JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

REVIEWS

Bioactivity and Analysis of Biophenols Recovered from Olive Mill Waste

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Biophenols have attracted increasing attention during the past few years due to their biological activities and natural abundance and are potential targets for the food and pharmaceutical industries. Olive mill waste (OMW) is rich in biophenols and typically contains 98% of the total phenols in the olive fruit, making value addition to OMW an attractive enterprise. The phenolic profile of OMW is complex, yet this complexity has not been fully exploited in the valorization of the waste. Most work on the bioactivity of OMW has focused on antioxidant and antimicrobial activities. The analytical techniques used to identify and quantify active biophenols are also reviewed.

Keywords: Olive mill waste; biophenols; bioactivity; analysis; value addition; antioxidant; antimicrobial

INTRODUCTION

The use of natural products as a source of new leads in the pharmaceutical industry was applied extensively in the 1800s and 1900s before experiencing a sharp decline from the early 1960s until the late 1980s. The decline in natural products research can be attributed to several factors (*1*). The isolation, fractionation, and separation of bioactive compounds from plants was a slow and expensive process (*2*), and identification of previously characterized compounds at the end was frustrating. The complexity of the extract probably contributed to the failure to detect some biologically active compounds that were present in trace amounts (*3*). In addition, there were scaling-up problems (*2*), and the reproducibility of bioassay results on sample recollection was often poor, particularly for plant and marine sources (*4*) due to large variability in the biochemical profiles of plants among different cultivars, harvesting times, and cultivation conditions (*5*). The introduction of combinatorial and computational chemistry as alternatives to drug discovery from nature (*6*) also contributed to the decline.

The renaissance of natural products research (*5*, *7*) began with the success of Taxol (paclitaxel) as an effective antineoplastic drug of natural origin (*8*). The past decade has witnessed a renewed recognition of the importance of natural products research, following the introduction of vinca alkaloids (antineoplastic) and artemisinin (antimalarial and antineoplastic) (*2*, *8*). The renewed interest in natural products has been supported by advances in chromatographic and spectroscopic techniques that have greatly facilitated drug discovery from plants. The application of hyphenated techniques such as high-performance liquid chromatography−mass spectrometry (HPLC-MS) and high-performance liquid chromatography−nuclear magnetic spectrometry (LC-NMR) has rejuvenated the opportunities for chemists to recommence their exploration of the realm of natural sources (*9*). Nature is now recognized anew as a unique and incomparable source of both novel bioactive molecules and templates for optimization by combinatorial and computational approaches.

Phenols are a major group of natural products that occur in all higher plants. Of the various plants, olive (*Olea europaea* L.) (*10*) has been recognized as a source of biophenols (*11*, *12*), and most drug compendia (European, Japanese, British, and French pharmacopoeias and The United States National Formulary) contain olive oil monographs, whereas the French pharmacopoeia also includes an olive leaves monograph (*13*). Epidemiological studies have correlated the low incidence of coronary heart disease, atherosclerosis, and some types of cancer (colorectal and breast cancer) with olive oil consumption in the Mediterranean diet (*11*, *14*). A number of reports have linked the health benefits of olive oil with its phenolic content (*15*−*23*). Olive biophenols are now recognized as potential targets for the food and pharmaceutical industries (*24*, *25*).

The fruit, leaves, and oil from olive have all attracted considerable attention as sources of biophenols (*26*−*31*). The olive leaf, for example, is a well-known source of biophenols that is marketed under multiple trade names as a nutraceutical.

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10.1021/jf048569x CCC: $30.25
Published on Web 01/21/2005

The biophenolic fraction of olive oil comprises only 2% of the total phenolic content of the olive fruits, with the remaining 98% being lost in olive mill waste (OMW) (*32*). Thus, OMW is also potentially a rich source of a diverse range of biophenols with a wide array of biological activities. The reported biological activities of major biophenols in OMW are shown in **Table 1**. OMW biophenols may be either naturally occurring in the fruits (see **Figure 1** for representative structures) or formed during processing, where they partition between oil and waste phases. Although a general review on the potential of agroindustrial waste as a source of biophenols has recently appeared (*25*), the present review focuses on olive and considers the generation of OMW and the bioactivity and analysis of OMW for biophenols and addresses valorization of OMW with emphasis on the recovery of biophenols. Many terms are used to refer to these compounds, but biophenols is the preferred term (*26*, *115*, *116*).

OLIVE MILL WASTE

Two processes are used for the extraction of olive oil: the two-phase and the three-phase systems. OMW produced by the latter is composed of two main byproducts, an aqueous liquid known as olive mill wastewater (OMWW) and a solid waste (pomace). The newer two-phase system produces only a semisolid waste known as alperujo (*35*, *73*, *117*, *118*). Prior to the 1970s farmers were the only group with any interest in OMW as they faced a significant disposal problem. The expansion of the industry, with increased production of noxious waste, presented a serious environmental concern (*34*, *47*, *94*, *95*, *119*−*123*). Sustainable development of the industry meant that waste management protocols required careful consideration.

Papers examining OMW began to appear in the 1960s motivated by scientific curiosity and the possible agricultural benefits of the waste. The literature on the composition and characteristics of OMW is vast but mostly in Spanish and Italian (*124*). OMW management options have been reviewed elsewhere (*125*) from an environmental perspective. Initial research targeted biophenols as undesirable substances and endeavored to dephenolize the OMW (*121*, *126*−*134*). Although various uses have been proposed, including use as a potentially renewable energy source (*119*, *135*−*137*) or fertilizer (*138*, *139*), it is value addition that offers greatest potential. Thus, OMW has been proposed as a low-cost substrate for production of xanthan (*140*), ethanol (*141*), and phenol oxidases (*142*, *143*). The production of biologically active compounds (*33*) such as phenolic antioxidants from OMW constitutes a viable alternative for valorizing this problematic waste.

Following recognition of the antioxidant activity of OMW (*67*, *144*) and the association of oxidative stress with many diseases, it was logical to consider OMW as a potential source of biophenolic antioxidants. Thus, research on the valorization of OMW increased significantly in the 1990s (*33*, *35*, *67*). The European Union initiated a project, "Natural antioxidants from olive oil processing wastewaters" (*50*), investigating the extraction of biophenols from OMW (*33*), and much of this work is covered by patents. The unstable nature of the waste due to enzymatic and microbial activities must be considered when one is dealing with agroindustrial waste as a potential source of bioactive compounds. This consideration may ultimately determine the viability of any strategy with a particular waste.

Phenolic Content. Servili et al. (*38*) compared the phenolic profiles of olive fruit (cv. Frantoio) and the corresponding oil, pomace, and OMWW obtained by laboratory scale press (**Figure 2**). The HPLC profiles of the fruit showed similarity to that of the pomace where secoiridoid glycosides were present in high concentration. In contrast, secoiridoid derivatives, namely, hydroxytyrosol and oleuropeindial (see **Figure 1**), were dominant in OMWW. The poor correlation between the biophenolic content of the fruits and the OMWW has been ascribed to the effects of processing (*50*). Nonetheless, OMW is rich in biophenols (*67*), and the complexity of the biophenolic fraction was demonstrated by Bianco et al. (*80*), who identified 20 biophenols in OMWW using HPLC-MS-MS. They were able to quantify 16 of these compounds.

The occurrence of specific biophenols in OMW depends on the fruit (e.g. cultivar, maturity), climatic conditions, and storage time, in addition to the processing technique (*66*, *95*, *145*). For example, oleuropein was not identified in OMWW sampled from late-harvest olives (*66*) but was a major component in other studies (*146*). The biophenolic content is closely dependent on the extraction process as this determines the partitioning behavior of the biophenols and hence their distribution between the oil and waste fractions. Most olive biophenols have low partition coefficients (oil/water) ranging from 6×10^{-4} to 1.5 (*32*), and so they favor partitioning into the wastewater. Both the processing temperature and the quantity of water affect the partitioning significantly. The higher the temperature, the higher is the partitioning of the biophenols into the oil, whereas the more water is added, the more the biophenols are lost in wastewater (*32*).

Varietal and processing effects were examined using OMWW collected from commercial and experimental mills with different varieties from four European countries (*50*). The OMWW was lyophilized and dissolved in acidified water (pH 2.2, formic acid) prior to processing by solid-phase extraction (SPE) using *n*-hexane, ethyl acetate, and acidic methanol (pH 2.2, formic acid) in exhaustive sequential elution. The ethyl acetate fraction contained 90−100% of the total biophenols, elenolic acid and its derivatives being the main compounds (28.8−80% of the total biophenols). Lesage-Meessen et al. (*73*) also studied the biophenolic composition of OMW as a function of the extraction system (three-phase and two-phase mills). Ethyl acetate provided efficient recovery of simple biophenols from the dry acidified (pH 3; HCl) residues. The phenolic profiles identified by HPLC at 280 nm were similar for residues from two-phase and three-phase mills, but the contents of individual biophenols (hydroxytyrosol, tyrosol, caffeic acid, ferulic acid, and *p*-coumaric acid) (see **Figure 1**), with the exception of vanillic acid, were higher for the two-phase system. The extract from the two-phase system also exhibited higher antiradical activity consistent with its biophenolic content. Hydroxytyrosol and tyrosol were the major compounds detected. Biophenols that have been frequently reported in OMWW such as protocatechuic acid, veratric acid, syringic acid, cinnamic acid, 4-hydroxyacetic acid, and oleuropein (see **Figure 1**) were not detected in the ethyl acetate extract.

The effect of storage on the biophenol content is seen in the loss of biophenols from composted OMW (147). Total phenols as measured by the Folin−Ciocalteu reagent decreased by 93% after 12 months of composting. This was confirmed by thin-layer chromatography (TLC). The majority of the biophenols identified by GC-MS in the fresh OMW disappeared after 12 months.

BIOACTIVITY

Bioactivity-guided fractionation is the most successful approach both for the investigation of new plants and as a novel tool to re-evaluate known plants (*2*, *6*, *7*). The advent of more

Table 1. Major Biophenols in OMW and Their Reported Activities

biophenol	bioactivity	remarks (ref)
hydroxytyrosol (*33–38*)	antioxidant	isolated from OMWW, antioxidant in rat plasma (*39*) antioxidant in rat liver (*40*) protects human erythrocytes against oxidative damage (*41*)
	cardioprotective and antiatherogenic	multiple effects (*18*, *27*) scavenges and reduces superoxide anion production in human promonocyte cells (*42*)
	chemopreventive	inhibition of peroxynitrite-dependent DNA damage (*43*) induces cytochrome C-dependent apoptosis (*44*) inhibition of the proliferation of tumor cells (*45*)
	antimicrobial	human pathogens (*46*) agricultural pathogenic bacteria (*47*)
	anti-inflammatory	prostaglandin sparing and antioxidant activity also were detected (*48*) inhibition of leukocytes leukotriene B_4 (*17*)
	skin bleaching	topical and bath preparation (cited in ref *49*)
oleuropein (*37*, *38*, *50*)	antioxidant	olive cake extract (*51*) in vivo and in vitro activity (*52*) radical scavenging activity within biomembrane (*53*)
	antiatherogenic and cardioprotective	inhibition of LDL oxidation and platelets aggregation (*16*, *54*) fatty acid composition of rat heart (*55*) enhances nitric oxide production (*56*)
	hypoglycemic	in rats (normal and diabetic) (*15*)
	antihypertensive	vasodilator (*57*)
	antimicrobial and antiviral	antibacterial (*46*, *58*) antimycoplasmal (*59*) antifungal effects (*60*) anti-HIV activity of olive leaf extract (*61*)
	anti-inflammatory	inhibition of 5-lipoxygenase (*48*)
	cytostatic	against McCoy cells (*62*)
	molluscicidal	South American snail *Biomphalaria glabratus* (*63*)
	endocrinal activity	thyroid stimulation (*64*); modulation of hypolipidemic-hypoglycemic activity
	enzyme modulation	activates pepsin and inhibits trypsin, lipase, glycerol dehydrogenase, glycerol-3-phosphate dehydrogenase, and glycerokinase (*65*)
tyrosol (*34*, *51*, *66*, *67*)	antioxidant	DPPH scavenging (*68*) protects against oxidized LDL (*69*) reduces DNA oxidation at high concentrations (*70*)
	anti-inflammatory	inhibition of 5-lipoxygenase (less active than HT) (*48*)
	antiatherogenic	in humans (*71*)
	cardioactive	antiarrhythmic and cardioprotective (*72*)
caffeic acid (*33*, *67*, *73*)	antioxidant	*tert*-butyl hydroperoxide induced oxidative stress (*74*) reactive species of oxygen and nitrogen (*75*)
	chemoprotective	inhibits DNA oxidation (less active than hydroxytyrosol but more efficient than tyrosol) in prostate cells (*70*)
	antiatherogenic	inhibition of LDL oxidation (*76*, *77*) pro-oxidant activity on LDL (*78*)
	antimicrobial	antibacterial and antifungal (*60*)
	anti-inflammatory	inhibition of 5-lipoxygenase (more active than tyrosol and less active than hydroxytyrosol and oleuropein) (*48*)
	antidepressive-like activity	unknown mechanism (*79*)
vanillic acid (*38*, *73*, *80*)	antioxidant	alkylperoxyl radical-scavenging (*81*)
	antimicrobial activity	antibacterial and antifungal (*60*)
verbascoside (*38*, *50*, *67*, *82*)	antioxidant	*Ballota nigra* extract (*83*)
	chemoprevention	reverse malignant phenotypic characteristics (*84*)
	cardioactive	chronotropic, inotropic, and coronary vasodilator mediated through cAMP (*85*)
	antihypertensive	angiotensin converting enzyme (ACE) inhibitor (*86*)
	anti-inflammatory	multiple mechanisms (*87*)
	antiatherogenic	plasma lipid peroxidation and erythrocyte membrane fluidity (*88*)
	sedative	*Ballota nigra* extract (*83*)
elenolic acid (*33*, *50*)	antimicrobial	antibacterial (*58*)
	antiviral	calcium elenolate (*89*)
p-coumaric acid (*37*, *73*, *80*)	antioxidant	protection of rat heart from oxidative stress of doxorubicin (*90*) hypochlorite scavenging activity (*91*)
	antimicrobial	antibacterial and antifungal (*60*, *92*)
	chemoprevention	antileukemic activity (*90*, *93*)
catechol (*37*, *94*, *95*)	phytotoxic	toxic to tomato plants (*94*)
	antimicrobial	against plant pathogens (*47*)
	carcinogenic	activity in rat stomach differs among strains (*96*)
	antioxidant and anticancer	contrasting effects (*97*)
rutin (*82*)	antioxidant	hepatoprotective (*98*) hemoglobin oxidation (*99*)
	antiatherogenic	less active than quercetin (*100*)
	anti-inflammatory	only in chronic inflammation (*101*, *102*)
	chemopreventive	blocking agent for heterocyclic amine-induced rat liver carcinogenicity (*103*)

