomarkers of lipid oxidative damage, such as plasma oxLDL, conjugated dienes, and hydroxyl fatty acids and provided good evidence for the antioxidant activity of olive polyphenols increased plasma hydroxytyrosol leves, showing that olive polyphenols are systemically available. The concentration of phenolic compounds in LDL was directly correlated with the phenolic concentration in the olive oils tested. Moreover, the increase in the phenolic content of LDL could account for the increase in resistance of LDL to oxidation, and the decrease of in vivo oxLDL, observed in the study.

The doses of olive phenolics showing an effect on plasma oxLDL levels ranged from 4 to 20 mg per day; consistent significant effets were observed with doses of about 10 mg per day of total olve phenolic compounds. Hydroxytyrosol of derivatives thereof represent about 50 % of the olive phenolic compounds. The data clearly support the protective effects of olive poliphenols against LDL oxidation.

F2- isoprostanes are produced by nonenzymatic, free. Radical-catalyzed peroxidation of arachidonic acid. They are considered as markers of lipid peroxidation and can also exert potent biological actions. They can be quantified in human bodi fluids such as plasma and urine. In several studies a trend toward a decrease in plasma isoprostan levels was observed with increasing intake of olive polyphenols (Covas-Nyyssonen et al., 2006). The olive polyphenols also tended to decrease the postprandial rise in plasma isoprostane levels (Covas-De la Torre et al., 2006). Howevere a significant decrease in plasma isoprostane levels between the groups receinving a low phenolic diet a a high phenolic diet was only observed in one study from ruano et al (Ruano, Lopez-Miranda et al., 2005). No effect was observed by Weinbrenner et al (Weinbrenner, Fito et al. 2004) in a small study. In another open study Leger et al (Leger-Carbonneau et al., 2005) reported that an olive poliphenolic extract had no effect on urinary isoprostane levels but significantly lowered serum thromboxane levels. Overall, the studies suggest that a modst effects on plasma isoprostane levels ma be observed after ingestion of olive polyphenols.

1.2.8) Hydroxytyrosol in vivo and in vitro activities studies

Within the total pool of olive poliphenols hydroxytyrosol is the most abundant (50% of total polyphenols).

It is claimed that hydroxytyrosol is formed in part as a result of hydrolysis of oleuropein (the major biophenol in many olive varieties) during oil extraction by the action of esterases (Capasso-Evidente et al., 1999). Moreover, the amount of hydroxytyrosol can be enriched by acid hydrolysis of secoiridoid derivatives and verbascoside. Recently, it has been made available commercially for research purposes and has also been introduced under different trade names as an anti- oxidant nutraceutical. Hydroxytyrosol is peculiar to olives (and, hence, to olive oil) and is being exploited as a potential supplement or preservative to be employed in the nutraceutical, cosmeceutical, and food industry.

The biological activities of hydroxytyrosol can be conveniently summarized as follows:

1) *Antioxidant activity.* Hydroxytyrosol is a potent inhibitor of metal-induced oxidation of low density lipoprotein (see above). In addition, metal-independent oxidation is also significantly retarded by hydroxytyrosol. The antioxidant activities of hydroxytyrosol, which has been proven to be more effective than BHT or vitamin E, were further confirmed, by the use of stable free radicals, such as DPPH, Also, hydroxytyrosol is a scavenger of superoxide anions generated by either human polymorphonuclear cells or by the xanthine/xanthine oxidase system. Furthermore, a scavenging effect of hydroxytyrosol was demonstrated with respect to hypochlorous acid, a

potent oxidant produced in vivo at the site of inflammation and a major component of chlorine-based bleaches that can often come into contact with food during manufacturing. Antioxidant activities have also been demonstrated versus DNA damage, hydrogen peroxide-induced insult to red blood cells. These results have been discussed above and can be found in several reviews, e.g. (Perez-Jimenez, Alvarez de Cienfuegos et al. 2005).

The signaling pathways involved in the biological activities of hydroxytyrosol are being elucidated (Corona-Deiana et al., 2009; Incani-Deiana et al., 2010) and will likely be the subject of several future investigations.

2) *Modulation of enzymes*. Hydroxytyrosol is able to modulate several enzymatic activities linked to cardiovascular disease. Among them, inhibition of platelet aggregation (Petroni-Blasevich et al., 1995) and pro- inflammatory enzymes such as 5-lipoxygenase (Kohyama-Nagata et al., 1997; Petroni, Blasevich et al., 1997; de la Puerta-Ruiz Gutierrez et al., 1999), and stimulation of the inducible form of nitric oxide synthase (Visioli-Bellosta et al., 1998; Maiuri-Sacchi et al.,2005) have been demonstrated in vitro. In vitro models, hydroxytyrosol is not able to upregulate the activity of the endothelial form of nitric oxide synthase, leaving its role in modulation of vasomotion unresolved (Schmitt-Handler et al., 2007). Notably, the vasomodulating effects of olive leaves extracts have been demonstrated in a rabbit model (Zarzuelo-Duarte et al., 1991), but the human relevance of these findings needs further investigations.

While the majority of data have been obtained in vitro, several experiments have been performed in laboratory animals. In addition, there are approximately 15 human experiments that compared olive oil with extra virgin olive oil (which, however, contains phenols other than hydroxytyrosol) (Covas et al., 2007). Finally, hydroxytyrosol and related olive phenols have been tested, as supplements, in humans. The most notable result is the inhibition of thromboxane B2 production by whole blood, suggesting antithrombotic activity in vivo (Leger-Carbonneau et al., 2005).

Animal experiments confirm, in vivo, most of the evidence obtained in vitro. In particular, hydroxytyrosol retains its antioxidant activity once ingested (though the human metabolic pathway has been elucidated and shows extensive glucuronidation and subsequent urinary excretion) (Visioli- Caruso et al., 2001), protects from second hand smoke induced oxidative damage (Visioli-Galli et al., 2000), inhibits platelet aggregation (Priora-Summa et al., 2008).ameliorates lipid profile and decreases atherosclerosis development (Gonzalez-Santiago, Martin-Bautista et al. 2006), increases brain cell resistance to oxidation and mitochondrial membrane potential (Schaffer-Podstawa et al., 2007).Further experiments confirmed

hydroxytyrosol's anti-inflammatory (Bitler-Viale et al., 2005), and anti-thrombotic potential (Leger-Carbonneau et al., 2005), and its ability to ameliorate osteoarthritis (Bitler-Matt et al., 2007). As a caveat, such experiments have been performed with mixtures of olive phenols in which Hydroxytyrosol was the most active ingredient, but not the exclusive one. Synergy with other olive phenols cannot, at present, be excluded.

1.3) Methods of extraction of phenolic compounds from OMW and olive leaves

Today's society, in which there is a great demand for appropriate nutritional standards, is characterized by cost rising and often decreasing availability or raw materials together with much concerns about environmental pollution.

This is particularly valid for the food and food processing industry in which wastes, effluent, residues, and by-products could be recovered and upgrated to higher value and useful products.

In the past many of those by-products were dumped or poorly used without any treatment for instance as cattle feed or fertilizers.

The discovery of chemical potential of some of these matrices (whey, OMW, grape skins and pomaces), together with commercial applications of extractable substances (whey proteins, polyphenols and flavonoids), gave new perspective to industrial development of process technologies and provide novel solution for food, cosmetics and nutraceutical specialties.

Consequently there is a considerable emphasis on recovering, recycling and upgrading wastes and residues from olive oil industry. Some of these approaches are here described.

Olive Mill Wastewater (OMW)

There are several works (patents) covering the industrial extraction of polyphenols:

(US2002198415 (A1); US2008090000 (A1); US2010216874 (A1)) describe, starting from olive oil production industry wastes, how to obtain polyphenol-based extracts, through acid treatment of the OMW and a prolonged storage thereof up to 12 months at a pH between 1 and 6 with the aim of determining the conversion of oleuropein into hydroxytyrosol. After incubation, the initial oleuropein was converted (about 75-90%) into hydroxytyrosol. (WO2007/013032) describes a process for recovering a concentrate rich in hydroxytyrosol from residues of the olive oli production industry, particularly vegetation waters and pruning residues (leaves). Said process provides for the use, after extraction using solvent (water or alcohol), of extraction systems with supercritical fluids, nanofiltration or, alternatively, reverse smosis for recovering hydroxytyrosol and minor polar compounds. The product thus obtained is an hydroxytyrosol based extract. (WO2008/090460)

describes a further example of a process from recovering the hydroxytyrosol component from the olive oil production industry residues, in which milling wastes are not used alone but rather also include a given amount of green olives with the aim of obtaining a product particularly enriched in hydroxytyrosol. This reference proposed a first acid hydrolysis treatment at a temperature greater than the reflux temperature for the initial material (pomaces and green olive pulps), followed by clarification of the resulting product (for example by filtration), in turn followed by a treatment on an ion exchange chromatography resin. The product adsorbed on such column, after elution, in turn may be supplied to a second chromatography column loaded with a non-ionic adsorbent resin. The product adsorbed on the latter resin, after elution, is further concentrated in hydroxytyrosol, if necessary, through a membrane tangential filtration, specifically reverse osmosis, in which retentate is the desired product.

The patent associated to this study (WO2005/123603 (A1)) describes a separation process based on membrane technologies specifically aimed a t recovering compounds of interest from OMW. In such process, to the various tangential membrane filtration separation operations, there was introduced an initial filtration aimed at maximising the commercial useful polyphenol (such as hydroxytyrosol). Pre-treatment consists in acidifying still fresh OMW to a pH of about 3-4.5, followed by enzymatic hydrolysis. After separating the liquid product thus treated by centrifuge, there follows a series of cascade tangential filtration operations, obtaining from the various retentates polyphenol fractions with different degrees of purification and from the reverse osmosis permeate purified water that can be used for producing beverages.

Olive Leaves

Japon-Lujan et al., in two papers 2006 a-b, proposed microwave and ultrasound assjstance to accelerate ethanol-water extraction of biophenols from olive leaves. (Japon-Lujan and de Castro et al., 2007), report extraction condition for the recovery of biophenols not only from the leaves but also from the small branches (fibrous softwood) of the olive tree. (Briante et al., 2002a) proposed a method of production of hydroxytyrosol based on the use of nonhomogenous hyperthermophilic beta-glycosidase immobilized on chitosan. Accordin to the authors the method is simple and provided a natural, nontoxic product. Leaves with high oleuropein content have to be selected to obtain a good substrate for biotransformation. The bioreactor eluates were examined as substitutes for synthetic antioxidants commonly used to increase the shelf life of food products and also for their possible effect in human cell.-(Salta et al., 2007) and co-workers suggested a technique based on ethanol extraction and fractionation by Short Path Distillation.

A recent report, (Rada et el., 2006) tried to optimize a process to extract and isolate hydroxytyrosol

from olive leaves, since the compound has a high added value with applications in cosmetics, pharmaceutical products, and food supplements.

Recent study, (De Leonardis et al., 2008) laboratory method was developed to produce an olive leaf extract of hydroxytyrosol content. The procedure involved an acid steam cooking of the integral olive leaf to directly hydrolyse the native complex phenols contained therein; succesively, the phenols were recovered by a liquid-liquid extraction with ethyl acetate.

1.4) Membrane separation technologies

Membrane technology is a term that refers to a number of different filtration processes that are used to separate substances. With this technology, membranes are used as filters in separation processes, with a wide variety of applications, both industrial and scientific. They provide effective alternatives to related technologies such as adsorption, ion exchangers, and sand filters. The membranes used in membrane technology may be regarded as barriers separating two fluids and allowing certain substances to be transported across the membrane.

At its simplest, the technological use of membranes may consist of setting up a permeable membranous filter which allows water to flow through, but traps suspended solids. There are various forces which may be used to cause water to penetrate through the membrane. These may include gravity, pressure, electrical current, or maintaining a concentration gradient across the membrane.

One of the major uses of this technology is in the field of water filtration and purification. This includes desalination, or creation of drinking water from salt water, as well as purification of ground waters or waste waters. Other areas of industry that utilize membrane technologies include biotechnology, food and drink manufacturing, and medical uses such as dialysis for kidney failure patients.

For well understand the characteristics of this technologies is interesting to know its recent historical development .

1.4.1) Historical development

Membrane separation technology, was born from a single landmark event: the development of the synthetic asymmetric membrane in 1960 at the University of California, Los Angeles by Sourirajan and Loeb.