sensitive, specific, and robust bioscreening methods makes high-throughput screening a reality (*2*, *148*). The success of bioactive compound discovery programs is based on the structural diversity of the tested compounds and the reliability of the screening techniques applied (*8*). The bioactivity of raw waste has been examined (*122*), but Capasso et al. (*47*) suggested that

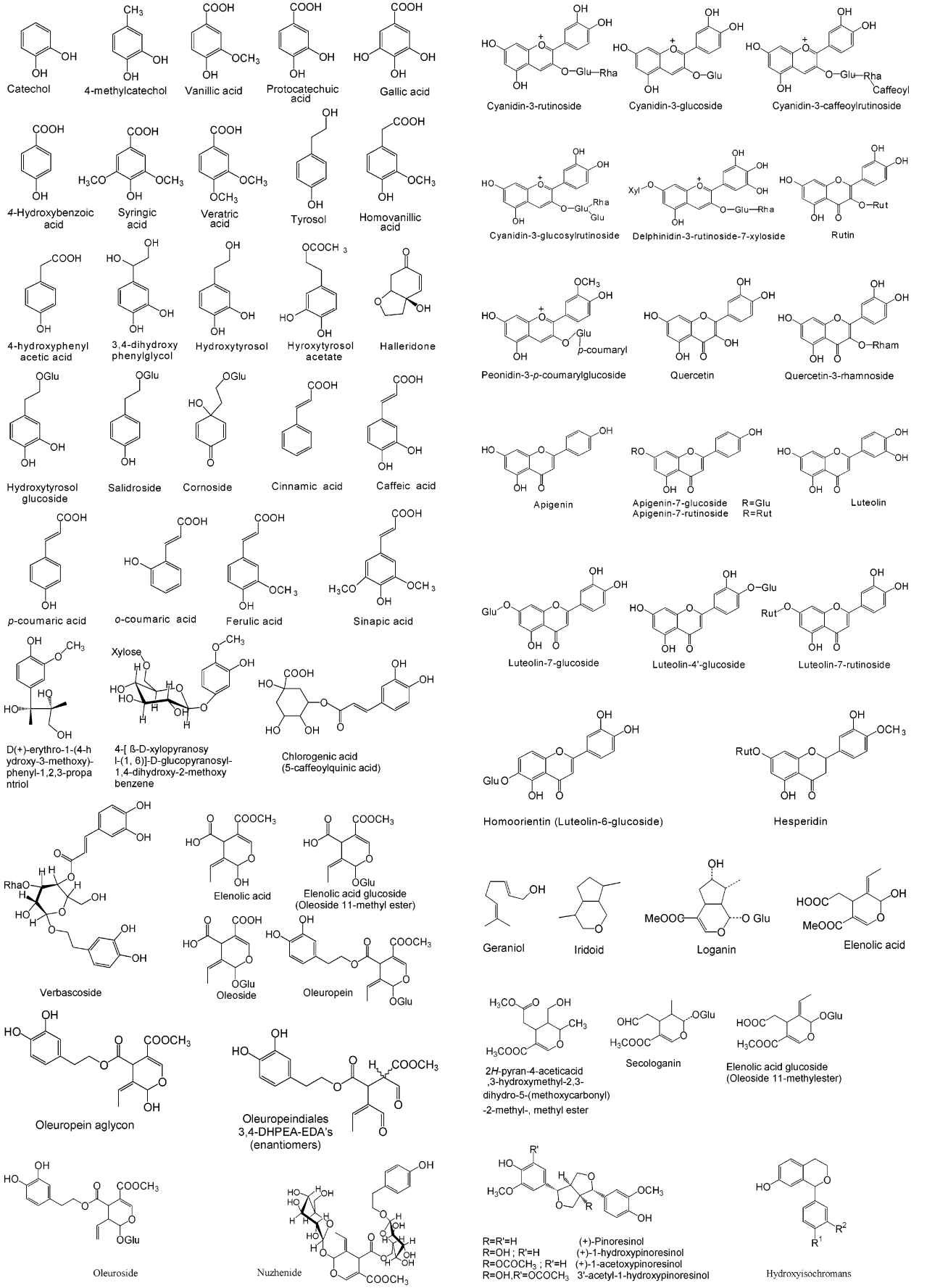

Figure 1. Biophenols and related compounds identified in olive or olive mill waste (*29*, *94*, *104–113*). Iridoids are monoterpenes (C_{10}) arising from the folding of geraniol (*114*), characterized by a bicyclic fused ring system (six-membered heterocyclic ring fused to cyclopentane ring). Secoiridoids are obtained through opening of the five-membered ring of iridoids. The parent of this group of metabolites is secologanin. Oleosides are oleaceae-specific secoiridoids characterized by an exocyclic 8,9-olifinic bond.

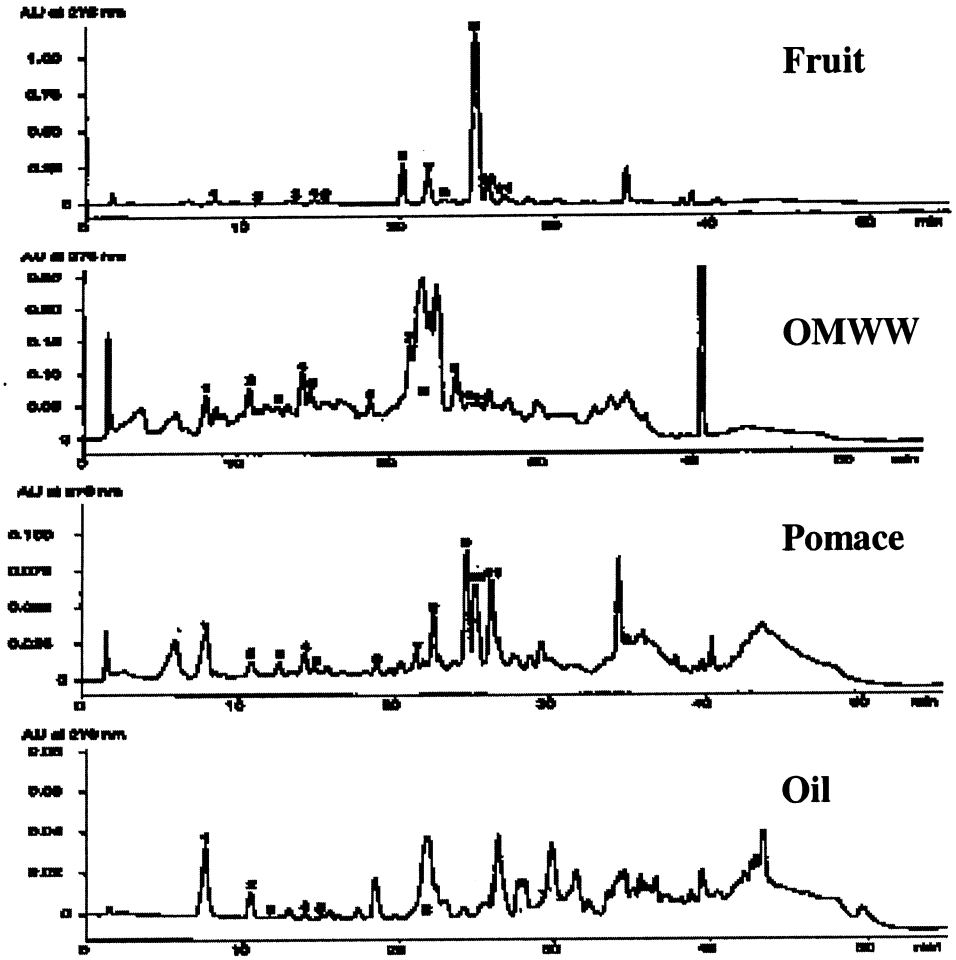

Figure 2. HPLC chromatograms comparing the phenolic profiles of olive drupes (cv. Frantoio) and the corresponding olive mill wastewater, pomace, and olive oil obtained by laboratory scale press. Reprinted with permission from ref *38*. Copyright 1999 American Oil Chemists' Society.

the use of raw OMWW in bioactivity tests should be avoided because of its phytotoxicity (and toxicity on human cell line). Thus, prior fractionation and separation of active constituents is regarded as desirable, and research has frequently involved various extracts and/or isolated biophenols from the OMW (*33*, *42*, *46*, *149*). This section examines the bioactivity of raw OMW and of biophenols either isolated from or demonstrated to be present in OMW.

Antioxidant Activity. The most extensively studied activity of olive biophenols is their antioxidant action (*33*, *73*, *150*). The inhibition of different types of oxidative damage has been investigated (*31*, *40*−*43*) using various measures of antioxidant activity, which have been reviewed elsewhere (*151*, *152*).

One of the earliest papers dealing with OMW as a possible source for antioxidants was published in 1988 (*144*). Olive waste was extracted sequentially with hexane, acetone, and ethanol using a Soxhlet apparatus. The ethanolic fraction contained the highest phenolic content and provided the highest protection against oxidative deterioration of refined olive oil and soybean oil. In contrast, Ranalli et al. (*150*) studied the antioxidant potency of phenolic standards associated with OMWW and grouped the phenolic standards into three categories according to their antioxidant potency as strong, medium, or weak. Antagonistic and synergistic effects of nonphenolic compounds (L-proline, chlorophylls, and tocopherols) were investigated.

The antiradical activities [1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging] of OMW have been examined (*73*). In one study, OMW (olive cake) extracts were investigated for antioxidant and radical scavenging activities (*51*). The residue left after evaporation of the ethanolic extract was dissolved in water and sequentially extracted with hexane, chloroform, and butanol. The butanol extract was fractionated on a silica gel column, and nine fractions were collected. Partial identification of biophenols in the fractions revealed coumaric acid in fractions 2 and 3, oleuropein in fraction 4, protocatechuic acid in fraction 7, cinnamic acid in fraction 8, and ferulic and caffeic acids in fraction 9. The fractions were examined using various measures of antioxidant activity [iron(III) reduction; inhibition of oxidation in refined soybean oil; DPPH radical scavenging] and, consistent with previous reports (*153*), the antioxidant activity varied according to the test method. For example, the first four fractions showed higher antioxidant activity than butylated hydroxytoluene (BHT) in refined soybean oil, whereas fractions 9 and 8 were the most effective free radical scavengers. The authors concluded that OMW extract may be useful in cosmetics as antiaging and antiwrinkle products due to its antioxidant activities (*51*).

Antioxidant and anti-inflammatory activity were measured in a comprehensive study of recovery and bioactivity (*33*) of biophenols in OMWW. The waste was obtained from a benchtop mill using olives from three different European countries. Biophenols were extracted using an XAD 1180 resin column (extract 1), liquid−liquid extraction (extract 2), and gel filtration by Sephadex LH-20 column (extract 3). Multiple antioxidant assays (LDL oxidation, DPPH radical scavenging activity, superoxide anion scavenging, and protection of catalase against

hypochlorous acid) and an anti-inflammatory activity assay (leukotriene B_4 production by human neutrophils) were performed. Extract 1 exhibited low antioxidant activity and no anti-inflammatory activity and was characterized by high molecular weight polymers. The secoiridoid elenolic acid was the major compound in extract 2 with some cinnamic acid derivatives. This extract showed good antioxidant and excellent anti-inflammatory activities. Extract 3 contained hydroxytyrosol, tyrosol, and a hydroxytyrosol derivative and exhibited the most potent antioxidant activity and a reasonable anti-inflammatory activity. The authors suggested that the extracts acted mainly as metal chelators and also had a potent free radical scavenging activity. They also concluded that hydroxytyrosol was the most active component in OMWW.

Lipid peroxidation in the copper sulfate−low-density lipoprotein (LDL) system was inhibited by hydroxytyrosol obtained by ethyl acetate extraction of lyophilized OMWW (*67*). Other compounds identified in the extract were tyrosol, verbascoside, oleuropein, caffeic acid, vanillic acid, and 4-hydroxybenzoic acid. Superoxide anion scavenging activity of four phenolic extracts from OMWW was measured in cultured human promonocyte cells (THP-1) (*42*). The extracts also lowered superoxide anion production by the cells, supporting the antiatherogenic activity of OMW, and the authors concluded that hydroxytyrosol was likely to be the active component. As the four extracts showed similar activity, and two of these were derived from a starting extract, the results suggest that the matrix had no effect (*42*).

Antimicrobial Activity. An early paper identified the antibacterial activity of OMWW constituents (*154*). Papers on antibacterial activity generally focused on the ecological impacts and degradability of OMWW in an endeavor to find methods to detoxify the biophenols (*123*). The antibacterial activity of OMWW was confirmed by both Ramos-Cormenzana et al. (*123*) and Gonzalez et al. (*155*) and was exhaustively reviewed by Moreno et al. (*124*), who noted antibacterial and phytotoxic activity of low molecular mass biophenols and the phytotoxicity of phenolic acids. A hexane extract of OMW was not active, so bioactivity was associated with the hydrophilic constituents and was correlated with the phenolic content of OMW (*156*). OMWW constituents were more effective on bacteria than yeast (*124*), whereas the antibacterial activity was higher on Gram-positive than on Gram-negative bacteria. Ethyl acetate and *n*-propanol extracts were the most active against *Bacillus megaterium* (*156*). Propanol was the solvent of choice to extract the antibacterial biophenols from OMWW (*157*), although the propanolic extract was less active than OMWW itself (*124*).

Capasso et al. (*47*) studied the antibacterial activity of OMWW, the major biophenols in the waste, and some synthetic analogues. 4-Methylcatechol was the most effective bactericidal compound, whereas hydroxytyrosol was active against *Pseudomonas savastanoi* only. Tyrosol did not show activity on any of the microorganisms. Compounds found in OMW that exhibited antibacterial activity were oleuropein and hydroxytyrosol (*46*), 4-hydroxybenzoic acid, vanillic acid, and *p*-coumaric acid (*72*). Hydroxytyrosol was generally more active than oleuropein (*46*). The latter and ligstroside showed no activity against *Bacillus subtilis*, *Saccharomyces cerevisiae*, or *Escherichia coli*, whereas in the presence of β-glucosidase both compounds were able to inhibit the growth of *B. subtilis* (*158*). However, the alkaline hydrolysis products of oleuropein were totally inactive. Federici and Bongi (*159*) developed an HPLC method for the isolation of oleuropein hydrolysis products and showed that two fractions (peaks) inhibited the growth of *Lactobacillus plantarum*. Contradictory data have been attributed to the use of rich assay media (*160*), and bactericidal action was demonstrated (*160*) with oleuropein and its heat-treated product on nine strains of *L. plantarum* in a medium devoid of any organic nitrogen.

Other Bioactivities. A diverse range of other bioactivities have been demonstrated, either for OMW itself or for phenols that have been reported to be present in the waste. OMWW (*161*) and its phenolic extracts (*161*, *162*) showed deterrent action at high concentrations to ovipostion by *Dacus oleae* (*Bactrocera oleae*) (Gmelin) females. Catechol was found to be the most potent repulsive phenol, whereas tyrosol and hydroxytyrosol were inactive (*161*).

OMW exhibited phytotoxicity for seed germination on radish and wheat (*163*) and on tomato and vegetable marrow (*94*). In both cases, toxicity was related to a synergistic action of the biophenols and unidentified compounds present in the waste. Catechol, 4-methylcatechol, tyrosol, and hydroxytyrosol were isolated as the four main phenolic compounds of OMWW, and phytotoxicity studies showed that catechol and 4-methylcatechol exerted the most harmful effects, whereas hydroxytyrosol and tyrosol showed selective toxicity (*94*).

Bioactivities of phenols reported in olive waste are given in **Table 1** and include enzyme inhibition (*11*, *65*), antiviral activity (*61*, *72*, *164*), and molluscicidal activity (*158*). Antiprotozoal activity has not been assessed, despite the facts that olive leaf extracts were used for treating malaria in the 1800s in the British colonies (*72*) and that olive leaves and waste will share some common biophenols.

Of the biophenols in OMW, hydroxytyrosol is the most exhaustively studied due to its presence in all OMW and its superior and diverse biological activities (**Table 1**). It has many promising applications in foods, cosmetics, and medicine, and there are numerous studies of its recovery and applications (*35*, *165*, *166*). The following section summarizes the data that are available.

HYDROXYTYROSOL

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 (*36*). 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 (approximate cost $US 1000−2000 per gram) and has also been introduced under different trade names as an antioxidant nutraceutical.

Various procedures for recovery of hydroxytyrosol from OMWW have been described. For example, continuous counter current extraction (*66*) yielded 1.0 g of purified hydroxytyrosol from 1 L of OMWW, whereas middle-pressure LC and preparative scale TLC gave a yield of 91 mg/L (purity = 80%) (*49*). The poor yield of the latter approach was attributed to retention of hydroxytyrosol on the stationary phase and its tendency to undergo chemical modifications. Hydroxytyrosol was also purified from alperujo (*35*) by hydrothermal treatment of the alperujo using a flash hydrolysis laboratory pilot unit and a simple chromatographic purification system that is currently patented. The reduction in the antioxidant amount present in the fruit compared to that found in the alperujo was attributed mainly to condensation reactions and other intermolecular reactions between biophenols and polysaccharides and glycoprotein rather than the most widely invoked explanation of chemical and enzymatic oxidative degradation during crush-

ing, malaxation, and processing. Hydroxytyrosol showed unexpected stability against the harsh conditions used in its extraction. The optimum conditions for recovery were steam at 200−220 °C and sulfuric acid catalyst (1.0−1.5%). Higher acid concentrations caused degradation of hydroxytyrosol. The isolated pure hydroxytyrosol was stable for 5 days under ambient temperature, exposed to light, in a direct continuous air current. The alternative synthetic route from 3,4-dihydroxyphenylacetic acid gave an 80% yield of hydroxytyrosol in a one-step reduction reaction using lithium aluminum hydride in dry tetrahydrofuran (*49*). The authors concluded that the synthetic route was superior to isolation from OMWW due to better yield and lower cost. However, the synthetic method involved the use of large volumes of organic solvents and relatively expensive starting materials.

The oxidative chemistry of hydroxytyrosol has been investigated theoretically (*167*) and practically (*168*) in an attempt to understand its antioxidant structure−activity relationship (SAR) and mechanism. The pharmacokinetics (absorption, distribution, metabolism, and excretion) (*169*−*172*) of hydroxytyrosol have also been studied, reflecting the emphasis that has been placed on this biophenol.

ANALYSIS OF OMW BIOPHENOLS

Analytical methods for OMW biophenols have been devised for a variety of situations including the detection of biophenols in OMW and the isolation and purification of these compounds. In the following discussion, distinction is not made among methods on the basis of analytical intent but rather on the analytical approach. The first attempt to analyze and identify OMWW (alpechin) biophenols was in 1967 by Ragazzi and Veronese (cited in ref *37*). Biophenols were partitioned between ethyl ether and aqueous bicarbonate. Caffeic acid and protocatechuic acid were identified in the bicarbonate layer, whereas in the ether layer catechol, tyrosol, and hydroxytyrosol were the main components. An often cited paper that presents a valuable phytochemical study of the alpechin biophenols is that of Vázquez Roncero et al. (*37*). Despite the limitations of the available instrumentation at that time, they were able to separate and identify 18 phenolic compounds in the ethyl acetate and *n*-propanol extracts of the alpechin using two-dimensional TLC and paper chromatography.

Sample Handling. Recovery of biophenols from OMW is a difficult analytical task for several reasons. Biophenols are reactive chemical species, vulnerable to oxidation, conjugation, hydrolysis, polymerization, and complexation. This is compounded by direct contact with enzymes and their substrates as the cells are no longer intact. OMW is a complex matrix that offers a reaction medium (water), catalysts (enzymes, organic acids, and metals) and substrates (proteins, polysaccharides, metals, small molecular weight reactive compounds, and phenols themselves) all contained under an umbrella of oxygen (air). Olive biophenols comprise a vast range of phenolic compounds with different structures and different physicochemical properties (solubility and partitioning) that makes any attempt to optimize the extraction a difficult task.

In many instances, the nature of the sample and details of sample handling prior to extraction are omitted (*34*). In those cases where details are provided, there is great diversity. For instance, Visioli et al. (*33*) used fresh OMWW derived from benchtop milling of frozen olives, whereas Capasso et al. (*49*) used fresh commercial OMWW. The immediate analysis of the fresh sample (*34*, *80*, *94*) is always the ideal situation, due to possible changes in the chemical composition during sample manipulation. Unfortunately, this is rarely achievable, and sample transfer to the laboratory, preservation, and storage may be unavoidable (*173*). The fidelity of the phenolic profile between the sample and the final extract is the measure for the success of the analysis, so sample manipulations should be as mild as possible to avoid artifacts (*115*).

If storage is unavoidable, the recommended practice is to collect the sample in liquid nitrogen with subsequent freeze-drying (*38*, *173*). Although lyophilization without prefreezing (*50*) has also been used, it is generally assumed that prefreezing stops enzyme action, and subsequent drying is then achieved with minimal thermal degradation. Alternatively, commercial alperujo was immersed in sodium fluoride solution prior to freezing and lyophilization (*174*). The presence of residual oil in the OMW prolongs the freeze-drying process due to formation of troublesome emulsions. The freeze-dried powder should be kept in a dry place, as the lyophilized samples tend to be hygroscopic (*173*). Storage may be at room temperature or, more commonly, at subzero temperatures such as −30 °C (*38*). Alternatively, Lesage-Meessen et al. (*73*) used ethanol (30% v/v) to stabilize fresh commercial OMW that was then clarified and stored in the dark at 4 °C. It was claimed that the phenolic composition of the samples was stable under these conditions for several months.

Extraction. The aim of extraction generally is to quantitatively recover all phenolic compounds from the sample while minimizing the coextraction of matrix components (*173*). The problem with phenolic extraction is that some of the biophenols remain attached to cell walls while others are in the cytoplasmic vacuoles. The choice of the extraction method, extraction solvent, extraction time, and extraction temperature is critical (*175*). The addition of antioxidant or enzyme inhibitor and pH adjustment may be employed. However, the stability of many biophenols is pH dependent, and alkaline conditions will favor oxidation and polymerization. Addition of acid, to a pH of 2−3, can be used to precipitate proteins (*34*, *73*), but as the phenolic group is weakly acidic, the pH will affect partitioning behavior during sample handling. Extraction methods show great diversity, ranging from simple filtration and direct injection into the HPLC without any further treatment (*82*) through liquid extraction to more advanced and complicated methods (*33*). The extraction method must be compatible with subsequent analysis procedures.