The phenomenon of osmosis, known about since 1748, when Abble Nollet observed the water diffusion from a diluted solution to a more concentrated one, when separated by a semipermeable membrane, represents the basic theory for the development of membrane Technology (Boddeker et al.,

1995). In fact, desalination and water treatment by reverse osmosis is probably the earliest and best-known practical application in a very small laboratory scale.

In 1855, Fick developed the first synthetic membrane, made apparently from nitrocellulose. Pfeffer reported in 1877 the successful manufacture of membrane made by precipitating copper ferrocyanide in the porous of porcelain. Than Bechhold, around the years 1907, developed methods for controlling the pore size of cellulosic polymers (collodion), controlling the rate of evaporation of the solvent and by water washing of the film.

The period of 1870-1920 saw the rapid development of theories of thermodynamics of solutions, most notably those of van't Hoff and his theory of diluted solutions, and Gibbs, whose work led to the primary relationship between osmotic pressure and other thermodynamic properties.

Membrane filters became commercially available in 1927 from the Sartorius Company in Germany, manufactured using the Zsigmondy process.

In 1957 the United State Public Health Service official adopted membrane filtration procedure for drinking water analysis.

Simultaneously with the development of microfiltration membrane, especially for bacterial removal in aqueous media, there was considerable interest in developing membrane for reverse osmosis applications, especially for desalination of sea water and purification of brackish water. The semipermeable membrane used by the early investigation was obtained from animals or from vegetable, as pig's bladders, onion skins, etc.

Consequently these membrane would be impractical for these purpose due to the high pressure necessary for practical desalination process.

Only in the 1957 Breton and Reid independently obtained good results (99% NaCl rejection), using a homogeneous cellulose acetate membrane.

In the following many researchers was involved in the study of reverse osmosis area in order to obtain commercially feasible permeate flux reducing the thickness of the membrane.

From1958 to 1960, Sourirajan and Sidney Loeb, attempted to modify commercial cellulose acetate membranes by heating them under water , in the apparent hope that this would expand the pores, thus increasing the flux.

But happened exactly the opposite: heating contracted the pores, consequently increase the salts rejection and reduce both the permeate flux, and the membrane thickness. In addition the heating created a phenomenon known as asymmetry in the ultrastructure of membrane characterized by a thin "skin" on one surface of the membrane, usually 0.1-0.2 micrometers in thickness, while the main body of the membrane named "sponge-like" shows many porous voids and a thickness of 100-150 microns.

Actually (2013) the commercial synthetic membrane shows an asymmetric or symmetric cross-section structure.

Generally, the membrane structures are functional to the membrane applications, in particular characterize the different membrane techniques as Microfiltration (symmetric) of ultrafiltration, nanofiltration and reverse osmosis (asymmetric).

Fundamentally membrane are manufactured from inorganic materials as alumina, zirconia, or metals, or organic materials as carbon, cellulose, or complex polymeric molecules such as polysulphone (PS) or polytetrafluoroethilene (PTFE), polyamide (PA), etc.

These materials are used to produce different membrane geometry as flat sheet, tubular, hollow fiber, or spiral wound. The choice of the membrane chemical composition and of the membrane geometry depends from the specific application process, in relation with different parameters as the chemicals composition of the feed matrix, the chemical-physic characteristic, temperature , pH, etc.

Membrane technology has become a dignified separation technology over the past decennia.

1.4.2) Membrane general characteristics

The main force of membrane technology is the fact that it works without the addition of chemicals, with a relatively low energy use and easy and well-arranged process conductions. Membrane technology (MT) is a generic term for a number of different, very characteristic separation processes. These processes are of the same kind, because in each of them a membrane is used. Membranes are used more and more often for the creation of process water from groundwater, surface water or wastewater. Membranes are now competitive for conventional techniques. The membrane separation process is based on the presence of semi permeable membranes. The principle is quite simple: the membrane acts as a very specific filter that will let water flow through, while it catches suspended solids and other substances. There are various methods to enable substances to penetrate a membrane. Examples of these methods are the applications of high pressure, the maintenance of a concentration gradient on both sides of the membrane and the introduction of an electric potential. Membranes occupy through a selective separation wall. Certain substances can pass through the membrane, while other substances are caught. Membrane filtration can be used as an alternative for flocculation, sediment purification techniques, adsorption (sand filters and active carbon filters, ion exchangers), extraction and distillation. There are two factors that determine the affectivity of a membrane filtration process; selectivity and productivity. Selectivity is expressed as a parameter called retention or separation factor. Productivity is expressed as a parameter called flux (expressed by the unit L/m^2h). Selectivity and productivity are membrane-dependent.

Membrane filtration can be divided up between micro and ultra filtration on the one hand and nanofiltration and Reverse Osmosis (RO or hyper filtration) on the other hand.
When membrane filtration is used for the removal of larger particles, microfiltration and ultra filtration are applied. Because of the open character of the membranes the productivity is high while the pressure differences are low.
When salts need to be removed from water, nanofiltration and Reverse Osmosis are applied. Nanofiltration and RO membranes do not work according to the principle of pores; separation takes place by diffusion through the membrane. The pressure that is required to perform nanofiltration and Reverse Osmosis is much higher than the pressure required for micro and ultra filtration, while productivity is much lower.
Membrane filtration has a number of benefits over the existing water purification techniques:

- It is a process that can take place while temperatures are low. This is mainly important because it enables the treatment of heat-sensitive matter. That is why these applications are widely used for food production;

- It is a process with low energy cost. Most of the energy that is required is used to pump liquids through the membrane. The total amount of energy that is used is minor, compared to alternative techniques, such as evaporation.

- The process can easily be expanded.

Microfiltration and Ultrafiltration

MF and UF are the two processes that are most often associated with the term "membrane filtration". MF and UF are characterized by their ability to remove suspended or colloidal particles via a sieving mechanism based on the size of the membrane pores relative to that of the particulate matter. However, all membranes have a distribution of pore sizes, and this distribution will vary according to the membrane material and manufacturing process. When a pore size is stated, it can be presented as either nominal (i.e., the average pore size), or absolute (i.e., the maximum pore size) in terms of microns (µm). MF membranes are generally considered to have a pore size range of 0.1 – 0.2 µm (nominally 0.1 µm), although there are exceptions, as MF membranes with pores sizes of up to 10 µm are available. For UF, pore sizes generally range from 0.01 – 0.05 µm (nominally 0.01 µm) or less, decreasing to an extent at which the concept of a discernable "pore" becomes inappropriate, a point at which some discrete macromolecules can be retained by the membrane material. In terms of a pore

size, the lower cutoff a UF membrane is approximately 0.005 µm. Because some UF membranes have the ability to retain larger organic macromolecules, they have been historically characterized by a molecular weight cutoff (MWCO) rather than by a particular pore size. The concept of the MWCO (expressed in Daltons, a unit of mass) is a measure of the removal characteristic of a membrane in terms of atomic weight (or mass) rather than size. Because such organic macromolecules are morphologically difficult to define and typically found in solution rather than as suspended solids, it may be convenient in conceptual terms to use a MWCO rather than a particular pore size to define UF. Typical MWCO levels for UF membranes range from 10,000 to 500,000 Daltons, with most membranes used for water treatment at approximately 100,000 MWCO. However, UF membranes remove particulate contaminants via a size exclusion mechanism and not on the basis of weight or mass. UF membranes used for drinking water treatment are also characterized according to pore size with respect to microbial and particulate removal capabilities. In fig 1.4.2.1 MF and UF behaviour is reported in relation to milk and whey fractionation.

Nanofiltration and Reverse Osmosis

NF and RO constitute the class of membrane processes that is most often used in applications that require the removal of dissolved contaminants, as in the case of softening or desalination. The typical range of MWCO levels is less than 100 Daltons for RO membranes, and between 200 and 1,000 Daltons for NF membranes. While NF and RO are sometimes referred to as "filters" of dissolved solids, NF and RO utilize semi-permeable membranes that do not have definable pores. NF and RO membranes are designed to remove dissolved solids through the process of reverse osmosis. Osmosis is the natural flow of a solvent, such as water, through a semi-permeable membrane (acting as a barrier to dissolved solids) from a less concentrated solution to a more concentrated solution. This flow will continue until the chemical potentials (or concentrations, for practical purposes) on both side of the membrane are equal. The amount of pressure that must be applied to the more concentrated solution to stop this flow of water is called the osmotic pressure. An approximate rule of thumb for the osmotic pressure of fresh or brackish water is approximately 1 psi for every 100 mg/L difference in total dissolved solids (TDS) concentration on opposite sides of the membrane. NF and RO are pressure-driven separation processes that utilize semi-permeable membrane barriers. NF differs from RO only in terms of its lower removal efficiencies for dissolved substances, particularly for monovalent ions. This results in unique applications of NF, such as the removal of hardness ions at lower pressures than would be possible using RO. Consequently, NF is often called "membrane softening". Semi-permeable NF and RO membranes are not porous, they have the ability to screen microorganism and particulate matter in the feed water. However, they are not necessarily absolute barriers. NF and RO membranes

are specifically designed for the removal of TDS and not particulate matter, and thus the elimination of all small seal leaks that have only a minor impact on the salt rejection characteristics is not the primary focus of the manufacturing process. Consequently, NF and RO spiral-wound elements are not intended to be sterilizing filters and some passage of particulate matter may occur despite the absence of pores in the membrane, which can be attributed to slight manufacturing imperfections (Meltzer et al., 1997). In fig 1.4.2.1 NF and RO behaviour is reported in relation to milk and whey fractionation

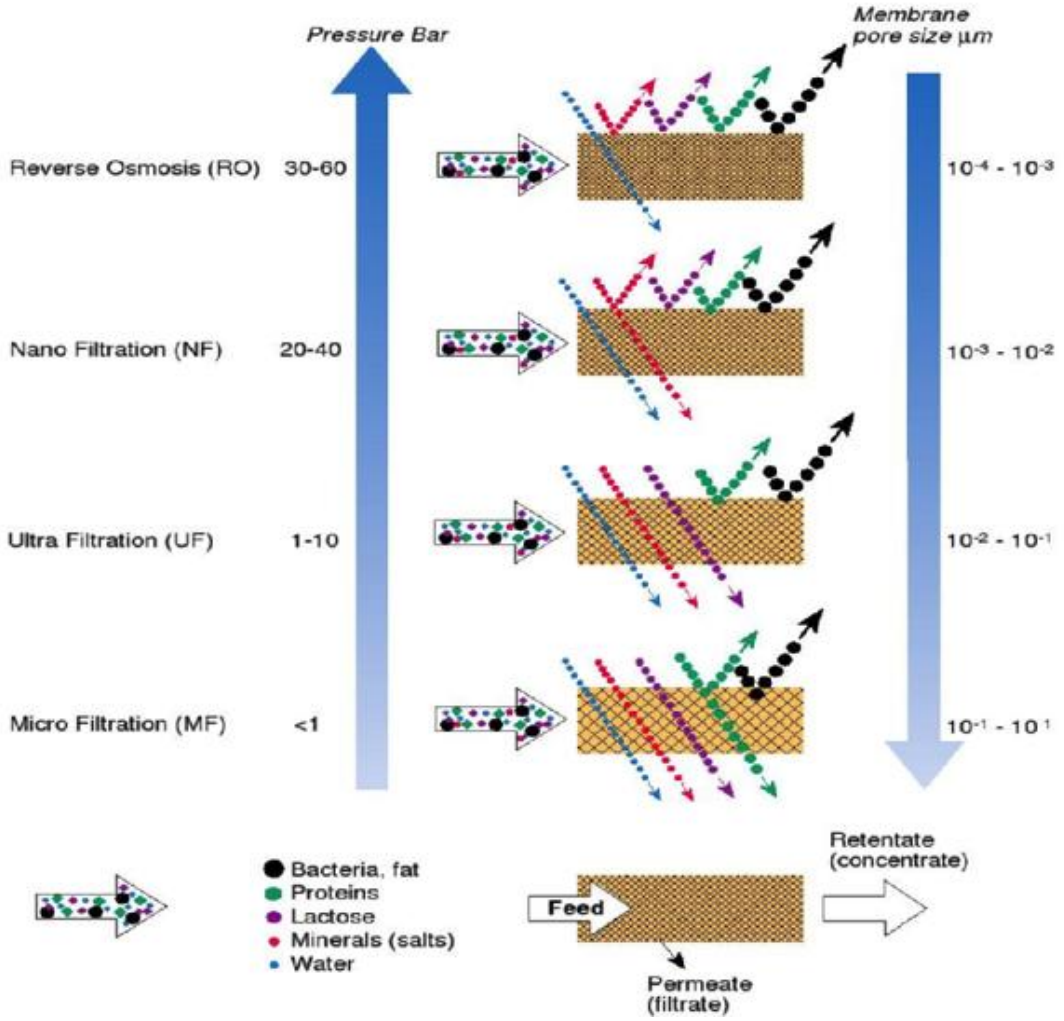

Figure 1.4.2.1 synoptic graphic of membrane filtration techniques and specific response to different molecules (milk and whey fractionation)

1.4.3) DEPTH or screen filtration

All the membrane are manufactured from a variety of materials using several method, but they can all be generally classified into two categories: depth filters or screen filters.