Solvent Extraction. The simplicity and convenience of solvent extraction contribute to its popularity (*115*, *173*, *176*). A vast range of solvents has been used for the extraction of biophenols from OMW including water (*108*), methanol (*174*, *177*), ethanol (*51*), ethyl acetate (*73*), and, less commonly, *n*-butanol (*94*, *178*), *n*-propanol (*37*), and *tert*-butyl methyl ether (*80*). Hydroalcoholic solutions such as methanol− or ethanol−water mixtures with different relative concentrations are often the popular choice to extract biophenols from pomace (*82*). Methanol is able to disrupt cell walls and inhibit enzyme action, and its mixture with water provides a very good solvent for most phenolic compounds (*173*). A simple strategy to suppress enzyme action is to add methanol first followed by water (*80*).

Aqueous ethanolic solutions are next to methanolic ones in popularity. The advantage of methanol over ethanol is its lower boiling point; it can be easily evaporated under vacuum at moderate temperatures to facilitate the recovery of extracted biophenols. However, ethanol is more lipophilic than methanol, so it is superior in dealing with polymeric and hydrophobic biophenols. Ethyl acetate is frequently used to extract biophenols from aqueous matrices such as OMWW. Ethyl acetate extraction

may result in the loss of some glycosidal biophenols (*82*). Capasso et al. (*178*) found that for quantitative extraction of OMWW biophenols, ethyl acetate was superseded by *n*-butanol, whereas qualitatively there was no difference. In contrast, ethyl acetate was chosen as the most suitable solvent in a comprehensive study targeting hydroxytyrosol (*66*) which demonstrated that extraction power decreased in the order ethyl acetate > methyl isobutyl ketone > methyl ethyl ketone > diethyl ether. This study is also notable for the use of gel filtration to demonstrate the presence of three phenolic fractions in OMWW, namely, a high molecular mass fraction (M > 250 kDa), a medium molecular mass fraction (M = 13 kDa), and monomeric phenols. It was further shown that best recovery of monomeric phenols occurred for an OMWW pH of 2. *o*-Diphenols were more extractable than monohydroxylated phenols due to more favorable distribution coefficients.

Extraction has mostly been carried out at ambient temperature, although elevated temperature was used (*144*) with Soxhlet extraction, whereas a subambient temperature of 0 °C has also been employed (*82*, *179*). The use of antioxidants to protect the biophenols during extraction is not uncommon. Different antioxidants can be applied, but the added antioxidant should be of higher antioxidant activity than the extracted biophenols, and its concentration should be adjusted; otherwise, it will act as an oxidizing agent or pro-oxidant. Sodium metabisulfite (sodium disulfite) (*180*, *181*) and sodium bisulfite (*182*) have been reported in the extraction of olive fruit phenolics, but their use for OMW has not been described. Phenolic antioxidants such as *tert*-butylhydroquinone (TBHQ) and butylated hydroxyanisole (BHA) have the disadvantage of interference with the detection of the biophenols (*183*). Servili et al. (*38*) compared the use of ascorbic acid and sulfur dioxide as antioxidants and working under a nitrogen blanket on biophenol recovery. Ascorbic acid showed pro-oxidant activity for most phenolic compounds, particularly hydroxytyrosol and oleuropein, and has also been reported to interfere with the detection of early-eluting biophenols (*183*). Sulfur dioxide may precipitate aldehydic phenolic compounds by a nucleophilic addition reaction (*38*). Thus, the concentration of hydroxytyrosol−elenolic acid dialdehyde in pomace was reduced significantly by SO_2 addition, whereas extraction under nitrogen significantly increased the recovery of *p*-hydroxybenzoic acid, caffeic acid, and vanillic acid (*38*). The addition of diethyldithiocarbamic acid sodium salt, a polyphenol oxidase and lipoxygenase inhibitor, is also reported (*38*, *82*). Enzyme activity may also be inhibited by precipitation using absolute alcohols (as for olive pulp) (*80*) and low-pH conditions (pH 2−3) (*34*).

Solid-Phase Extraction (SPE). SPE has the ability to both fractionate and clean up the sample. It has been used for the recovery of biophenols from olive pomace (*38*, *82*) and OMWW (*38*, *50*). There has been no systematic examination of the use of different cartridges for OMW, although C_{18} cartridges have been common (*38*, *82*). Sequential extraction with *n*-hexane, ethyl acetate, and acidic methanol (formic acid, pH 2.2) was employed (*50*) for the recovery of biophenols from freeze-dried OMWW. In the case of pomace, SPE gave higher recoveries of biophenols than liquid−liquid extraction (*38*). With SPE, higher recoveries were achieved by elution with methanol than by elution with diethyl ether or ethyl acetate. For OMWW, highest recoveries of biophenols were achieved by SPE with diethyl ether. The main biophenols in the OMWW were hydroxytyrosol and hydroxytyrosol−elenolic acid dialdehyde. The dissolution of quinones and melanoidins in methanol resulted in high background noise in HPLC chromatograms (*38*).

Solid-phase microextraction (SPME) is gaining acceptance as a phenol extraction technique but has not been applied to the recovery of biophenols from OMW. However, volatile phenols of wine have been determined by SPME and GC (*184*), suggesting that it should be applicable to the aqueous matrix of OMW.

Cleanup and Preconcentration. After extraction, cleanup is usually necessary before chromatography to remove coextracted matrix components that may interfere in subsequent steps or reduce column lifetime. Solvent partitioning with hexane, petroleum ether, or chloroform has been used to remove fatty residues, although hexane is generally preferred and probably superior (*33*, *174*). Ethyl acetate is claimed to be selective to low and medium molecular weight biophenols (*33*), but Ceccon et al. (*34*) obtained low and variable recovery of biophenols (notably caffeic and vanillic acids) using ethyl acetate. The ethyl acetate extract is usually evaporated, and the residue is reconstituted in methanol or ethanol. Acetone has been added to precipitate colloids (*34*). Alternatively, classical column chromatography has used columns of silica gel (*49*), Sephadex LH-20 for gel filtration, and adsorptive polymeric resins such as Amberlite XAD 1180 resin (*33*). However, SPE is preferred (*38*, *50*) as it offers simultaneous cleanup and preconcentration (*108*).

Identification and Quantification. *Total Phenols.* Spectrophotometric methods are generally used to quantify total biophenols. These methods are very simple but are nonspecific for phenols and usually tend to overestimate the amount of biophenols present. The Folin−Ciocalteu (FC) method is the most common quantification method. It depends on measuring the absorbance of the blue reduction product of a phosphotungstic−phosphomolybdic complex in alkaline solution at 760 nm (*173*). The results are expressed as gallic acid, caffeic acid, tannic acid, or tyrosol equivalents. Any substance that is able to reduce the phosphotungstic−phosphomolybdic complex will interfere, including ascorbic acid, tocopherols, carotenes, reducing sugars, and phenolic amino acids (i.e., phenylalanine and tyrosine). The FC method responds differently to different biophenols, and its use for measuring overall phenolic quantity is sometimes criticized (*185*).

HPLC methods for determining total phenols have been based on the summation of individual peak responses (*33*, *50*) using calibration curves, available standards, and different wavelengths. One (*38*) or more (*50*) standard compounds have been employed as a reference and, in cases where commercial standards were not available, molecular weight correction factors were applied (*33*). The use of syringic acid as an internal standard and quantification by response factors (area of reference compound/area of syringic acid) have been tried (*82*). The complexity of the HPLC chromatogram and the number of overlapping peaks are the major limiting factors for the reliability of this technique for the quantification of total phenols (*27*). Nevertheless, further investigation of this approach is recommended as an alternative to frequently reported spectrophotometric methods. LC-MS may be of value, particularly if used in tandem mode as LC-MS-MS (*27*, *80*, *186*), which allows for the quantification of daughter ion products and minimizes the impact of overlapping peaks.

Chromatographic Methods. Olive biophenols are polar compounds and of limited volatility (*28*) so derivatization is often mandatory in gas chromatography (GC). A very complex chromatogram resulted when GC−flame ionization detection (FID) was applied to an extract of OMWW (*34*) after derivatization with bis(trimethylsilyl)trifluoroacetamide. FID was

extensively employed in early work, but carbohydrate interference was a critical problem (*34*), and most GC work is now done using mass spectral detectors or tandem mass spectrometry (GC-MS-MS). GC-MS was applied for the identification of the sugar part of a new glycosidal biophenol in OMWW (*105*) after methylation, hydrolysis, reduction, and acetylation. For large phenolic molecules, derivatization may increase the molecular mass of the analytes beyond the analyzing capacity of the mass detector. Thermal degradation, failure of derivatization of high molecular weight biophenols, and unsuitability for preparative scale analysis are other drawbacks. Hence, GC is not a popular technique for routine use in biophenol analysis. It is more suitable for profile generation or structure elucidation, where its excellent resolving power is required (*28*).

TLC is a simple and versatile technique that can be used for the identification, separation, and isolation of biophenols on both analytical and semipreparative scales. The chromatogram contains the actual compounds, not their response. This permits subsequent elution and identification of each spot. Capasso et al. (*178*) have detected the major biophenols in OMWW using reversed phase C_{18}-TLC (C_{18}-RPTLC) and normal phase high-performance TLC. Spots were visualized under UV light at 254 nm, by spraying with 10% sulfuric acid in methanol followed by phosphomolybdic acid (3% in methanol) and heating, and by spraying with aqueous ferric chloride and heating. They recommended the use of both reagents to combine the high sensitivity of the first reagent and the specificity of the second reagent. Oleuropein was not detected in this sample. One-dimensional C_{18}-RPTLC proved to be more satisfactory for the analysis of OMWW biophenols. In the course of their work on OMWW biophenols, Ragazzi and Veronese (*187*) introduced a method for the quantification of phenolic compounds using the FC reagent after TLC separation.

TLC can also be used for the screening of biological activity in a technique known as bioautography. It is widely applied for screening antibacterial, antifungal, and radical scavenging activities. For antibacterial and antifungal activities agar diffusion, direct application, and agar-overlay are the methods for application (*188*). This method has not been applied to OMW.

HPLC has been the standard for analysis of biophenols, on both analytical and semipreparative scales, against which other techniques are judged. The typical conditions for HPLC are reversed phase chromatography using an octadecyl silica column with a suitable guard column and a binary pumping system, linear gradient elution, and photodiode array detector (PDA) (*50*). The mobile phase typically contains various combinations of water, methanol, or acetonitrile in different proportions and adjusted to an acidic pH by the addition of acetic acid, formic acid, or phosphoric acid. Different column brands and chromatographic systems (the pump and the detector) show different resolution abilities (*82*). In one study, the high polarity of representative biophenols from OMW caused problems with conventional reversed phase systems (*189*). A porous graphitic carbon column with a tetrahydrofuran gradient was used as an alternative. Spectrophotometric detection has been common, and for routine work 280 nm is a good compromise. The low wavelengths, 225 and 250 nm, are more universal, but the high background noise is a potential problem (*175*). The use of PDA detectors overcomes the problem of finding a wavelength suited to all phenols. Alternatively, MS has been applied to OMW samples for qualitative and quantitative work. The use of electron impact ionization and fast atom bombardment mass spectrometry (EI-MS and FAB-MS) of olive waste biophenols and their acetyl derivatives has been reviewed (*190*). FAB-MS in the positive mode proved to be more versatile and more satisfactory than EI-MS. However, the advantages of soft ionization techniques were demonstrated by the detection of biophenols using atmospheric pressure chemical ionization (APCI-MS) (*189*). Aramendía et al. (*191*) applied negative ion APCI-MS to qualitatively and quantitatively analyze 15 phenolic compounds found in OMWW. Bianco et al. (*80*) showed the high selectivity of HPLC-MS-MS in the analysis of OMWW. Elecrospray ionization (ESI)-MS was performed in the negative ion mode. Despite lower intensity peaks in negative ion mode than in positive mode, negative ion mode was also chosen in another study (*174*) because cleaner spectra were obtained. The latter study demonstrates the utility of ESI spectra, particularly in MS^n mode.

The potential of a number of detectors that have not been applied to OMW biophenols is demonstrated by their application to fruit phenolics. Fluorescence detection provides enhanced selectivity and sensitivity for some compounds (*112*, *192*). In contrast, the evaporative light scattering detector (*193*) is a bulk property detector that provides nonselective, universal detection with approximately the same response for all nonvolatile solutes (*194*). Electrochemical (coulometric) detection is a sensitive and selective method for phenolic acids (*195*) and olive oil phenolic antioxidants (*196*), and the electrochemical behavior may provide some structural information about compounds with similar UV spectra (e.g., hydroxytyrosol and its 4-β-D-glucoside) (*82*).

Peak identification in chromatography involves comparing the retention time with a standard and spiking. PDA and MS are useful for confirmation of the identity. Spectroscopic identification of biophenols has been the standard practice for their identification. Nowadays spectroscopic techniques provide very efficient systems for identification and structural elucidation, particularly when coupled with high-performance chromatography. Spectroscopic techniques include UV, NMR, and MS used either on-line or off-line. For instance, Limiroli et al. (*107*) identified both free and glucosidal phenols from the vegetation water (water-soluble constituents) of olive fruits by ^{1}H NMR. DellaGreca et al. (*105*) have identified four new compounds in OMWW using LC-MS and off-line NMR including two-dimensional NMR. The main problem that is usually encountered in the identification and quantification is the unavailability of standards for a large number of biophenols (*197*). Synthesis and/or separation from plant material may be mandatory.

Capillary Zone Electrophoresis (CZE). CZE depends on the relative migration of ions under an electric field (*198*). OMWW has been analyzed by CZE and ESI-MS detection in the negative ion mode and compared with CZE/UV (*199*). The total run time was 30 min. Quantitative analysis using *p*-chlorophenol as internal standard was carried out with a limit of detection of 1 pg for certain biophenols. The drawback of CZE is the use of buffer of high pH, which may be a problem for compounds unstable under these conditions such as anthocyanins. A similar technique, which is noteworthy in this context, is micellar electrokinetic capillary chromatography (MECC). The main similarity between CZE and MECC is the instrumentation; MECC is a hybrid between HPLC and CE where both neutral and ionic species can be separated by the difference in the distribution between the moving buffer and the capillary coating (electroosmotic flow) (*198*). Pomponio et al. (*200*) have used MECC for the separation of 10 phenolic acids, but it has not been applied to OMW.

CONCLUSION

OMW comprises a rich source of biophenols with a wide array of biological activities. Various procedures have been patented, but most rely on maximizing the recovery of one compound, hydroxytyrosol, and thus the complexity of the biophenols may be underutilized. Furthermore, the complete valorization of OMW—involving recovery of biophenols and other compounds prior to targeting the remaining byproducts to low technology uses such as animal feed, fuel or fertilizer—remains elusive.

Just as varietal, seasonal, geographical, and agricultural factors affect the phenol profile in olive fruit, so too will they affect the profile in OMW. This knowledge will be important if full utilization of the biophenols is to be achieved. Also of significance is the fact that OMW represents a highly chemically and microbiologically active medium, and storage prior to phenol extraction has the potential to significantly alter the phenol profile. An intriguing possibility is that chemical and/or microbiological changes during storage will generate new compounds with greater bioactivity than those presently reported, which warrants further investigation.

Analysis of biophenols in OMW generally parallels that for phenols from other sources, and a variety of procedures have been reported. However, the fact that OMW is the result of a process that breaks cell walls and exposes the matrix to enzymes, oxygen, and mild heat (during malaxation of the paste) means that the phenol profile undergoes significant changes prior to sampling. This represents a challenge to stabilize the profile at the point of sampling to minimize further changes prior to analysis. This aspect of OMW analysis has not been systematically addressed.

ACKNOWLEDGMENT

We acknowledge Dr. Radwan Kharabshah and his wife, Seniora Fatima, for their assistance in providing translation from Spanish.

LITERATURE CITED

(1) Kupchan, S. M. Drugs from natural products-plant sources. In *Drug Discovery Science and Development in a Changing Society*; American Chemical Society: Washington, DC, 1971.
(2) Kingston, D. G. I. Natural products as pharmaceuticals and sources for lead structures. In *The Practice of Medicinal Chemistry*; Wermuth, C. G., Ed.; Academic Press Harcourt Brace: London, U.K., 1996.
(3) Poulev, A.; O'Neal, J. M.; Logendra, S.; Pouleva, R. B.; Timeva, V.; Garvey, A. S.; Gleba, D.; Jenkins, I. S.; Halpern, B. T.; Kneer, R.; Cragg, G. M.; Raskin, I. Elicitation, a new window into plant chemodiversity and phytochemical drug discovery. *J. Med. Chem.* **2003**, *46*, 2542−2547.
(4) Cordell, G. A. Biodiversity and drug discovery—a symbiotic relationship. *Phytochemistry* **2000**, *55*, 463−480.
(5) Raskin, I.; Ribnicky, D. M.; Komarnytsky, S.; Ilic, N.; Poulev, A.; Borisjuk, N.; Brinker, A.; Moreno, D. A.; Ripoll, C.; Yakoby, N. Plants and human health in the twenty-first century. *Trends Biotechnol.* **2002**, *20*, 522−531.
(6) Waterman, P. G. Natural products for drug discovery: an overview. In *Advances in Drug Discovery Techniques*; Harvey, L. A., Ed.; Wiley: Chichester, U.K., 1998.
(7) Kinghorn, A. D. Pharmacognosy in the 21st century. *J. Pharm. Pharmacol.* **2001**, *53*, 135−148.
(8) Grabley, S.; Thiericke, R. The impact of natural products on drug discovery. In *Drug Discovery from Nature*; Grabley, S., Thiericke, R., Eds.; Springer-Verlag: Berlin, Germany, 1999.
(9) Wolfender, J.-L.; Ndjoko, K.; Hostettmann, K. Liquid chromatography with ultraviolet absorbance-mass spectrometric detection and with nuclear magnetic resonance spectrometry: a powerful combination for the on-line structural investigation of plant metabolites. *J. Chromatogr. A* **2003**, *1000*, 437−455.
(10) Pennington, J. A. T. Food composition databases for bioactive food components. *J. Food Compos. Anal.* **2002**, *15*, 419−434.
(11) Visioli, F.; Galli, C. Biological properties of olive oil phytochemicals. *Crit. Rev. Food Sci. Nutr.* **2002**, *42*, 209.
(12) Blekas, G.; Psomiadou, E.; Tsimidou, M.; Boskou, D. On the importance of total polar phenols to monitor the stability of Greek virgin olive oil. *Eur. J. Lipid Sci. Technol.* **2002**, *104*, 340−346.
(13) Part 2. Supplementary drugs and other substances. In *Martindale, The Complete Drug Reference*, 33rd ed.; Sweetman, S. C., Ed.; Pharmaceutical Press: London, U.K., 2002.
(14) Martin-Moreno, J. M. The role of olive oil in lowering cancer risk: is this real gold or simply pinchbeck? *J. Epidemiol. Commun. H* **2000**, *54*, 726.
(15) Trovato, A.; Forestieri, A. M.; Iauk, L.; Barbera, R.; Mnoforte, M. T.; Galati, E. M. Hypoglycemic activity of different extracts of *Olea europaea* L. in the rats. *Plant Med. Phytother.* **1993**, *26*, 300−308.
(16) Petroni, A.; Blasevich, M.; Salami, M.; Papini, N.; Montedoro, G. F.; Galli, C. Inhibition of platelet aggregation and eicosanoid production by phenolic components of olive oil. *Thromb. Res.* **1995**, *78*, 151−160.
(17) Petroni, A.; Blasevich, M.; Papini, N.; Salami, M.; Sala, A.; Galli, C. Inhibition of leukocyte leukotriene B4 production by an olive oil-derived phenol identified by mass-spectrometry. *Thromb. Res.* **1997**, *87*, 315−322.
(18) Visioli, F.; Bellosta, S.; Galli, C. Cardioprotective properties of olive oil-derived polyphenols. *Atherosclerosis* **1997**, *134*, 336.
(19) Owen, R. W.; Giacosa, A.; Hull, W. E.; Haubner, R.; Wurtele, G.; Spiegelhalder, B.; Bartsch, H. Olive-oil consumption and health: the possible role of antioxidants. *Lancet Oncol.* **2000**, *1*, 107−112.
(20) Owen, R. W.; Giacosa, A.; Hull, W. E.; Haubner, R.; Spiegelhalder, B.; Bartsch, H. The antioxidant/anticancer potential of phenolic compounds isolated from olive oil. *Eur. J. Cancer* **2000**, *36*, 1235−1247.
(21) Visioli, F.; Caruso, D.; Galli, C.; Viappiani, S.; Galli, G.; Sala, A. Olive oils rich in natural catecholic phenols decrease isoprostane excretion in humans. *Biochem. Biophys. Res. Commun.* **2000**, *278*, 797−799.
(22) Visioli, F.; Galli, C.; Bornet, F.; Mattei, A.; Patelli, R.; Galli, G.; Caruso, D. Olive oil phenolics are dose-dependently absorbed in humans. *FEBS Lett.* **2000**, *468*, 159−160.
(23) Vissers, M. N.; Zock, P. L.; Wiseman, S. A.; Meyboom, S.; Katan, M. B. Effect of phenol-rich extra virgin olive oil on markers of oxidation in healthy volunteers. *Eur. J. Clin. Nutr.* **2001**, *55*, 334−341.
(24) Moure, A.; Cruz, J. M.; Franco, D.; Dominguez, J. M.; Sineiro, J.; Dominguez, H.; Jose Nunez, M.; Parajo, J. C. Natural antioxidants from residual sources. *Food Chem.* **2001**, *72*, 145−171.
(25) Schieber, A.; Stintzing, F. C.; Carle, R. By-products of plant food processing as a source of functional compounds recent developments. *Trends Food Sci. Technol.* **2001**, *12*, 401−413.
(26) Uccella, N. Olive biophenols: biomolecular characterization, distribution, and phytolexin histochemical localization in the drupes. *Trends Food Sci. Technol.* **2001**, *11*, 315−327.
(27) Visioli, F.; Galli, C.; Galli, G.; Caruso, D. Biological activities and metabolic fate of olive oil phenols. *Eur. J. Lipid Sci. Technol.* **2002**, *104*, 677−684.
(28) Ryan, D.; Robards, K. Phenolic compounds in olives. *Analyst* **1998**, *123*, 31R−44R.
(29) Bianco, A. D.; Muzzalupo, I.; Piperno, A.; Romero, G.; Uccella, N. Bioactive derivatives of oleuropein from olive fruits. *J. Agric. Food Chem.* **1999**, *47*, 3531−3534.