Depth filters derive their name from the fact that filtration or particles removal occurs within the depths of the filter material. Depth filtration consist of a matrix of randomly oriented fibers or beads that are bonded together to form a tortuous maze of flow channels (figure 1.4.3.1)

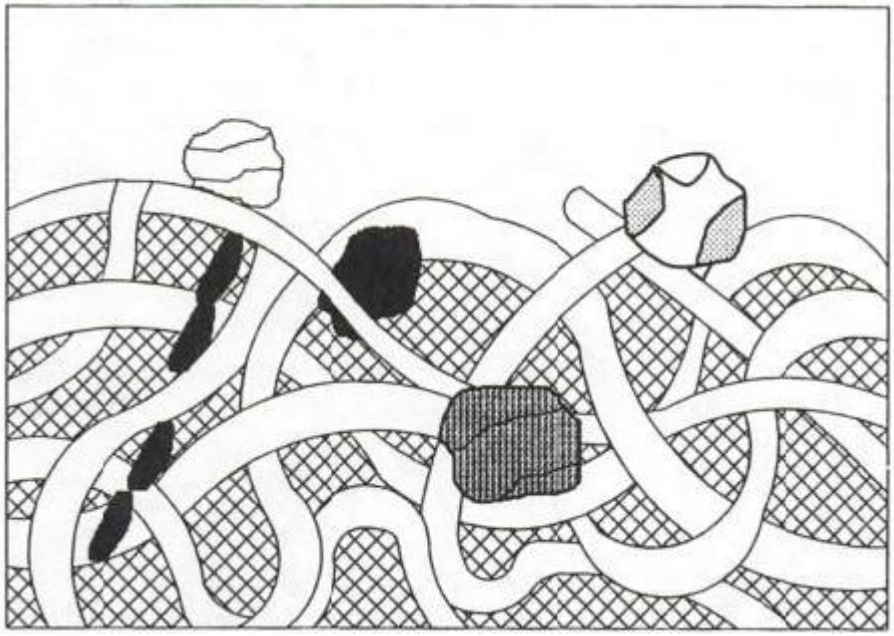

Figure 1.4.3.1 Schematic of depth filter, showing the randomly oriented fibers trapping particles on its surface and within its matrix

Particulates which are insoluble or colloidal in nature are removed from a fluid by entrapment or adsorption to the filter matrix.

A screen filter, in contrast , separates by retaining particles on its surfaces, in much the same manner as a sieve (figure 1.4.3.2)

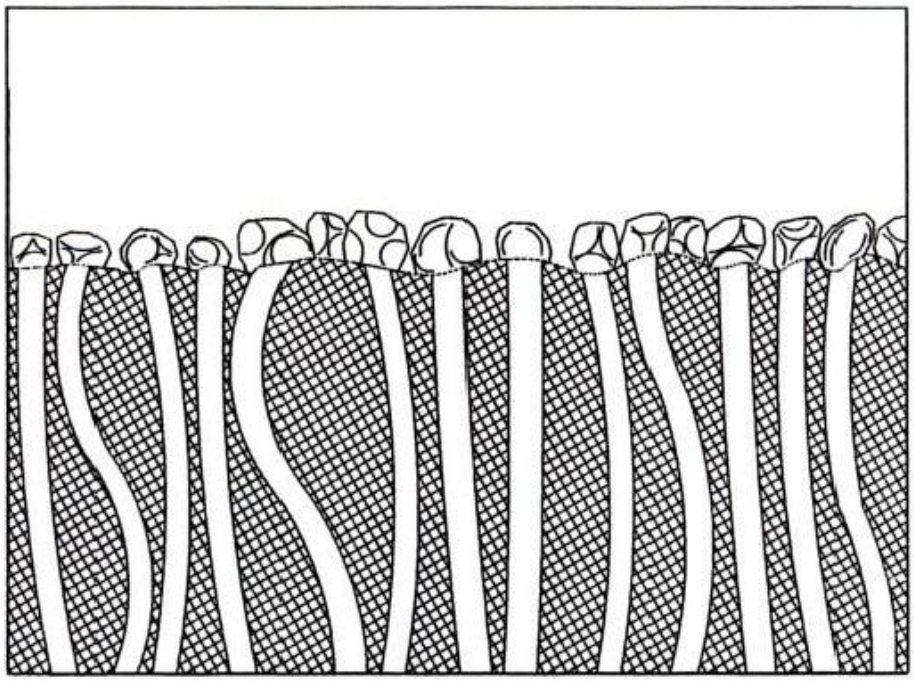

Figure 1.4.3.2 Schematic of screen filter, which retains particles on its surface

The structure is usually more rigid, uniform and continuous, with pore size more accurately controlled during manufacture. Membrane filters fall into this category. Unlike depth filters, there is almost no danger on material migration, and "grow-through" of microorganisms is not normally a problem. Because screen filters have a defined pore size, they can be given an absolute, or quantitative, rating. A further advantage of screen filters is that much higher recoveries of the retained materials are possible. This may be important in the chemicals recovery or in cell harvesting applications.

Screen filters are also classified according to their ultrastructure as either microporous or asymmetric. Microporous membrane are further classified as isotropic (with pore of uniform size throughout the body of the membrane) or anisotropic (where the change in size from one surface to other).

Generally the pore size of microporous membrane is about 0,45 micron or less. Asimmetric membrane, whose manufacture and characterized by a thin "skin" on the surface of the membrane, consequently solute rejection occurs only in the surfaces with skin layer (Cheryan et al., 1986).

1.4.4) Membrane Fouling

Fouling is a major cause of reduction in flux during several membrane separations and in particular microfiltration and ultrafiltration, where feed pretreatment is not used (of reverse osmosis and electrodialysis). Fouling is the essentially the deposition of retained particles (colloids, salts, macrolecules etc) onto the membrane surface or in the pores. The build up of this deposit causes a continuous decline in flux with time, which in microfiltration particularly can fall to values an order of

magnitude less than the initial values. The fouling behaviour is specific to the system of interest and consequently varies according to contaminant of foulant, whether organic macromolecules, biological, inorganic salts, particulates.

The main difference between the types of fouling (colloidal fouling, organic fouling, scaling and biofouling) is the nature of the particles that cause the fouling. In addition, fouling can be divided into reversible and irreversible fouling based on the attachment strength of particles to the membrane surface. Reversible fouling can be removed by means of strong shear force or backwashing. Formation of a strong matrix of fouling layer with the solute during continuous filtration process will resultin reversible fouling being transformed into irreversible fouling layer. Irreversible fouling is normally caused by strong attachment of particles, which is impossible to be removed by physical cleaning method (Choi et al., 2005).

Colloidal particles are ubiquitous in natural waters. Colloids cover a wide size range, from a few nano-meters to a few micrometers (examples: colloidal silica, aluminium, iron and manganese oxides, calcium carbonate precipitates etc). For reverse osmosis, nanofiltration and "tight" ultrafiltration membranes, colloidal fouling is caused by the accumulation of particles on the membrane surface in a so-called cake layer. For microfiltration membranes, pore plugging by colloidal particles can be an important fouling mechanism, in addition to particle accumulation on the membrane surface. The extent of pore plugging and cake layer formation depends on the relative size of the particles compared to the membrane pore size (Zhu-Elimelech et al., 1997).

The term organic fouling is applied for these substances that are dissolved in the feed solution and that tend to stick to the surface of the membrane. Foulants like oil, macromolecules, proteins, anti-foaming agents are all contributing to an organic gel layer on top of the membrane or in pores.

Biofouling is a special class of organic fouling and is the result of complex interactions between the membrane material, dissolved substances, fluid flow parameters and microorganisms.

Scaling or precipitation fouling involves crystallization of solid salts, oxides and hydroxides from solutions. Through changes in temperature, or water removal (as in reverse osmosis), the concentration of salts may exceed the saturation, leading to a precipitation of salt crystals. Precipitation fouling is not only a problem in reverse osmosis, but is also a very common problem in boilers and heat exchengers operating with hard water and often results in limescale.

1.4.5) Membrane cleaning

The methods for cleaning membranes after fouling has occurred based on hydraulic, mechanical, chemical and electrical methods.

Chemical cleaning is the most important and extensively used method for controlling fouling in membrane separations. The choice of agent(s) depends upon the type pf membrane, the type of foulant and the severity of fouling. Membrane manufacturers generally recommend appropriate agents for their specific products. The types of agents are:

- Acids (strong or weak);
- Alkalis(NaOH);
  detergents;
- Enzymes;
- Complexing agents (EDTA);
- Disinfectants.

Chemical cleaning is usually performed as clean in place (CIP) techniques by filling the retentate channels of the membrane module with cleaning solution from a separate cleaning tank. The module is generally exposed to the cleaning solution for a period of several hours. In most cases the plant feed and circulation pumps are used to recirculate the cleaning liquid though the module at high velicity and low operating pressure. In the food industry, cellulose acetate reverse osmosis membranes are usually cleaned with enzymatic detergent solutions, which are able to break down protein layers, followed by sanitisation with an oxidising solution such as sodium hypochlorite or hydrogen peroxide. The majority of ultrafiltration and microfiltration membranes, and also some of the newer (noncellulose acetate) types of reverse osmosis membranes can be cleaned with more aggressive and cheaper chemicals. Membranes in the food and dairy are frequently cleaned sequentially with hot strong alkali (NaOH at 50 °C) and hot acid (HNO_3 at 45 °C). The membranes are water washed and finally sanitised with an oxidant e.g. sodium hypochlorite, ethylene oxide or hydrogen peroxide. The sanitisation step will generally destroy most, but not all, micro-organisms in the system. Certain modules can as an alternative be steam sterilised at 120 °C which is likely to destroy all micro-organisms in the system. In hygienic applications cleaning it is likely to be carried out every day and results in a considerable downtimeof the plant.

Cleaning formulations and the frequency and duration of cleaning cycle depend on the stream being processed, the degree of fouling, the extent of the concentration, etc. In general, cleaning should be performed at low pressure and high velocity, at temperatures of 45 to 50 °C.

The hydraulic cleaning of the membrane is typically achieved with back flushing of the permeate through the membrane. The process is carried out by reversing the direction of flow of the permeate, usually for a few minutes, at a pressure which can be as large as the filtration pressure. This effectively

dislodges the foulant from the membrane and restores the flux to a value close to the initial (or previous high) value. Back flushing is carried out repeatedly at regular intervals and leads to a saw-tooth type of flux behaviour, as shown in Figure 1.4.5.1

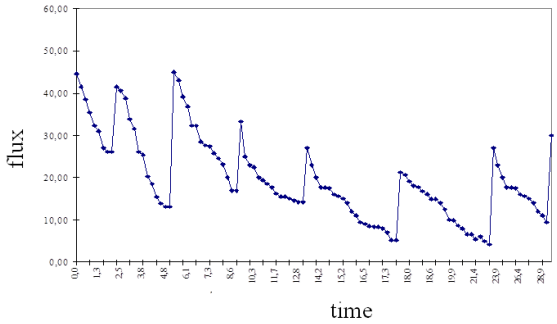

Figure 1.4.5.1 Effect of back flushing on membrane flux-rate

The average flux rate generally still shows a gradual declin with time and membrane cleaning will still be necessary.

The procedure is routinely carried out with MF membranes and hollow fibre and tube UF membranes, both types are able to withstand the reversal in pressure difference. The method is effective with many kinds of foulants, particularly larger particles, but less so with material which forms adherent films. Ceramic membranes are particularly suitable for backflushing being mechanically very strong. An alternative method of hydraulic cleaning is by back-pulsing, ie short bursts of back pressure, alternative pressurising and depressurising and reversing the feed flow direction with the permeate exit closed (this induces back pressure).