(30) Markin, D.; Duek, L.; Berdicevsky, I. In vitro antimicrobial activity of olive leaves. *Mycoses* **2003**, *46*, 132−136.
(31) Paiva-Martins, F.; Gordon, M. H. Isolation and characterization of the antioxidant component 3,4-dihydroxyphenylethyl 4-formyl-3-formylmethyl-4-hexenoate from olive (*Olea europaea*) leaves. *J. Agric. Food Chem.* **2001**, *49*, 4214−4219.
(32) Rodis, P. S.; Karathanos, V. T.; Mantzavinou, A. Partitioning of olive oil antioxidants between oil and water phases. *J. Agric. Food Chem.* **2002**, *50*, 596−601.
(33) Visioli, F.; Romani, A.; Mulinacci, N.; Zarini, S.; Conte, D.; Vincieri, F. F.; Galli, C. Antioxidant and other biological activities of olive mill wastewaters. *J. Agric. Food Chem.* **1999**, *47*, 3397−3401.
(34) Ceccon, L.; Saccu, D.; Procida, G.; Cardinali, S. Liquid chromatographic determination of simple phenolic compounds in wastewaters from olive oil production plants. *J. AOAC Int.* **2001**, *84*, 1739−1744.
(35) Fernandez-Bolanos, J.; Rodriguez, G.; Rodriguez, R.; Heredia, A.; Guillen, R.; Jimenez, A. Production in large quantities of highly purified hydroxytyrosol from liquid−solid waste of two-phase olive oil processing or "Alperujo". *J. Agric. Food Chem.* **2002**, *50*, 6804−6811.
(36) Capasso, R.; Evidente, A.; Visca, C. Production of hydroxytyrosol from olive oil vegetation waters. *Agrochimica* **1994**, *38*, 165−171.
(37) Vázquez Roncero, A.; Maestro Durán, R.; Graciani Constante, E. Componentes fenólicos de la aceituna. II. Polifenols del alpechin. *Grasas Aceites* **1974**, *25*, 341−345.
(38) Servili, M.; Baldioli, M.; Selvaggini, R.; Miniati, E.; Macchioni, A.; Montedoro, G. High-performance liquid chromatography evaluation of phenols in olive fruit, virgin olive oil, vegetation waters, and pmace and 1D-and 2D-nuclear magnetic resonance characterization. *J. Am. Oil Chem. Soc.* **1999**, *76*, 873−882.
(39) Visioli, F.; Caruso, D.; Plasmati, E.; Patelli, R.; Mulinacci, N.; Romani, A.; Galli, G.; Galli, C. Hydroxytyrosol, as a component of olive mill wastewater, is dose-dependently absorbed and increases the antioxidant capacity of rat plasma. *Free Radical Res.* **2001**, *34*, 301−305.
(40) Casalino, E.; Calzaretti, G.; Sblano, C.; Landriscina, V.; Tecce, M. F.; Landriscina, C. Antioxidant effect of hydroxytyrosol (DPE) and Mn^{2+} in liver of cadmium-intoxicated rats. *Comp. Biochem. Phys. C* **2002**, *133*, 625−632.
(41) Manna, C.; Galletti, P.; Cucciolla, V.; Montedoro, G.; Zappia, V. Olive oil hydroxytyrosol protects human erythrocytes against oxidative damages. *J. Nutr. Biochem.* **1999**, *10*, 159−165.
(42) Leger, C. L.; Kadiri-Hassani, N.; Descomps, B. Decreased superoxide anion production in cultured human promonocyte cells (THP-1) due to polyphenol mixtures from olive oil processing wastewaters. *J. Agric. Food Chem.* **2000**, *48*, 5061−5067.
(43) Deiana, M.; Aruoma, O. I.; Bianchi, M. D. P.; Spencer, J. P. E.; Kaur, H.; Halliwell, B.; Aeschbach, R.; Banni, S.; Dessi, M. A.; Corongiu, F. P. Inhibition of peroxynitrite dependent DNA base modification and tyrosine nitration by the extra virgin olive oil-derived antioxidant hydroxytyrosol. *Free Radical Biol. Med.* **1999**, *26*, 762−769.
(44) Della Ragione, F.; Cucciolla, V.; Borriello, A.; Della Pietra, V.; Pontoni, G.; Racioppi, L.; Manna, C.; Galletti, P.; Zappia, V. Hydroxytyrosol, a natural molecule occurring in olive oil, induces cytochrome C-dependent apoptosis. *Biochem. Biophys. Res. Commun.* **2000**, *278*, 733−739.
(45) Fabiani, R.; De Bartolomeo, A.; Rosignoli, P.; Servili, M.; Montedoro, G. F.; Morozzi, G. Cancer chemoprevention by hydroxytyrosol isolated from virgin olive oil through G^1 cell cycle arrest and apoptosis. *Eur. J. Cancer Prevention* **2002**, *11*, 351−358.
(46) Bisignano, G.; Tomaino, A.; Lo Cascio, R.; Crisafi, G.; Uccella, N.; Saija, A. On the in-vitro antimicrobial activity of oleuropein and hydroxytyrosol. *J. Pharm. Pharmacol.* **1999**, *51*, 971−974.
(47) Capasso, R.; Evidente A.; Schivo, L.; Orru, G.; Marcialis, M. A.; Cristinzio, G. Antibacterial polyphenols from olive oil mill wastewaters. *J. Appl. Bacteriol.* **1995**, *79*, 393−398.
(48) De La Puerta, R.; Gutierrez, V. R.; Hoult, J. R. S. Inhibition of leukocyte 5-lipoxygenase by phenolics from virgin olive oil. *Biochem. Pharmacol.* **1999**, *57*, 445−449.
(49) Capasso, R.; Evidente, A.; Avolio, S.; Solla, F. A highly convenient synthesis of hydroxytyrosol and its recovery from agricultural wastewaters. *J. Agric. Food Chem.* **1999**, *47*, 1745−1748.
(50) Mulinacci, N.; Romani, A.; Galardi, C.; Pinelli, P.; Giaccherini, C.; Vincieri, F. F. Polyphenolic content in olive oil wastewaters and related olive samples. *J. Agric. Food Chem.* **2001**, *49*, 3509−3514.
(51) Amro, B.; Aburjai, T.; Al-Khalil, S. Antioxidative and radical scavenging effects of olive cake extract. *Fitoterapia* **2002**, *73*, 456−461.
(52) Speroni, E.; Guerra, M. C.; Minghetti, A.; Crespi-Perellino, N.; Pasini, P.; Piazza F.; Roda, A. Oleuropein evaluated in vitro and in vivo activity as an antioxidant. *Phytother. Res.* **1998**, *12*, S98−S100.
(53) Saija, A.; Trombetta, D.; Tomaino, A.; Lo Cascio, R.; Princi, P.; Uccella, N.; Bonina, F.; Castelli, F. "In vitro" evaluation of the antioxidant activity and biomembrane interaction of the plant phenols oleuropein and hydroxytyrosol. *Int. J. Pharm.* **1998**, *166*, 123−133.
(54) Andrikopoulos, N. K.; Antonopoulou, S.; Kaliora, A. C. Oleuropein inhibits LDL oxidation induced by cooking oil frying by-products and platelet aggregation induced by platelet-activating factor. *Lebensm. Wiss. Technol.* **2002**, *35*, 479−484.
(55) Ruiz Gutierrez, V.; Muraina, F. J. G.; Maestro, R.; Graciani, E. Oleuropein on lipid and fatty acid compostion of rat heart. *Nutr. Res.* **1995**, *15*, 37−51.
(56) Visioli, F.; Bellosta, S.; Galli, C. Oleuropein, the bitter principle of olives, enhances nitric oxide production by mouse macrophages. *Life Sci.* **1998**, *62*, 541−546.
(57) Duarte, J.; Perez, O.; Zarzuelo A.; Jimenez, J.; Perez-Vizcaino, F.; Tamargo, J. Effects of oleuropeoside in isolated guinea-pig atria. *Planta Med.* **1993**, *59*, 318−322.
(58) Fleming, H. P.; Walter, W. M.; Etchells, J. L. Antimicrobial properties of oleuropein and products of its hydrolysis from green olives. *Appl. Microbiol.* **1973**, *26*, 777−782.
(59) Furneri, P. M.; Marino, A.; Saija, A.; Uccella, N.; Bisignano, G. In vitro antimycoplasmal activity of oleuropein. *Int. J. Antimicrob. Agents* **2002**, *20*, 293−296.
(60) Aziz, N. H.; Farag, S. E.; Mousa, L. A. A.; Abo-Zaid, M. A. Comparative antibacterial and antifungal effects of some phenolic compounds. *Microbios* **1998**, *93*, 43−54.
(61) Lee-Huang, S.; Zhang, L.; Huang, P. L.; Chang, Y. T.; Huang, P. L. Anti-HIV activity of olive leaf extract (OLE) and modulation of host cell gene expression by HIV-1 infection and OLE treatment. *Biochem. Biophys. Res. Commun.* **2003**, *307*, 1029−1037.
(62) Saenz, M. T.; Garcia, M. D.; Ahumada, M. C.; Ruiz, V. Cytostatic activity of some compounds from the unsaponifiable fraction obtained from virgin olive oil. *Farmaco* **1998**, *53*, 448−449.
(63) Kubo, I.; Matsumoto, A. Molluscicides from olive *Olea europaea* and their efficient isolation by countercurrent chromatography. *J. Agric. Food Chem.* **1984**, *32*, 687−688.
(64) Al-Qarawi, A. A.; Al-Damegh, M. A.; ElMougy, S. A. Effect of freeze-dried extract of *Olea europaea* on the pituitary-thyroid axis in rats. *Phytother. Res.* **2002**, *16*, 286−287.
(65) Polzonetti, V.; Egidi, D.; Vita, A.; Vincenzetti, S.; Natalini, P. Involvement of oleuropein in (some) digestive metabolic pathways. *Food Chem.* **2004**, *88*, 11−15.
(66) Allouche, N.; Fki, I.; Sayadi, S. Toward a high yield recovery of antioxidants and purified hydroxytyrosol from olive mill wastewaters. *J. Agric. Food Chem.* **2004**, *52*, 267−273.

(67) Visioli, F.; Vinceri, F. F.; Galli, C. Wastewaters from olive oil production are rich in natural antioxidants. *Experientia* **1995**, *51*, 32−34.
(68) Damiani, E.; Belaid, C.; Carloni, P.; Greci, L. Comparison of antioxidant activity between aromatic indolinonic nitroxides and natural and synthetic antioxidants. *Free Radical Res.* **2003**, *37*, 731−741.
(69) Giovannini, C.; Straface, E.; Modesti, D.; Coni, E.; Cantafora, A.; De Vincenzi, M.; Malorni, W.; Masella, R. Tyrosol, the major olive oil biophenol, protects against oxidized-LDL-induced injury in Caco-2 Cells. *J. Nutr.* **1999**, *129*, 1269−1277.
(70) Quiles, J. L.; Farquharson, A. J.; Simpson, D. K.; Grant, I.; Wahle, K. W. J. Olive oil phenolics: Effects on DNA oxidation and redox enzyme mRNA in prostate cells. *Br. J. Nutr.* **2002**, *88*, 225−234.
(71) Marrugat, J.; Covas, M. I.; Fito, M.; Schroder, H.; Miro-Casas, E.; Gimeno, E.; Lopez-Sabater, M. C.; De La Torre, R.; Farre, M.; SOLOS Investigators. Effects of differing phenolic content in dietary olive oils on lipids and LDL oxidation − a randomized controlled trial. *Eur. J. Nutr.* **2004**, *43*, 140−147.
(72) Soler-Rivas, C.; Espin, J. C.; Wichers, H. J. Oleuropein and related compounds. *J. Sci. Food. Agric.* **2000**, *80*, 1013−1023.
(73) Lesage-Meessen, L.; Navarro, D.; Maunier, S.; Sigoillot, J. C.; Lorquin, J.; Delattre, M.; Simon, J. L.; Asther, M.; Labat, M. Simple phenolic content in olive oil residues as a function of extraction systems. *Food Chem.* **2001**, *75*, 501−507.
(74) Nardini, M.; Pisu, P.; Gentili, V.; Natella, F.; Di, M.; Piccolella, F.; Scaccini, C. Effect of caffeic acid on *tert*-butyl hydroperoxide-induced oxidative stress in U937. *Free Rad. Biol. Med.* **1998**, *25*, 1098−1105.
(75) Kono, Y.; Kobayashi, K.; Tagawa, S.; Adachi, K.; Ueda, A.; Sawa, Y.; Shibata, H. Antioxidant activity of polyphenolics in diets: Rate constants of reactions of chlorogenic acid and caffeic acid with reactive species of oxygen and nitrogen. *Biochim. Biophys. Acta* **1997**, *1335*, 335−342.
(76) Meyer, A. S.; Heinonen, M.; Frankel, E. N. Antioxidant interactions of catechin, cyanidin, caffeic acid, quercetin, and ellagic acid on human LDL oxidation. *Food Chem.* **1998**, *61*, 71−75.
(77) Nardini, M.; D′Aquino, M.; Tomassi, G.; Gentili, V.; Di Felice, M.; Scaccini, C. Inhibition of human low-density lipoprotein oxidation by caffeic acid and other hydroxycinnamic acid derivatives. *Free Radical Biol. Med.* **1995**, *19*, 541−552.
(78) Yamanaka, N.; Oda, O.; Nagao, S. Prooxidant activity of caffeic acid, dietary non-flavonoid phenolic acid, on Cu^{2+}-induced low-density lipoprotein oxidation. *FEBS Lett.* **1997**, *405*, 186−190.
(79) Takeda, H.; Tsuji, M.; Inazu, M.; Egashira, T.; Matsumiya, T. Rosmarinic acid and caffeic acid produce antidepressive-like effect in the forced swimming test in mice. *Eur. J. Pharmacol.* **2002**, *449*, 261−267.
(80) Bianco, A.; Buiarelli, F.; Cartoni, G.; Coccioli, F.; Jasionowska, R.; Margherita, P. Analysis by liquid chromatography-tandem mass spectrometry of biophenolic compounds in olives and vegetation waters, Part I. *J. Separation Sci.* **2003**, *26*, 409−416.
(81) Sawa, T.; Nakao, M.; Akaike, T.; Ono, K.; Maeda, H. Alkylperoxyl radical-scavenging activity of various flavonoids and other phenolic compounds: Implications for the anti-tumor-promoter effect of vegetables. *J. Agric. Food Chem.* **1999**, *47*, 397−402.
(82) Romero, C.; Brenes, M.; Garcia, P.; Garrido, A. Hydroxytyrosol 4-β-D-glucoside, an important phenolic compound in olive fruits and derived products. *J. Agric. Food Chem.* **2002**, *50*, 3835−3839.
(83) Daels-Rakotoarison, D. A.; Seidel, V.; Gressier, B.; Brunet, C.; Tillequin, F.; Bailleul, F.; Luyckx, M.; Dine, T.; Cazin, M.; Cazin, J. C. Neurosedative and antioxidant activities of phenylpropanoids from *Ballota nigra*. *Arzneim.-Forsch.* **2000**, *50*, 16−23.
(84) Li, J.; Zheng, Y.; Zhou, H.; Su, B. N.; Zheng, R. L. Differentiation of human gastric adenocarcinoma cell line MGC80-3 induced by verbascoside. *Planta Med.* **1997**, *63*, 499−502.
(85) Pennacchio, M.; Alexander, E.; Syah, Y. M.; Ghisalberti, E. L. The effect of verbascoside on cyclic 3′,5′-adenosine monophosphate levels in isolated rat heart. *Eur. J. Pharmacol.* **1996**, *305*, 169−171.
(86) Kang, D. G.; Lee, Y. S.; Kim, H. J.; Lee, Y. M.; Lee, H. S. Angiotensin converting enzyme inhibitory phenylpropanoid glycosides from *Clerodendron trichotomum*. *J. Ethnopharmacol.* **2003**, *89*, 151−154.
(87) Diaz, A. M.; Abad, M. J.; Fernandez, L.; Silvan, A. M.; De Santos, J.; Bermejo, P. Phenylpropanoid glycosides from *Scrophularia Scorodonia:* in Vitro Anti-inflammatory activity. *Life Sci.* **2004**, *74*, 2515−2526.
(88) Liu, M. J.; Li, J. X.; Guo, H. Z.; Lee, K. M.; Qin, L.; Chan, K. M. The effects of verbascoside on plasma lipid peroxidation level and erythrocyte membrane fluidity during immobilization in rabbits: a time course study. *Life Sci.* **2003**, *73*, 883−892.
(89) Renis, H. E. In vitro antiviral activity of calcium elenolate. *Antimicrob. Agents Chemother.* **1969**, 167−172.
(90) Abdel-Wahab, M. H.; El-Mahdy, M. A.; Abd-Ellah, M. F.; Helal, G. K.; Khalifa, F.; Hamada, F. M. A. Influence of p-coumaric acid on doxorubicin-induced oxidative stress in rat's heart. *Pharmacol. Res.* **2003**, *48*, 461−465.
(91) Firuzi, O.; Giansanti, L.; Vento, R.; Seibert, C.; Petrucci, R.; Marrosu, G.; Agostino, R.; Saso, L. Hypochlorite scavenging activity of hydroxycinnamic acids evaluated by a rapid microplate method based on the measurement of chloramines. *J. Pharm. Pharmacol.* **2003**, *55*, 1021−1027.
(92) Wen, A. M.; Delaquis, P.; Stanich, K.; Toivonen, P. Antilisterial activity of selected phenolic acids. *Food Microbiol.* **2003**, *20*, 305−311.
(93) Chiang, L. C.; Chiang, W.; Chang, M. Y.; Ng, L. T.; Lin, C. C. Antileukemic activity of selected natural products in Taiwan. *Am. J. Chinese Med.* **2003**, *31*, 37−46.
(94) Capasso, R.; Cristinzio, G.; Evidente, A.; Scognamiglio, F. Isolation, spectoscopy and selective phytotoxic effects of polyphenols from vegetable wastewaters. *Phytochemistry* **1992**, *31*, 4125−4128.
(95) Fiorentino, A.; Gentili, A.; Isidori, M.; Monaco, P.; Nardelli, A.; Parrella, A.; Temussi, F. Environmental effects caused by olive mill wastewaters: toxicity comparison of low-molecular-weight phenol components. *J. Agric. Food Chem.* **2003**, *51*, 1005−1009.
(96) Tanaka, H.; Hirose, M.; Hagiwara, A.; Imaida, K.; Shirai, T.; Ito, N. Rat strain differences in catechol carcinogenicity to the stomach. *Food Chem. Toxicol.* **1995**, *33*, 93−98.
(97) Weisburger, J. H. Mutagenic, carcinogenic, and chemopreventive effects of phenols and catechols. In *Phenolic Compounds in Food and Their Effects on Health II: Antioxidants and Cancer Prevention*; Ho, C.-T., Lee, C. Y., Huang, M.-T., Eds.; American Chemical Society: Washington, DC, 1992.
(98) Janbaz, K. H.; Saeed, S. A.; Gilani, A. H. Protective effect of rutin on paracetamol- and CCl_4-induced hepatotoxicity in rodents. *Fitoterapia* **2002**, *73*, 557−563.
(99) Grinberg, L. N.; Rachmilewitz, E. A.; Newmark, H. Protective effects of rutin against hemoglobin oxidation. *Biochem. Pharmacol.* **1994**, *48*, 643−649.
(100) Hou, L.; Zhou, B.; Yang, L.; Liu, Z.-L. Inhibition of human low-density lipoprotein oxidation by flavonols and their glycosides. *Chem. Phys. Lipids* **2004**, *129*, 209−219.
(101) Rotelli, A. E.; Guardia, T.; Juarez, A. O.; de la Rocha, N. E.; Pelzer, L. E. Comparative study of flavonoids in experimental models of inflammation. *Pharmacol. Res.* **2003**, *48*, 601−606.
(102) Guardia, T.; Rotelli, A. E.; Juarez, A. O.; Pelzer, L. E. Anti-inflammatory properties of plant flavonoids. Effects of rutin, quercetin and hesperidin on adjuvant arthritis in rat. *Farmaco* **2001**, *56*, 683−687.
(103) Hirose, M.; Takahashi, S.; Ogawa, K.; Futakuchi, M.; Shirai, T. Phenolics: blocking agents for heterocyclic amine-induced carcinogenesis. *Food Chem. Toxicol.* **1999**, *37*, 985−992.

(104) Ryan, D.; Robards, K.; Lavee, S. Changes in phenolic content of olive during maturation. *Int. J. Food Sci. Technol.* **1999**, *34*, 265−274.
(105) DellaGreca, M.; Previtera, L.; Temussi, F.; Zarrelli, A. Low-molecular-weight components of olive oil mill wastewaters. *Phytochem. Anal.* **2004**, *15*, 184−188.
(106) Vlahov, G. Flavonoids in three olive (*Olea europaea*) fruit varieties during maturation. *J. Sci. Food Agric.* **1992**, *58*, 157−159.
(107) Limiroli, R.; Consonni, R.; Ranalli, A.; Bianchi, G.; Zetta, L. ^{1}H NMR study of phenolics in the vegetation water of three cultivars of *Olea europaea*: similarities and differences. *J. Agric. Food Chem.* **1996**, *44*, 2040−2048.
(108) Servili, M.; Baldioli, M.; Selvaggini, R.; Macchioni, A.; Montedoro, G. Phenolic compounds of olive fruit: one- and two-dimensional nuclear magnetic resonance characterization of nüzhenide and its distribution in the constitutive parts of fruit. *J. Agric. Food Chem.* **1999**, *47*, 12−18.
(109) Amiot, M.-J.; Fleuriet, A.; Macheix, J.-J. Accumulation of oleuropein derivatives during olive maturation. *Phytochemistry* **1989**, *28*, 67−69.
(110) Akasbi, M.; Shoeman, D. W.; Csallany, A. S. High-performance liquid chromatography of selected phenolic compounds in olive oil. *J. Am. Oil Chem. Soc.* **1993**, *70*, 367−370.
(111) De Nino, A.; Mazzotti, F.; Pia Morrone, S.; Perri, E.; Raffaelli, A.; Sindona, G. Characterization of cassanese olive cultivar through the identification of new trace components by ionspray tandem mass spectrometry. *J. Mass Spectrom.* **1999**, *34*, 10−16.
(112) Robbins, R. J. Phenolic acids in foods: an overview of analytical methodology. *J. Agric. Food Chem.* **2003**, *51*, 2866−2887.
(113) Bianco, A.; Coccioli, F.; Guiso, M.; Marra, C. The occurrence in olive oil of a new class of phenolic compounds:hydroxy-isochromans. *Food Chem.* **2001**, *77*, 405−411.
(114) Dewick, P. M. *Medicinal Natural Products: A Biosynthetic Approach*; Wiley: Chichester, U.K., 1997.
(115) Tura, D.; Robards, K. Sample handling strategies for the determination of biophenols in food and plants. *J. Chromatogr. A* **2002**, *975*, 71−93.
(116) Saija, A.; Uccella, N. Olive biophenols: functional effects on human wellbeing. *Trends Food Sci. Technol.* **2000**, *11*, 357−363.
(117) Ranalli, A.; Angerosa, F. Integral centrifuges for olive oil extraction. The qualitative characteristics of products. *J. Am. Oil Chem. Soc.* **1996**, *73*, 417−422.
(118) Madejon, E.; Galli, E.; Tomati, U. Bioremediation of olive mill pomaces for agricultural purposes. *Fresenius' Environ. Bull.* **1998**, *7*, 873−879.
(119) Bravo, J. *The By-products of Oil Processing*; Bravo, J., Ed.; IOOC: Madrid, Spain, 1990.
(120) Martin Garcia, A. I.; Yanez Ruiz, D. R.; Moumen, A.; Molina Alcaide, E. Effect of polyethylene-glycol on the chemical composition and nutrient availability of olive (*Olea europaea* var. europaea) by-products. *Anim. Feed Sci. Technol.* **2004**, *114*, 159−177.
(121) Rivas, F. J.; Beltran, F. J.; Gimeno, O.; Frades, J. Treatment of olive oil mill wastewater by Fenton's reagent. *J. Agric. Food Chem.* **2001**, *49*, 1873−1880.
(122) Fodale, A. S.; Mule, R.; Bati, B. The antifungal activity of olive oil wastewater on isolates of *Verticillium dahliae* Kleb. in vitro. *Acta Hortic.* **1999**, *474*, 753−756.
(123) Ramos-Cormenzana, A.; Juarez-Jimenez, B.; Garcia-Pareja, M. P. Antimicrobial activity of olive mill wastewaters (Alpechin) and biotransformed olive oil mill wastewater. *Int. Biodeter. Biodegr.* **1996**, *38*, 283−290.
(124) Moreno, E.; Quevedo-Sarmiento, J.; Ramos-Cormenzana, A. Antibacterial activity of wastewaters from olive oil mills. In *Encyclopedia of Environmental Control Technology*; Cheremisoff, P. N., Ed.; Gulf Publishing: Houston, TX, 1989.
(125) Azbar, N.; Bayram, A.; Filibeli, A.; Muezzinoglu, A.; Sengul, F.; Ozer, A. A review of waste management options in olive oil production. *Crit. Rev. Environ. Sci. Technol.* **2004**, *34*, 209−247.
(126) Della Monica, M.; Agostiniano, A.; Ceglie, A. An electrochemical sewage treatment process. *J. Appl. Electrochem.* **1980**, *10*, 527−533.
(127) Hamdi, M. Toxicity and biodegradability of olive mill wastewaters in batch anaerobic digestion. *Appl. Biochem. Biotechnol.* **1992**, *37*, 155−163.
(128) Di Giacomo, G.; Brandani, V.; Del Re, G. Evaporation of olive oil mill vegetation waters. *Desalination* **1991**, *81*, 249−259.
(129) Turano, E.; Curcio, S.; De Paola, M. G.; Calabro, V.; Iorio, G. An integrated centrifugation-ultrafiltration system in the treatment of olive mill wastewater. *J. Membr. Sci.* **2002**, *209*, 519−531.
(130) Alonso, J. B. D.; Anton, J. T.; Rodriguez, J. G.; Vargas, J. R. D. Ozone treatment of olive mill wastewater. *Grasas Aceites* **2000**, *51*, 301−306.
(131) Aktas, E. S.; Imre, S.; Ersoy, L. Characterization and lime treatment of olive mill wastewater. *Water Res.* **2001**, *35*, 2336−2340.
(132) Longhi, P.; Vodopivec, B.; Fiori, G. Electrochemical treatment of olive oil mill wastewater. *Ann. Chim.* **2001**, *91*, 169−174.
(133) Casa, R.; D'annibale, A.; Pieruccetti, F.; Stazi, S. R.; Sermanni, G. G.; Lo Cascio, B. Reduction of the phenolic components in olive-mill wastewater by an enzymatic treatment and its impact on Durum wheat (*Triticum durum* Desf.) germinability. *Chemosphere* **2003**, *50*, 959−966.
(134) Greco, Jr., G.; Toscanoa, G.; Cioffi, M.; Gianfreda, L.; Sannino, F. Dephenolization of olive mill wastewaters by olive husk. *Water Res.* **1999**, *33*, 3046−3050.
(135) Tekin, A. R.; Dalgic, A. C. Biogas production from olive pomace. *Resources, Conservation Recycling* **2000**, *30*, 301−313.
(136) Dally, Bassam, and Mullinger, P. In *Utilization of Olive Husks for Energy Generation: A Feasibility Study*; South Australian State Energy Research Advisory Committee: 2002.
(137) Vitolo, S.; Petarca, L.; Bresci, B. Treatment of olive oil industry wastes. *Bioresour. Technol.* **1999**, *67*, 129−137.
(138) Haddadin, M. S.; Abdulrahim, S. M.; Al-Khawaldeh, G. Y.; Robinson, R. K. Solid-state fermentation of waste pomace from olive processing. *J. Chem. Technol. Biotechnol.* **1999**, *74*, 613−618.
(139) Haddadin, M. S.; Al-Natour, R.; Al-Qsous, S.; Robinson, R. K. Bio-degradation of lignin in olive pomace by freshly-isolated species of basidiomycete. *Bioresour. Technol.* **2002**, *82*, 131−137.
(140) Lopez, M. J.; Ramos-Cormenzana, A. Xanthan production from olive-mill wastewaters. *Int. Biodeter. Biodegr.* **1996**, *38*, 263−270.
(141) Ballesteros, I.; Oliva, J. M.; Ballesteros, M. Ethanol production from lignocellulosic byproducts of olive oil extraction. *Appl. Biochem. Biotechnol.* **2001**, *91*−*93*, 237−252.
(142) Kahraman, S.; Yesilada, O. Industrial and agricultural wastes as substrates for laccase production by white-rot fungi. *Folia Microbiol.* **2001**, *46*, 133−136.
(143) Fenice, M.; Sermanni, G. G.; Federici, F.; D'annibale, A. Submerged and solid-state production of laccase and Mn-Peroxidase by *Panus tigrinus* on olive mill wastewater-based media. *J. Biotechnol.* **2003**, *100*, 77−85.
(144) Sheabar, F. Z.; Neeman, I. Separation and concentration of natural antioxidants from rape of olives. *J. Am. Oil Chem. Soc.* **1988**, *65*, 990−993.
(145) Piperidou, C. I.; Chaidou, C. I.; Stalikas, C. D.; Soulti, K.; Pilidis, G. A.; Balis, C. Bioremediation of olive oil mill wastewater: Chemical alterations induced by *Azotobacter vinelandii*. *J. Agric. Food Chem.* **2000**, *48*, 1941−1948.
(146) Visioli, F.; Poli, A.; Galli, C. Antioxidant and other biological activities of phenols from olives and olive oil. *Med. Res. Rev.* **2002**, *22*, 65−75.