Elettrical methods of cleaning can utilise electrical pulsing which results in the movement of charged species (particles, molecules) away from the surface. The process is carried out while the membrane separation is in process, although special module designs are required to introduce the charge to the membrane surface, which is generally metal.

The method(s)s used depend upon the separation process and the configuration of the module.

1.4.6) Main application of membrane processes

Membrane technologies have had over the last 10 years an increasing application in many different industrial sectors ranging from biomedical (kidney dialysis) the agri-food industry, water treatment for drinking water production, applications in the engineering industry the production of drinking water from seawater. Table 1.4.6.1 summarizes main applications in food industry where has already been

demonstrated the technical feasibility and economic self-interest; as reported in figure 1.4.6.1 where is illustrated the growth of the membrane market world wide and in the United States for the next year and also industrial applications of membrane process. We can notice that 55% is related to wastewater treatment while 23 % concern food and beverage. Each industry apply different techniques (MF, UF, NF, RO) in accordance with production needs.

The application of innovative techniques such as Membrane Technologies (MT) in food transformation processes represent a real opportunity to increase and qualify the productivity and reduce the environmental pollution of farms. The commercial application of MT in food processing industry includes concentration of fruit juices, and treatment of a variety of food processing waste streams (Paulson et al., 1984; Jonsson et al., 1990).

In general MT's are applied in different unit operations: concentration, purification, clarification, recovery and upgrading of products (Van Der Horst et al., 1990; Pizzichini et al., 1991; Jiao et al., 1992).

These operations allow to increase the product quality (taste, aroma, appearance, etc.) the recovery of process byproducts and a high reduction of the process energy consumption. In addition, membrane technologies permit a strong reduction of chemicals used for clarification or flocculation of raw natural compounds such as wine, vinegar, beer, fruit juices, coffee, tea, etc.

Membrane processes such as cross-flow MF, UF, NF and RO, all pressure driven membrane processes, have been intensively exploited in the food industry. In this field many products, such as juice and milk, require removal of large quantities of water to concentrate the products for more efficient packaging or shipping.

The potential application of MF are numerous, e.g. to retain macromolecular solutes for concentration and /or clarification of milk, wine, juice, vegetable, brine, gelatin, whey, beer. The potential advantages of MF are to be found in product improvement and product innovation, in process economy and process control.

The UF process offers unique separation possibilities, primarily in fractionation of chemicals (like whey). Commercial applications of UF are numerous in many different fields: the chemical, electronic, food, pharmaceutical industry, the environmental pollution control, etc.

UF has been used in commercial-scale protein fractionation and concentration for more than 18 years, with substantial quantities of organic materials (proteins) fractionated and concentrated daily.

NF is important for concentration of saccharides, brackish water treatment, for concentration of acids in chemical or galvanoplastic application.

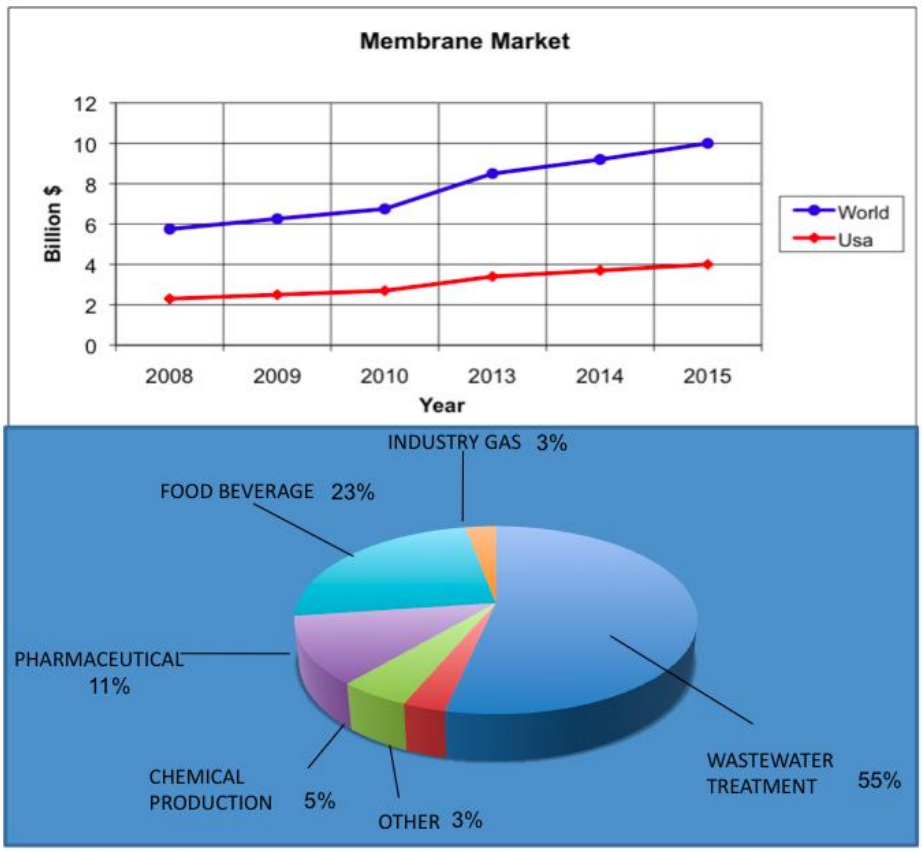

Fig. 1.4.6.1 trend of membrane market (worldwide and USA), and industrial application of membrane processes.

Applications of RO are much more widespread, including in particular sectors such as pharmaceuticals, where RO units are present both at small and medium scale.

In desalination and water purification systems, however, plant sizes are high (thousands of m^2 of membrane filtering surface applied). The limits of application of this technique are strongly dependent on processing costs and investment.

In food industry RO is generally used for product concentration, in many cases coupled with UF or MR processes.

Manufacturing sector	Type of application	Technique
Milk	Concentration of whole milk and skim milk	UF, OI
	Fractionation of milk and whey	UF, NF, OI
	Separation of bacteria	MF
	Removal of casein, fat and lactose	MF, UF, NF
	Hydrolysis of lactose	MR
Fruit juices	Clarification of apple and citrus	MF, UF
	Deacidification	UF
	Concentration	OI
	Sterilization before bottling	MF, UF
	Recovery of pectin in the cycle of candied	UF, OI
Wine	Recovery of the juice or wine residue (marc)	MF
	Sterilization of the must before fermentation	MF
	Dealcoholization of wine	OI
	Clarification of vinegar	MF, UF
Sugar	Treatment of molasses (color and hexoses)	OI
	Concentration of cane juice	OI
	Desalination molasses	OI, ED
Proteins	Concentration of egg	UF
	Concentration of soy protein and emulsions	UF
	Removing odors from soy milk	UF, OI
	Purification of proteins from soy	UF
	Concentration of gelatin	UF
	Recovery of waste water from food process	UF, OI
Coffee - Tea	Extraction, concentration, decaffeination	OI
Tomato	Concentration of juice and tomato puree	MF + OI
	Water treatment washing the tomato	UF + OI

Table 1.4.6.1 Main applications of membrane technology in the agri-food sector

Table 1.4.6.1 summarize some specific application of membrane processes in food industry, where membrane undertake the separation and concentration of specific class of substance of interest.

Other interesting process can be advised in literature where are reported specific applications the tanning industry (Pizzichini et al., 1996b), in sewage effluent (Pizzichini et al., 1998) and in the desalination of 'sea water (Pizzichini et al., 2002).

1.5) References

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2) AIMS OF THE STUDY

The aim of this work consists in verifying technical and practical feasibility of a two treatment approaches to olive industry by-products : olive mill wastewater (OMW), and Olive leaves in order to recovery purified polyphenols and reduce at the same time the environmental impact related to the disposal of these matrices.
Membrane filtration techniques as: microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO), are adopted as main technological approach, in a sequential manner.
The treatment process of raw matrices solid ones (olive leaves) and liquid ones (OMW) are conducted in two different ways:

- OMW Chemical and Enzymatic pre-treatment followed by sequential tangential filtration (MF, UF, NF, OI)

- Olive Leaves water extraction followed by sequential tangential filtration. (MF, OI)

Both processes allows to recovery liquid polyphenol enriched fractions and ultrapure water. Process fractions most interesting for commercial exploitation were charachterized and analyzed.

3) RESULTS AND DISCUSSION

3.1) The new OMW treatment process

The treatment of OMW is based on the application of membrane technology with the aim of extracting and recovering the maximum amount of polyphenols in order to facilitate the disposal of these wastes as described in (WO2005123603 (A1)).
It is known that membrane technologies employ special filters (membrane) that are operated in a special fluido-dynamic condition (tangential flow) that reduce the filter fouling and consequently assures a high permeate flux as a function of time (Cheryan et al., 1986). These technologies are defined BAT (Best Available Technology) from the EPA and also recognized by the European Union (UE).The membrane technology is largely applied in the word not only for wastewater treatment but especially for dispersed solutes recovery, often pollutant, and to generate purified water.
Five treatment steps are necessary: a chemical and enzymatic pre-treatment of the row OMW matrix, followed by four tangential flow membrane filtration steps in sequence as: micro-filtration (MF), ultra-

filtration (UF), nano-filtration (NF) and reverse osmosis (RO). The MF section is equipped with ceramic tubular membrane with a molecular weight cut-of (MWCO) 0.14 µm. All the other membrane sections are equipped with spiral wound polymeric membranes with MWCO ranging between 20,000 and 200 kDaltons. The filtration processes allow the recovery of five main liquid fractions in different volumetric percentages, all of them suitable for commercial use in the energy compartment (biogas), in the food industries (novel food), in the nutraceutical and cosmetic industries.

3.2) Materials and method OMW

Experiments were performed both in Reserch Center of ENEA Casaccia Rome, and in the PhenoFarm Company of Rome (www.phenofarm.it). OMW used were supplied from an olive mill in the north of Roma (Olive mill Santa Barbara, Scandriglia, RI), operating in a continuous extraction process.

Pre-treatment

After the collection OMW were acidified at pH 3,5 using H_2SO_4 98% supplied by Carlo Erba. The enzymes used in the pre-treatment are provided by Natuzym WeissBiotech (Ascheberg, Germany). The enzyme reaction took place in a reactor thermostated at 37 ° C for 120-150 minutes.

Membrane filtration

Membrane filtration experiments were performed with MF, UF, NF and RO PhenoFarm pilot plants.

- MF is equipped with two centrifugal pump by Alfa Laval; two housing containing 12 ceramic tubular membrane (TAMI france); two flow-meters on the retentate and permeate; three manometers are installed, upstream and downstream the pressure vessel (ceramic membrane) and on permeate line; a cooling system controlled by an electrovalve regulated with a thermocouple with ranging from to ± 1 °C; a back pulse apparatus for membrane continuous cleaning with permeate stream at prefixed interval time.
- UF is equipped with centrifugal pump by Alfa Laval; two vessel containing polymeric membrane 4”x40”; two flow meters on the retentate and permeate; manometers are installed, upstream and downstream the pressure vessel; a cooling system controlled by an electrovalve regulated with a thermocouple with ranging from to of ± 1 °C;
- NF is equipped with two centrifugal pump by Alfa Laval; three vessel containing polymeric membrane 4”x40”; two flow meters on the retentate and permeate; two manometers are installed, upstream and downstream the pressure vessel; a cooling system controlled by an electrovalve regulated with a thermocouple with ranging from to of ± 1 °C;
- OI is equipped with two centrifugal pump by Alfa Laval; three vessel containing polymeric membrane 4”x40”; two flow meters on the retentate and permeate; two manometers are

installed, upstream and downstream the pressure vessel; a cooling system controlled by an electrovalve regulated with a thermocouple with ranging from to of ± 1 °C;

The specifics of the MF, UF, NF and RO membranes employed are reported in table 3.2.1

Table 3.2.1 Membrane specifics

Membrane	Process	Material	Conformation	Cut-off
Ceramic			Tubular	
Tami	MF	ZrO_2	23 canals	0,14 µm
			(3,6 mm □)	
Polymeric			Spiral-wound	
Osmonics	UF	PES	4''x40'' inches	20 Kd
			28 mils spacer	
Polymeric			Spiral-wound	
Nadir	NF	Polyamide	4''x40'' inches	150-300 Kd
		TFM	20 mils spacer	
Polymeric				99,5% salts rejection
Hydronauti	RO	Composite	Spiral-wound	
cs		polyamide	4''x40'' inches	

ZrO_2: Zirconium oxide

PES: Polyethersulfone

TFM: Thin Film Composite

Membrane cleaning

- Ceramic membranes were regenerated with chemical washes, using alkaline solutions (NaOH), even at high concentrations (0.8 M) and high temperatures (60-80 °C);
- The UF membranes were regenerated by washing Basic (0.3 M) at 40-45 ° C and subsequent rinsing by tap water.

Total solid measurements

The dry matter content was measured with Thermobalance mechanical infrared by Orma (Italy). 5 grams of each fraction were heated to 105 °C for 45 min. until reaching a 0,1% of residual humidity.

Phenols quantitation

Polyphenolic concentration was determined in HPLC/DAD (Gynkotec - Ramsey, USA) using an high pressure gradient pump Gynkotec with an UV/DAD (Diode Array Detector) operating at 280-350 nm. Chromatographic separation was performed using a Lichrosorb 5 RP18 column (250x4,6 mm) and the

eluting mobile phase constituted by water acidified (pH 3.2) with TFA (solvent A) and acetonitrile-methanol (solvent B).

Time (min.)	Water + TFA	Acetonitrile (80%) + Methanol (20%)
0	100	0
20	85	15
25	85	15
35	75	25
43	75	25
53	0	100
58	0	100
65	100	0

Table 3.2.2 Individual phenolic component identification was achieved by mean phenolic standards. All polyphenolic standards were purchased from Sigma Aldrich (Italy).

3.3) Results and discussion OMW

Freshly collected OMW were supplied from an olive milling factory adjacent to the production plant of the PhenoFarm company located in Scandriglia, (RI) operating in a continuous extraction process. Raw OMW were soon acidified with H_2SO_4 98% to reduce pH from 5,38 to 3,5 to prevent oxidation of polyphenols, and favour the activities of pectolytic enzyme necessary to increase the polyphenols extration in the aqueous medium.

According to proposed process, the acidified OMW promotes the hydrolysis of cellulose, emicellulose and microdispersed pectin by a pectinase enzyme complex, in order to increase the membrane separation performance. Raw OMW proteolytic and cellulolytic enzymatic treatment to rupture the adsorption link between polyphenols and organic compounds can also increase hydroxytyrosol yield in MF permeate stream. Successively the OMW are treated in the membrane plant following the hydraulic scheme reported in figure 3.3.1.

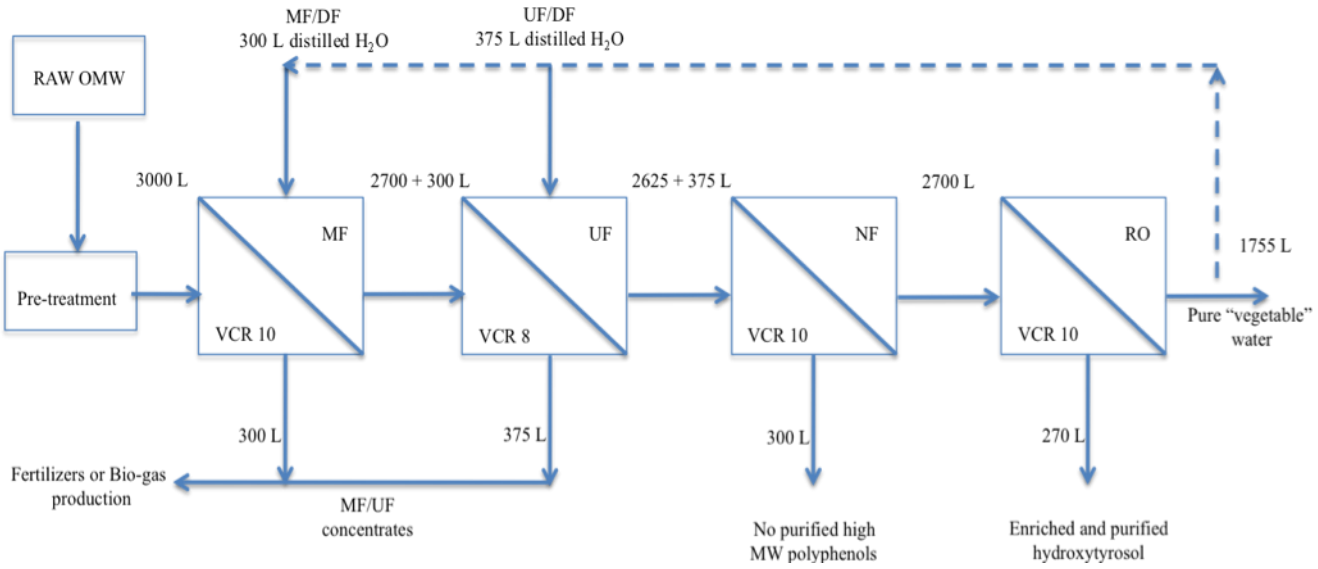

Figure 3.3.1 Process lay-out of membrane sections

Process parameters of MF, UF, NF and RO are reported in table 3.3.1

	MF	UF	NF	RO
Feed flow (m^3/h)	8-9	4-5	3-4	0,5-1
TMP or operative pressure (bar)	1,5-2	3,5	15	30-40
Temperature (°C)	25-30	25	25	25
Flow speed (m/s)	5-7	0,25	0,27	
VCR	10	8	10	10
DV	1	1	-	-

Table 3.3.1 Process parameters

Diafiltration process (DF) consisting in the addiction of an equal volume (dia.volume DV) of distilled water (generally RO permeate) to the concentrated fraction and continuing the filtration process in the same operational conditions in order to increase the passage of solutes in permeate fractions, was conducted in MF and UF.

3000 L of pretreated OMW were treated in MF 0,14 µm and 300 L of distilled water were added to the MF retentate (300 L) obtaining other 300 L of MF/DF permeate.

MF 0,14 µm holds all suspended solids in the retentate while rejection to polyphenols is respectively 30-40 %. DF on the MF retentate, was obtained adding 300 L of distilled water to 300 L of MF retentate. This procedures increases the yield of polyphenols in the permeate stream of about 20-25%.

The MF of the OMW is the most critical step of the whole process separative membrane, dealing with a stream characterized by an high content of total solids, colloidal macromolecules and residual oil.
As can be seen from the curve of permeability of Figure 3.3.2, after about 7 hours of work, the productivity of the membranes decreases by about 70-75%.

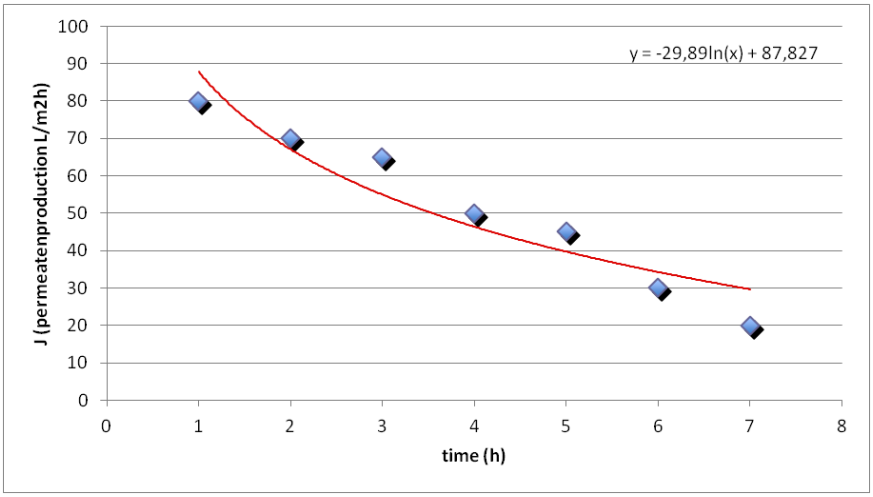

Figure 3.3.2 Productivity MF

After the washing procedure was carried out a rinsing with water and the membranes have regained the initial permeability. The MF and MF/DF permeates were successively treated in UF membrane section equipped with spiral wound membrane of 20 KDalton. The UF retentate stream retain about the 30% of dissolved solids contained in the MF/DF permeate. In this condition the UF permeate, obtained with 1 DV, contain generally the 50-55% of hydroxytyrosol contained in the UF retentate.
Figure 3.3.3, shows the productivity of UF.

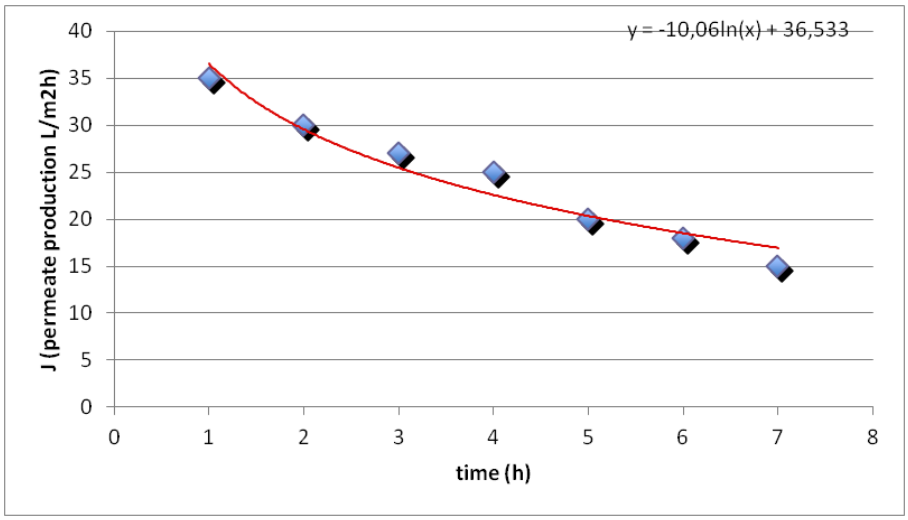

Figure 3.3.3 Productivity UF

The permeate of UF, contains colloids, macromolecules dissolved in solution which also determine a decreasing of about 55% in membrane productivity.

Successively the UF and UF/DF permeates were treated in NF membrane section equipped with spiral wound membrane having a MWCO of 200 Dalton. NF holds about 60% of dissolved solids contained in UF and U/DF permeates. Salts and low MW polyphenols, as the hydroxytyrosol, flows trough the NF membrane and were recovered in RO concentrate solution. RO retentate has a total solids continent of 10%.

In Figure 3.3.4, shows the liquid fractions separated from the OMW.

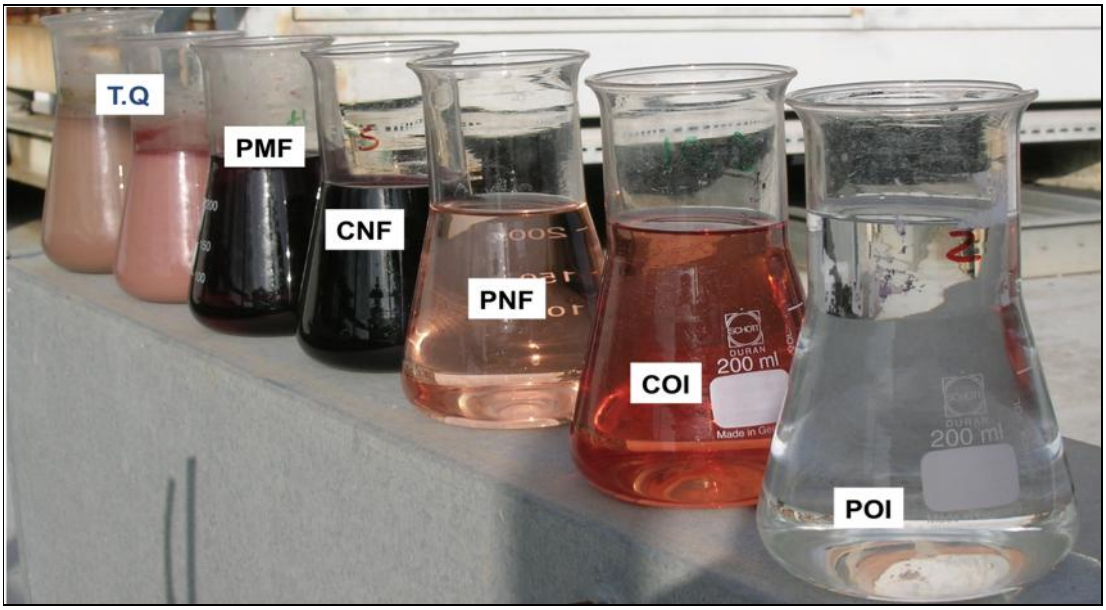

Figure 3.3.4 Liquid fractions separated from the OMW

NF and RO, in terms of productivity expressed as litres of permeate produced per init area and time (L/m^2 h) versus time run, are shown in figure 3.3.5 (a and b).

It is observed that the membrane of NF has a productivity of between 35 and 13 (L/m^2 h), with respect to the productivity of the RO that remains stable at values ranging between 35-30 L/m^2 h and the fouling of the membrane is minimal and the regeneration of the module is done through simple and repeated washing with water.

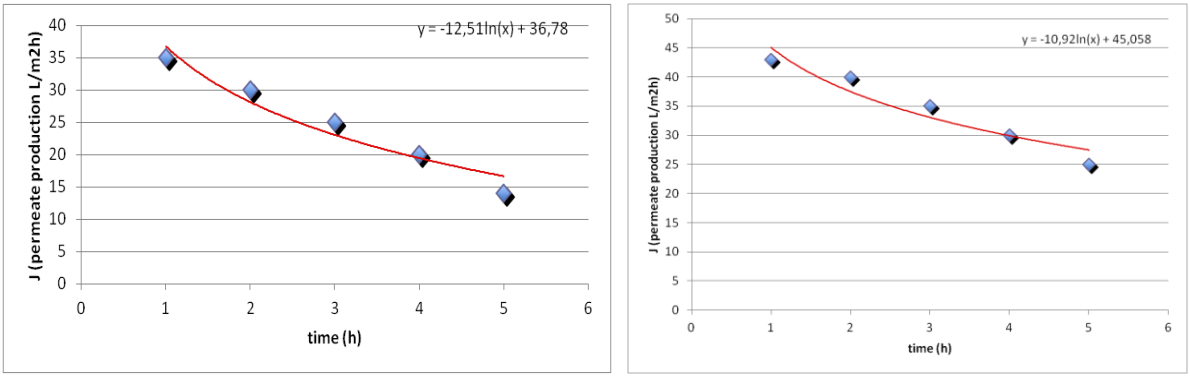

Figure 3.3.5 Productivity NF (a) and OI (b)

Analytical characterization

All Liquid fractions were analyzed for total polyphenols, and hydroxytyrosol yield by HPLC (see table 3.3.2) among them most interesting ones (NF retentate and RO retentate) for commercial exploitation chromatograms are reported below (see figures 3.3.6 and 3.3.7)

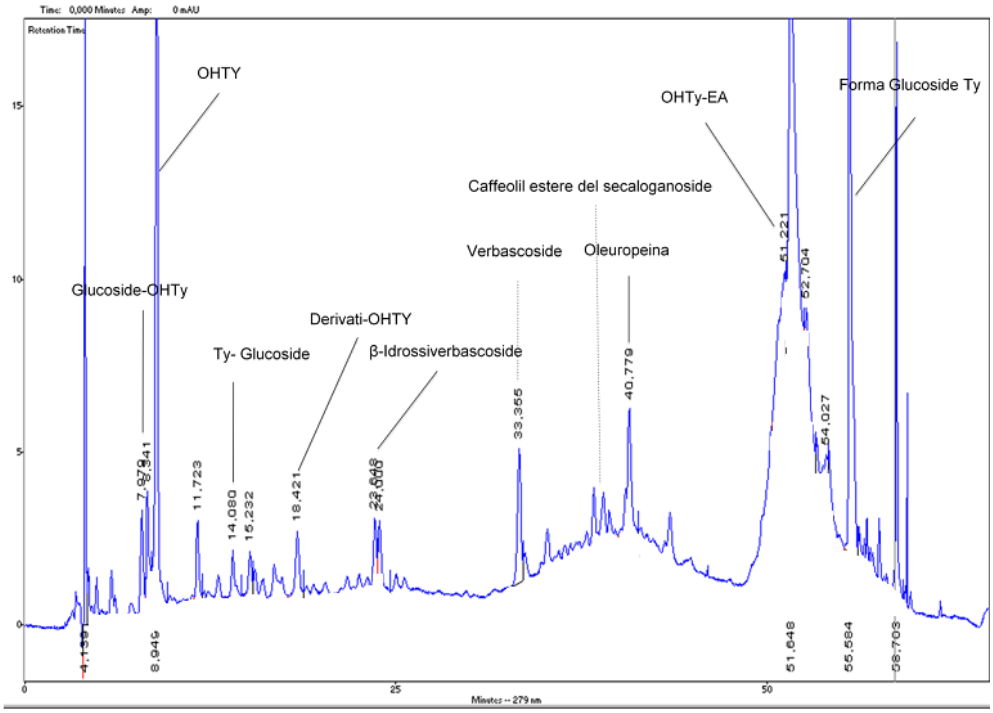

Figure 3.3.6 Polyphenolic chromatograms of NF

The chromatographic profile of NF retentate shows that most important contribution is due to Hidroxytyrosol tyrosol and their derivatives (glucosides) these molecules remain in equilibrium between permeate and retentate fraction if DF procedures are note conducted. But in NF retentates we

observe also peaks representing high molecular weight compounds as verbascoside, oleuropein and caffeic acid derivatives.

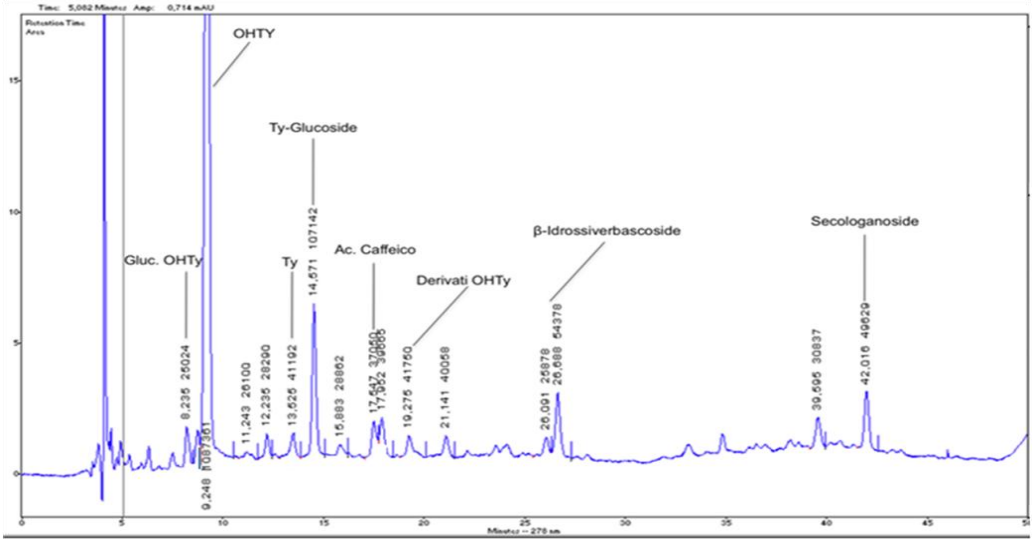

Figure 3.3.7 Polyphenolic chromatograms of OI

In RO retentate hydroxytyrosol is the most representative compound among poliphenolic pool it constitutes 94% of total polyphenols, also are present tyrosol, tyrosol glucosides and small amounts of verbascoside isomers, caffeic acid and its derivatives. These compounds can transit over membrane because small imperfections in membrane polymeric structure.

From Chromatograms evaluation we can confirm the concentration of high molecular phenolic compounds in NF and the specific separation of orthodiphenols (tyrosol, hydroxytyrosol and their glucosides) in RO retentate.

RO retentate all the components contained in NF permeates with rejection values greater than 98%. At the same time the RO permeate is a pure "vegetable water" with a total solids content of 0,004% , an electrical conductivity of 0,1 mS/cm, and a COD lower of 120 ppm of O_2. The RO retentate shows an hydroxytyrosol concentration of 14067,7 ppm (table 3.3.2).

Frazioni	Polifenoli Totali (g)	Idrossitirosolo (g)	Resa idrossitirosolo (%)
AV	9450	7276,5	100
MF Permeato	5386,5	4176,7	57,4
MF Retentato	3780	3230,8	44,4
MF/DF Permatoe	973,3	720,3	9,9
MF/DF Retentato	2891,7	2546,7	35
UF Feed	6350	4948,02	68
UF Permeato	4016,2	3398,1	46,7
UF Retentato	2258,5	1586,3	21,8
UF/DF Permeato	1105,6	924	12,7
UF/DF Retentato	1219	793,1	10,9
NF Permeato	4100	3900	53,6
NF Retentato	1011,1	400,4	5,5
RO permeato	97,3	94,6	1,3
RO Retentato	4000	3798,3	52,2

Table 3.3.2 Low MW polyphenols and hydroxytyrosol mass balance

3.4) Conclusions OMW

The treatment process based on multiple sequential membrane filtration steps seems compatible with an industrial process, we observed important volumetric amounts and acceptable productivity from all four section of filtration. Those volumes could be representative of a small-medium industrial production.

In terms of polyphenols (hydroxytyrosol) yield the greater amount is lost in the MF and UF retentates, respectively 35% and 11% of the initial hydroxytyrosol content, because of greater rejection to polyphenols due to their adsorption with other organic molecules contained in OMW and the greater membrane resistance due to fouling phenomena on its surface during filtration operation. To have a greater release of hydroxytyrosol in the MF permeate and reduce fouling during MF and UF is necessary to optimize the pre-treatment.

Diafiltration is important in the first process step to purify MF and UF retentates from polyphenol content and allowing an increasing polyphenolic yield in the permeate streams.

In NF is loss about 5% of the initial hydroxytyrosol contained in OMW.

RO concentrates hydroxytyrosol recovering about 97% of that contained in NF permeates. In RO retentate 52% of the initial hydroxytyrosol of OMW is recovered. In particular hydroxytyrosol, with a concentration of 14067,7 ppm, rapresents more than 90% of total polyphenols.

HPLC analysis and chromatographic profiling of NF and RO retentates confirm the performance of phenolic molecules separation: high moleculare weight compounds (>500 Da) are retained by NF while short molecules (< 200 Da) can pass in the permeate stream (orthodiphenols and derivatives) the RO has to be considered as a concentrated form of NF permeate cause only water is able to permeate in RO filtration (see below).

The membrane filtration process allow recovery of five main liquid fractions in different volumetric percentages, all of which are suitable for commercial applications.

MF and UF concentrates fractions rich in organic matter without or extremely depleted of the polyphenolic content, useful for agricultural ferilizer and bio-gas. NF retentate contains high molecular weight polyphenols not purified and could be reused in the food industry as integrator.

RO concentrate ideal for novel food (production of functional olive oils).

RO permeate is a pure "vegetable" water ideal as base for beverage formulations. Otherwise it can be reused in the same industrial process, for agricultural purposes or discharged into the river in agreement with environmental regulation.

This water shows a total dissolved matter lower then 0,04 g/L and a COD lower then100-120 ppm O_2. This value depends by the operating pressure of RO section, over 35 bar the COD remain in under 90 ppm O_2 . Our studies are now focused to utilize this water in the beverage sector, because it is sterile at the origin, shows a very good and aromatic taste, the salts are constituted mainly of potassium, with sodium concentration lower then 3 ppm/L. This water can represent a new potable water class (vegetable water) with hypotonic and antioxidant properties for the presence of traces of hydroxytyrosol and the typical olive flavours.

The process here described represents a realistic solution for solving the environmental problem of OMW disposal, in fact about 60-70% of initial OMW volume is transformed in a purified water having a C.O.D. of about 100 ppm of O_2, with the propertied of drinkable water.

The technical feasibility of the OMW treatment process is proved not only at membrane pilot plants, but also with industrial plants. Effectively the finals results measured with industrial plant not only confirms the previously results obtained in the ENEA pilot plant, but in many case, best membrane performance (productivity), and process performance was obtained .

For example an higher VCR is obtained in relation to the high volume of OMW treated into industrial membrane plants, in each filtration campaign .

However only few technical date obtained in the industrial plants are reported in this paper because the business restriction are imposed from the PhenoFarm Company.

However, the experimental shown very good results in terms of membrane productivity and in terms of polyphenols extracted quality.

3.5) References OMW

Cheryan M. (1986). Ultrafiltration Handbook. Technomic Publishing Comp. Inc.

Pizzichini M., Russo C. (2005). Process for recovering the components of olive mill wastewater with membrane technologies. WO2005123603 (A1).

3.6) Olive leaves treatment

The purpose of olive leaves treatment is to demonstrate the technical feasibility of a new process devoted to recovery and reuse of phenolic components derived from leaves of the olive tree, aiming to obtain standardized extracts in a suitable formulation for food or related applications.
Five manufacturing steps are needed:

1. water extraction of plant material (olive leaves);
2. re-filtering for suspended solid removal;

and two tangential flow membrane filtration steps in sequence:

3. micro-filtration (MF);
4. reverse osmosis (RO);
5. spray drying on RO retentate upon addition of maltodextrins (5%).

The raw extract is subjected to MF in order to remove all suspended solids and for clarification.
The permeate is concentrated by RO filtration. RO retentate is the only fraction of interest it contains the entire phenolic pool of olive leaf.
RO permeate is constituted by ultrapure water and it is recycled along the process.
The RO retentate was converted in fine powder by spray drying, adding 5% maltodextrins as a thickener. The dry extract (olive leaf powder) may be employed as ingredient in cosmetics or food products.
The overall scheme of the fractionation process is shown in figure 3.6.1

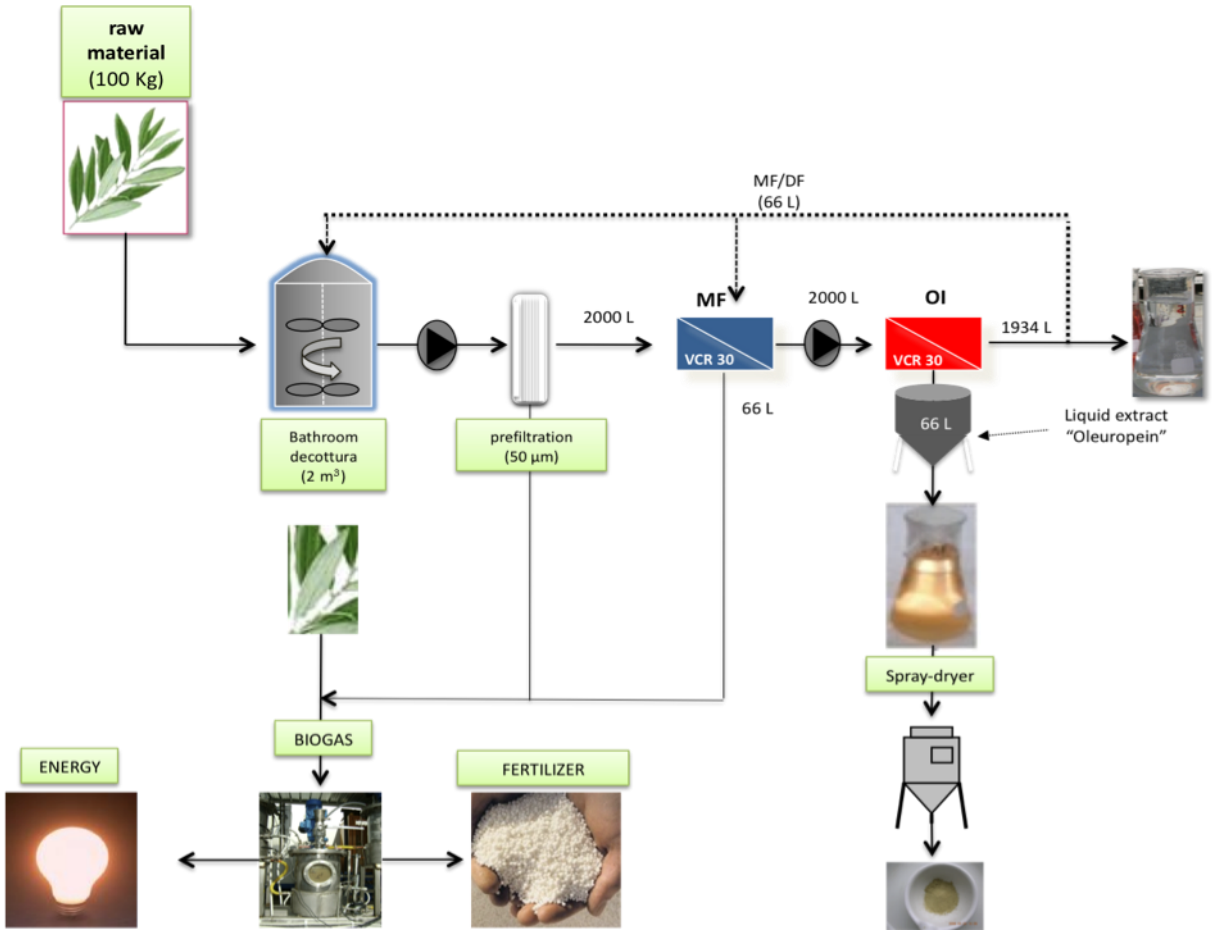

Figure 3.6.1 Process lay-out olive leaves

3.7) Materials and method olive leaves

Experiments were performed both in Reserch Center of ENEA Casaccia Rome, and in the PhenoFarm Company of Rome (www.phenofarm.it). Olive Leaves used were supplied from surrounding coltures the north of Roma (Olive mill Santa Barbara, Scandriglia, RI). Before any treatment Olive leaves are washed and soaked in demineralized water.

Water extraction and pre-filtering

Water extraction is performed in two cycle:

1. first cycle 100 kg fresh olive leaves were soaked in 1200 lt of RO demineralized water and mantained under hydraulic stirring for 90 minutes at 70-90°C. Raw extract was drained and recovered.
2. a new load of fresh demineralized water is added (1000 lt) the second cycle repeat the operative extraction conditions of first cycle .

Table 3.7.1 resume the operational condition adopted in water extraction.

Pre filtering cut-off 50 micron is performed on raw extract in order to remove corpuscular materials and gross particles.

Leaves/water ratio	1:20
Temperature (°C)	70-90
Extraction time (minutes)	180
Numbers of batch	2
Solvent	RO demineralized water

Table 3.7.1 Operational conditions of *Olea europaea* leaves water extraction

Membrane filtration

Membrane filtration experiments were performed with MF, and RO PhenoFarm pilot plants.

- MF is equipped with two centrifugal pump by Alfa Laval; two housing containing 12 ceramic tubular membrane (TAMI france); two flow-meters on the retentate and permeate; three manometers are installed, upstream and downstream the pressure vessel (ceramic membrane) and on permeate line; a cooling system controlled by an electrovalve regulated with a thermocouple with ranging from to ± 1 °C; a back pulse apparatus for membrane continuous cleaning with permeate stream at prefixed interval time.
- OI is equipped with two centrifugal pump by Alfa Laval; three vessel containing polymeric membrane 4"x40"; two flow meters on the retentate and permeate; two manometers are installed, upstream and downstream the pressure vessel; a cooling system controlled by an electrovalve regulated with a thermocouple with ranging from to of ± 1 °C;
- DF on the MF retentate, was obtained adding 66 L (DV) of distilled water to 66 L of MF retentate.

Membrane cleaning

- Ceramic membranes were regenerated with chemical washes, using alkaline solutions (NaOH), even at high concentrations (0.8 M) and high temperatures (60-80 °C);
- The UF membranes were regenerated by washing alkaline and acid solutions (0,1-0,2 M) at 40-45 ° C.

Spray drying

Spray drying trials were performed at ENEA research center.

The pilot unit "spray dryer" was made by the company ICF Marella (Pr) (figure 3.7.1). Dry extract are obtained operating at following conditions:

- 98° of inlet temperature;
- 78 ° of outlet temperature;
- feed stream 0,5 ml/min;
- evaporating pressure 2 bar;
- 5% of maltodextrins.

Figure 3.7.1 Spray dryer

Total solid measurements

The dry matter content was measured with Thermobalance mechanical infrared by Orma (Italy). 5 grams of each fraction were heated to 105 °C for 45 min. until reaching a 0,1% of residual humidity.

Phenols quantitation

Phenolic compounds concentration in liquid and dry extracts was estimated by Folin Ciocalteau colorimetric assay, as described Singleton & Rossi, 1965.

Polyphenolic profile in dry extract was determined by HPLC/DAD using an high pressure gradient pump Gynkotec with an UV/DAD (Diode Array Detector) operating at 280 nm. Chromatographic separation was performed using a Lichrosorb 5 RP18 column (250x4,6 mm) and the eluting mobile phase constituted by water acidified (pH 3.2) with TFA (solvent A) and acetonitrile-methanol (solvent B). Individual phenolic component identification was achieved by mean phenolic standards. All polyphenolic standards were purchased from Sigma Aldrich (Italy).

3.8) Results and discussion olive leaves

The Treatment of olive leaves for polyphenol recovery need a preliminary stage of water extraction.
In order to achieve a quantitative extraction of active principle, two different stages of extraction are needed, the first cycle determine a loosening of leave coating constituted by waxes, the second cycle allows a higher recovery of polyphenols.
Raw extract solution are yellow-green coloured, and lightly cloudy for the presence of suspended solids (colloids and insoluble fibers).
These solutions are pre-filtered (50 μm) in order to remove leaves fragments, soil particles and clots.
After the drainage of raw extract leaves contain still a 10 % of initial volum of water added.
The pre-filtered extract is subjected to the double stage of tangential filtration: microfiltration (MF) and MF permeate is concentrated by reverse osmosis (RO).
During MF (first stage of tangential filtration) the pre-filtered extract is purified from fine suspended solids as fibers and residual cells, polyphenols and active principles can cross the membrane and are presents both in permeate and concentrate .
MF permeate is a clear solution without any suspended solid containing all soluble components belonging to leaves olive.
In order to increase the polyphenol yield a further step of diafiltration (DF) is performed on the permeate.
DF consists in an addition an equal volume of solvent to the volume concentrate at the joining of final volume concentration ratio (VCR) and the further filtration until whole volume of solvent added crossed the membrane as permeate.
Each addition of solvent to the concentrate is a DF cycle and the volume of solvent added is a (dia-volume DV). Solvent consists of demineralized water. In this work after MF was performed a single DF cycle and one DV is added.
This procedures increases the yield of polyphenols in the permeate stream of about 20 %.
Ceramic membrane productivities, reaching a volume concentration ratio (VCR) of 30, are of about 35 L/m^2 h, as can be seen from the curve of permeability of Figure 3.8.1

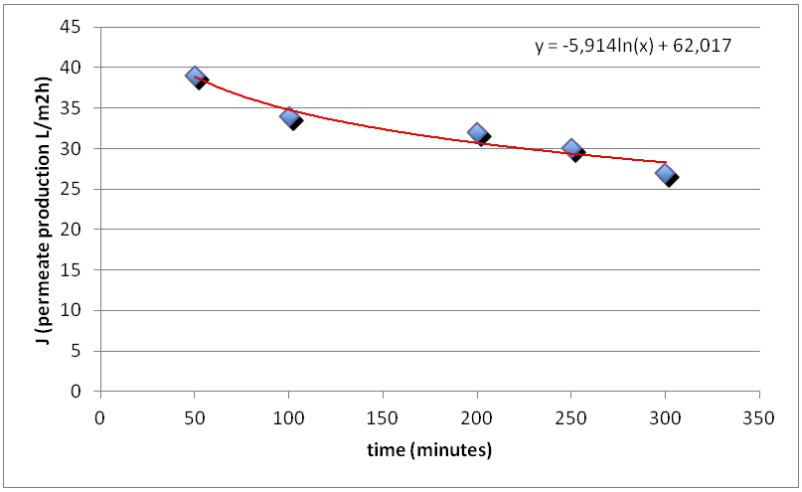

Figure 3.8.1 MF productivity

2000 L of MF/DF Permeate and related are concentrated in RO until VCR 30. RO productivity is reported in Figure 3.8.2

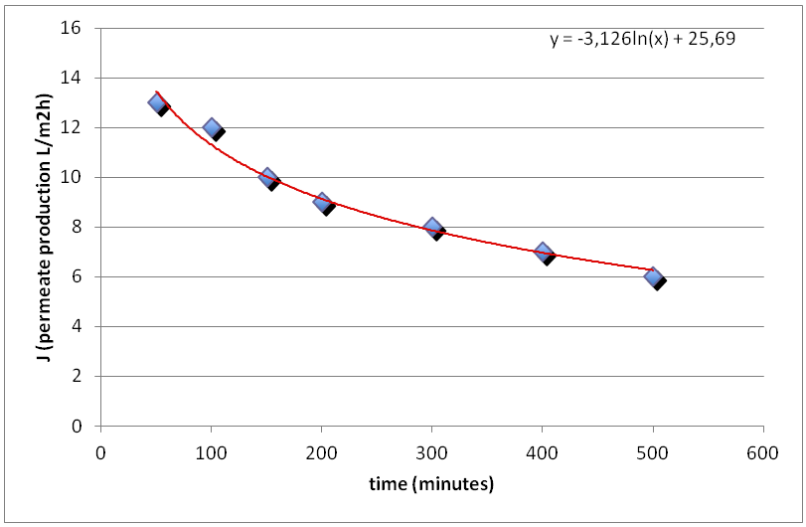

Figure 3.8.2 RO productivity

During RO a decrease of about 60 % in permeate flux was observed and it is this findings seems due to the elimination of a early stage of nanofiltration (NF) performed formerly on MF permeate. NF can concentrate all molecules with a molecular weight over 300 Da.

So it can be able to discriminate in the phenolic pool between large poliphenolic compounds (as oleuropein (540 Da)) and small ones (hydroxytyrosol (154 Da), tyrosol (138 Da)). The aim of this filtration consisted in obtaining a liquid concentrated fraction where whole polyphenolic pool is contained for this reason the NF step was removed.

It was observed (Scheepens et al., 2010) that polyphenols bioactivity are increased by synergistic interaction among several molecules.

So the goal of this experiment was the recovery of the entire pool of those substances. Polymeric membrane washing, with alkaline and acid solutions 0,1-0,2 M, is difficult and the membrane results irreversibly fouled. Only 60% of the initial permeability is recovered.

Reaching VCR 30, it is obtained a concentrate (66 L RO retentate) with 85 g/L of dried residue and 10 g/L of total polyphenols (gallic acid equivalent) as reported in table 3.8.1

RO permeate, about 90-95% of the initial feed volume, is a vegetable water suitable for beverage formulations.

Since our aim was the formulation of a stable polyphenolic extract with bioactive properties the liquid extract (RO retentate) was dryed and a fine powder was obtained figure 3.8.3. A 10 g of powder was obtained

Figure 3.8.3 Dry extract of Olea europaea leaves

Analytical characterization

Streams separated in membrane filtration steps are analytically characterized. by the dry residue and the total phenolic content (table 3.8.1)

As reported in table 3.8.1 it is observed that second extract has a 60-65% more polyphenols respect than first batch of water extraction in MF/DF retentate an important amount of polyphenols persists although the DF was conducted on MF retentate,

OI retentate show a 25-30% more polyphenols respect MF retentate and it exhibits about 3% of solid matter due to the high VCR reached. Powder as expected show the highest level of polyphenols (74 mg/g-GAE).

FRACTIONS	TOTAL POLYPHENOLS (GAE – g/L)	DRY MATTER (%)
EXTRACTION (1° BATCH)	0,6	0,4
EXTRACTION (2° BATCH)	1	0,4
FEED FILTRATION	0,8	0,4
RETENTATE MF/DF	7,7	2,7
RETENTATE OI	10	8,5
DRY EXTRACT	74	95

Table 3.8.1 Streams separated in membrane filtration steps are analytically characterized. by the dry residue and the total phenolic content.

HPLC analysis were also conducted on dried RO retentate in order to evaluate the polyphenolic profile of olive leaves see table 3.8.2. and figure 3.8.4, where is resumed the chromatographic profile HPLC analysis.

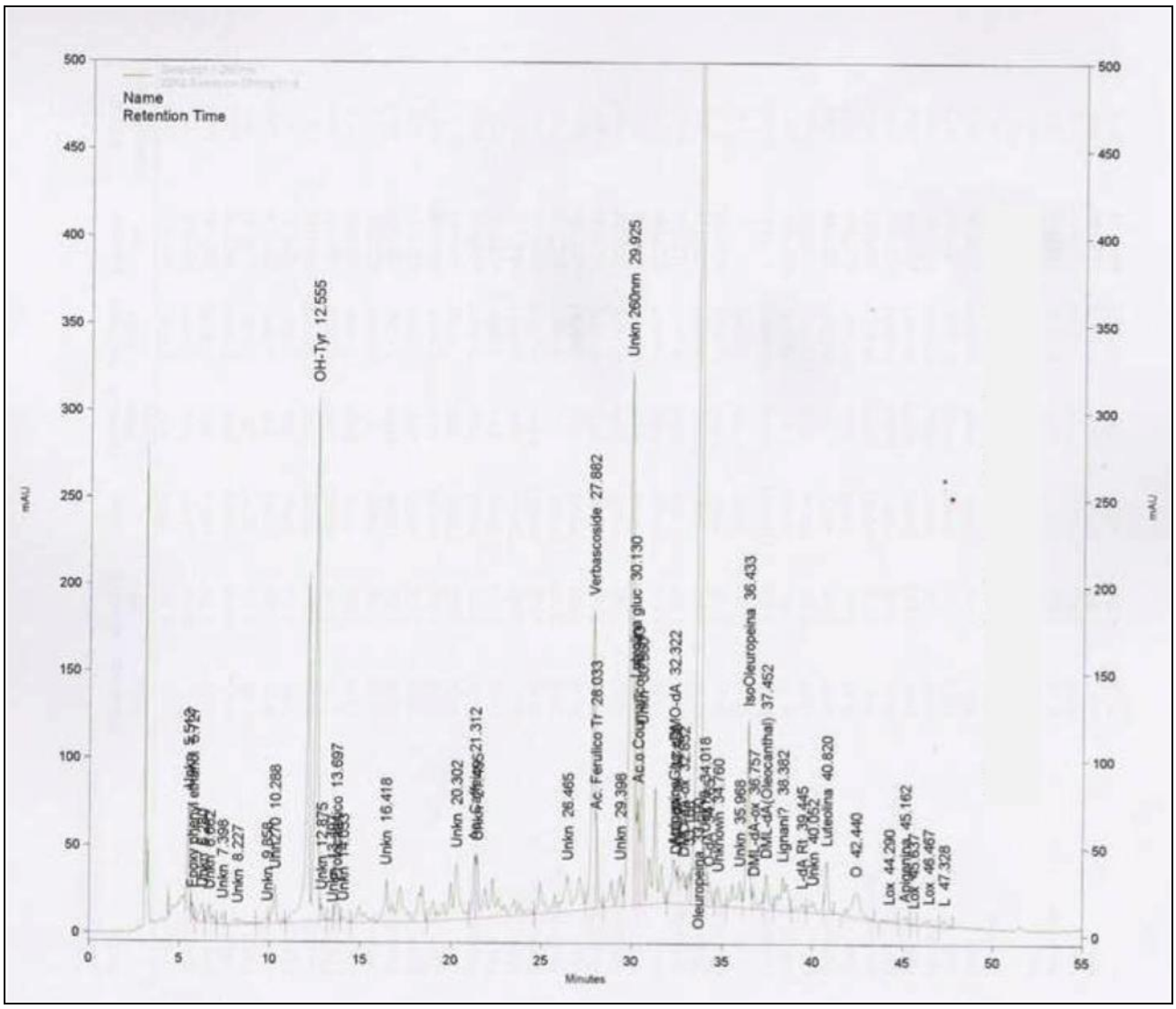

Figure 3.8.4 HPLC polyphenolic profile of olive leaves *Olea europaea*

The most abundant peak is associated , as expected , to Oleuropein and Hydroxytyrosol, and also some phenolic acids (ferulic acid and coumaric acid and protocathecuic acid) quantitation of main phenolic compounds is reported in table 3.8.2. Derivatives of oleuropein (elenolic acid, iso-oleuropein, dacolag) are associated with oleuropein for commercial purposes. It is noticed that a certain number of unknown species is revealed. The complete charachterization of olive phenolic pool was not achieved and it would need a further investigation and acquisition of specific standards .

DETERMINATION OF PHENOLS BY HPLC *	λ		
	nm	mg/Kg	
HYDROXYTYROSOL	280	mg/Kg	11458
OLEUROPEIN AND DERIVATIVES	280	mg/Kg	35833
LIGSTROSIDE AND DERIVATIVES	280	mg/Kg	514
VERBASCOSIDE	280	mg/Kg	2686
OLEOCANTHAL	280	mg/Kg	427
LIGNANS (acetoxypinoresinol and pinoresinol)	280	mg/Kg	779
TOTAL PHENOLIC ACIDS (Protocatechuic acid, caffeic acid, ferulic acid, O-Coumaric acid)	280	mg/Kg	2537
TOTAL FLAVONOIDS	280	mg/Kg	10550
TOTAL PHENOLIC	280	mg/Kg	**64.784**

Table 3.8.2 HPLC Analysis Olive leaf dry extract *Olea europaea. (*Data expressed using tyrosol as external standard)

HPLC profile of olive leaves polyphenols showed the major contribute of Hydroxytyrosol as single molecule (17%), oleuropein and derivatives are the 55% indicating the importance of this compounds and of processes concerning its hydrolysis other minor compounds are represented as verbascoside and oleocanhtal that contribute together for about 4,8% to phenolic pool.

Also flavonoids are present and they take into account for about 16%, phenolic acids (Protocatechuic acid, caffeic acid, ferulic acid, O-Coumaric acid) are represented for 4%. A minor percentage 1,2% is associated to lignans indicating that this secoiridoid and its species are poorly represented in this case.

3.9) Conclusions olive leaves

In this work we demonstrated the technical feasibility of the membrane process for the recovery of polyphenols from olive leaves.

The recovery of olive leaves polyphenols was achieved by obtaining of a water raw extract from fresh leaves. The purification of the raw extract is obtained with the MF that remove all the suspended solids constituted by small vegetable fractions coming from the partial leaves disruption with the heat .

Obviously these materials remain into the MF retentate, whereas the permeate stream contain all the polyphenols dissolved in a limpid solution.

In order to increase the polyphenols extraction yield one step of diafiltration is performed adding demineralised water into the MF retentate and proceed with the same filtration.

RO was conducted on MF/DF permeate in order to obtain a concentrated form it.

The filtration was easier respect OMW filtration in MF where, final productivities were increased of 13 % respect MF OMW, RO filtration showed a decrease of 75% in final productivity respect OMW filtration, due to the lacking of intermediate filtration as UF or NF.

The membrane fractionation of of olive leaves water extract allowed to obtain a single fraction of interest: the RO retentate.

RO permeate was constituted by ultrapure water and re- employed as solvent for next water extraction of fresh leaves.

In order to obtain a stable product the RO retentate was spray dried and a dry extract was obtained.

As final product with a 5% of maltodextrin added, the powder seem maintains physico-chemical features of original matrix as, for instance water solubility and organolectic properties.

The dry extract was analyzed for its content in poliphenolic substancs by HPLC analyses.

From the total polyphenolic pool 55% was represented by oleuropein and its derivatives. The Amount of Hydroxytyrosol depends on oleuropein hydrolysis and its related compounds (elenolic acid) during filtration procedures or while the storage of Olive leaves. Significant changes happens in phenolic composition of fruits and leaves during the ripening (Briante et al., 2002).

Minor compounds can be evidenced as Oleocanthal that show anti inflammatory activities in vitro.

50 g (more than three and a half tablespoons) of a typical extra virgin olive oil per day contains an amount of oleocanthal with similar in vitro anti-inflammatory effect as 1/10 of the adult ibuprofen dose. It is therefore suggested that long-term consumption of small quantities of oleocanthal from olive oil may be responsible in part for the low incidence of heart disease and Alzheimer's disease associated with a Mediterranean diet (Beauchamp et al., 2005; Abuznait et al., 2013).

However, 50 g is a great deal of olive oil for most consumers; moreover, the absorption, metabolism, and distribution of oleocanthal is not yet well-characterized, and it is not known whether these in vitro effects actually occur in the body. Against this background, the in vivo anti-inflammatory effects of dietary oleocanthal cannot be as relevant as hypothesized by Beauchamp et al (Fogliano-Sacchi et al., 2006).

The technological approach above described allows to obtain stable product for real commercial applications. The powder could be formulated different ways upon variation on concentration of polyphenolics in RO retentate and on maltodextrin percentage, even if the phenolic characterization is not complete and it needs a deeper investigation in order to clarify the nature of unknown species,

properties associated to oleuropeins and hydroxytyrosol make olive leaf dry extract a valid functional ingredient also for nutraceutical, or cosmetic purposes.

3.10) References olive leaves

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