(147) Baddi, G. A.; Hafidi, M.; Merlina, G.; Revel, J. C. Characterization and identification of polyphenols during composting of olive mill waste. *Agrochimica* **2003**, *47*, 161−172.
(148) Hill, D. C. Advanced screening technology and informatics for natural products drug discovery. In *Advances in Drug Discovery Techniques*; Harvey, A. L., Ed.; Wiley: Chichester, U.K.,1998.
(149) Tranter, H. S.; Tassou, S. C.; Nychas, G. J. The effect of the olive phenolic compound, oleuropein, on growth and enterotoxin B production by *Staphylococcus aureus*. *J. Appl. Bacteriol.* **1993**, *74*, 253−259.
(150) Ranalli, A.; Lucera, L.; Contento, S. Antioxidizing potency of phenol compounds in olive oil mill wastewater. *J. Agric. Food Chem.* **2003**, *51*, 7636−7641.
(151) Antolovich, M.; Prenzler, P. D.; Patsalides, E.; McDonald, S.; Robards, K. Methods of testing antioxidant activity. *Analyst* **2002**, *127*, 183−198.
(152) Robards, K.; Prenzler, P. D.; Tucker, G.; Swatsitang, P.; Glover, W. Phenolic compounds and their role in oxidative processes in fruits. *Food Chem.* **1999**, *66*, 401−436.
(153) McDonald, S.; Prenzler, P. D.; Antolovich, M.; Robards, K. Phenolic content and antioxidant activity of olive extracts. *Food Chem.* **2001**, *73*, 73−84.
(154) De Caro, M.; Galamini Ligori, M. Attivita antibiotica di un estratto desunto dalle acque di vegetazione delle olive. *Rend. Ist. Super. Sanita* **1959**, *22*, 233.
(155) Gonzalez, M. D.; Moreno, E.; Quevedo-Sarmiento, J.; Ramos-Cormenzana, A. Studies on antibacterial activity of wastewaters from olive oil mills (alpechin): Inhibitory activity of phenolic and fatty acids. *Chemosphere* **1990**, *20*, 423−432.
(156) Perez, J.; Delarubia, T.; Moreno, J.; Martinez, J. Phenolic content and antibacterial activity of olive oil wastewaters. *Environ. Toxicol. Chem.* **1992**, *11*, 489−495.
(157) Rodriguez, M. M.; Perez, J.; Ramos-Cormenzana, A.; Martinez, J. Effects of extracts obtained from olive oil mill wastewaters on Bacillus megaterium ATCC 33085. *J. Appl. Bacteriol.* **1988**, *64*, 219−226.
(158) Kubo, I.; Hanke, F. J. Chemically based resistance in plants. In *Chemically Mediated Interactions between Plants and Other Organisms*; Plenum: New York, 1985.
(159) Federici, F.; Bongi, G. Improved separation of bacterial inhibitors from oleuropein hydrolysis. *Appl. Environ. Microbiol.* **1983**, *46*, 509−510.
(160) Ruiz-Barba, J. L.; Gardio-Fernandez, A.; Jimenez-Diaz, R. Bactericidal action of oleuropein extracted from green olives against *Lactobacillus plantarum*. *Lett. Appl. Microbiol.* **1991**, *12*, 65−68.
(161) Capasso, R.; Evidente, A.; Trembaly, E.; Sala, A.; Santoro, C.; Cristinzio, G.; Scognamiglio, F. Direct and mediated effects on *Bactrocera oleae* (Gmelin) (Diptera: Tephritidae) on natural polyphenols and some of the related synthetic compounds: structure−activity relationships. *J. Chem. Ecol.* **1994**, *20*, 1189−1199.
(162) Scalzo, R. L.; Scarpati, M. L.; Verzegnassi, B.; Vita, G. *Olea europaea* chemicals repellent to *Dacus oleae* females. *J. Chem. Ecol.* **1994**, *20*, 1813−1823.
(163) Alliotta, G.; Fiorentino, A.; Oliva, A.; Tesmussi, F. Olive oil mill wastewater: isolation of polyphenols and their phytotoxicity in vitro. *Allelopathy J.* **2002**, *9*, 9−17.
(164) Ma, S. C.; He, Z. D.; Deng, X. L.; But, P. P. H.; Ooi, V. E. C.; Xu, H. X.; Lee, S. H. S.; Lee, S. F. In vitro evaluation of secoiridoid glucosides from the fruits of *Ligustrum lucidum* as antiviral agents. *Chem. Pharm. Bull.* **2001**, *49*, 1471−1473.
(165) Crea, R. Method of Obtaining a Hydroxytyrosol-Rich Composition from Vegetation Water. U.S. Patent 6,416,808, 2002.
(166) Cuomo, J.; Rabovskiy, A. B. Antioxidant compositions extracted from olives and olive by-products. U.S. Patent 6,358,542, 2002.
(167) Erkoc, F.; Keskin, N.; Erkoc, S. Theoretical investigation of hydroxytyrosol and its radicals. *J. Mol. Struct.−THEOCHEM* **2003**, *625*, 87−94.
(168) Vogna, D.; Pezzella, A.; Panzella, L.; Napolitano, A.; D'ischia, M. Oxidative chemistry of hydroxytyrosol: Isolation and characterization of novel methanooxocinobenzodioxinone derivatives. *Tetrahedron Lett.* **2003**, *44*, 8289−8292.
(169) D'angelo, S.; Manna, C.; Migliardi, V.; Mazzoni, O.; Morrica, P.; Capasso, G.; Pontoni, G.; Galletti, P.; Zappia, V. Pharmacokinetics and metabolism of hydroxytyrosol, a natural antioxidant from olive oil. *Drug Metab. Dispos.* **2001**, *29*, 1492−1498.
(170) Miro-Casas, E.; Covas, M. I.; Farre, M.; Fito, M.; Ortuno, J.; Weinbrenner, T.; Roset, P.; De La Torre, R. Hydroxytyrosol disposition in humans. *Clin. Chem.* **2003**, *49*, 945−952.
(171) Miro-Casas, E.; Covas, M. I.; Fito, M.; Farre-Albadalejo, M.; Marrugat, J.; De La Torre, R. Tyrosol and hydroxytyrosol are absorbed from moderate and sustained doses of virgin olive oil in humans. *Eur. J. Clin. Nutr.* **2003**, *57*, 186−190.
(172) Visioli, F.; Galli, C.; Grande, S.; Colonnelli, K.; Patelli, C.; Galli, G.; Caruso, D. Hydroxytyrosol excretion differs between rats and humans and depends on the vehicle of administration. *J. Nutr.* **2003**, *133*, 2612−2615.
(173) Waterman, P. G.; Mole, S. *Analysis of Phenolic Plant Metabolites*, 1st ed.; Blackwell Scientific Publications: Oxford, U.K., 1994.
(174) Cardoso, S. M.; Guyot, S.; Marnet, N.; Lopes-da-Silva, J. A.; Renard, C. M. G. C.; Coimbra, M. A. Characterization of phenolic extracts from olive pulp and olive pomace by electrospray mass spectrometry. *J. Sci. Food Agric.* **2005**, Web page.
(175) Robards, K. Strategies for the determination of bioactive phenols in plants, fruit and vegetables. *J. Chromatogr. A* **2003**, *1000*, 657−691.
(176) Shahidi, F.; Naczk, M. *Food Phenolics: Sources, Chemistry, Effects, Applications*; Technomic Publishing: Lancaster, PA, 1995.
(177) Gamel, T. H.; Kiritsakis, A. Effect of methanol extracts of rosemary and olive vegetable water on the stability of olive oil and sunflower oil. *Grasas Aceites* **1999**, *50*, 345−350.
(178) Capasso, R.; Evidente, A.; Scognamiglio, F. A simple thin layer chromatographic method to detect the main polyphenols occurring in olive oil vegetation water. *Phytochem. Anal.* **1992**, *3*, 270−275.
(179) Sampedro, I.; Romero, C.; Ocampo, J. A.; Brenes, M.; Garcia, I. Removal of monomeric phenols in dry mill olive residue by saprobic fungi. *J. Agric. Food Chem.* **2004**, *52*, 4487−4492.
(180) Brenes-Balbuena, M.; García-García, P.; Garrido-Fernandez, A. Phenolic compounds related to the black color formed during the procesing of ripe olives. *J. Agric. Food Chem.* **1992**, *40*, 1192−1196.
(181) Amiot, M.-J.; Fleuriet, A.; Macheix, J.-J. Importance and evaluation of phenolic compounds in olive during growth and maturation. *J. Agric. Food Chem.* **1986**, *34*, 823−826.
(182) Romani, A.; Mulinacci, N.; Pinelli, P.; Vincieri, F. F.; Cimato, A. Polyphenolic content in five Tuscany cultivars of *Olea europaea* L. *J. Agric. Food Chem.* **1999**, *47*, 964−967.
(183) Nuutila, A. M.; Kammiovirta, K.; Oksman-Caldentey, K. M. Comparison of methods for the hydrolysis of flavonoids and phenolic acids from onion and spinach for HPLC analysis. *Food Chem.* **2002**, *76*, 519−525.
(184) Castro Mejias, R.; Natera Marin, R.; de Valme Garcia Moreno, M.; Garcia Barroso, C. Optimization of headspace solid-phase microextraction for the analysis of volatile phenols in wine. *J. Chromatogr. A* **2003**, *995*, 11−20.
(185) Escarpa, A.; Gonzalez, M. C. Approach to the content of total extractable phenolic compounds from different food samples by comparison of chromatographic and spectrophotometric methods. *Anal. Chim. Acta* **2001**, *427*, 119−127.
(186) Caruso, D.; Colombo, R.; Patelli, R.; Giavarini, F.; Galli, G. Rapid evaluation of phenolic component profile and analysis of oleuropein aglycon in olive oil by atmospheric pressure chemical ionization-mass spectrometry (APCI-MS). *J. Agric. Food Chem.* **2000**, *48*, 1182−1185.

(187) Ragazzi, E.; Veronese, G. Quantitative analysis of phenolic compounds after thin-layer chromatographic separation. *J. Chromatogr.* **1973**, *77*, 369−375.

(188) Marston, A.; Hostettmann, K. Biological and chemical evaluation of plant extracts and subsequent isolation startegy. In *Bioassay Methods in Natural Product Research and Drug Development*; Bohlin, L., Bruhn, J. G., Eds.; Kluwer Academic Publications: Dordrecht, The Netherlands, 1999.

(189) Vial, J.; Hennion, M. C.; Fernandez-Alba, A.; Aguera, A. Use of porous graphitic carbon coupled with mass detection for the analysis of polar phenolic compounds by liquid chromatography. *J. Chromatogr. A* **2001**, *937*, 21−29.

(190) Capasso, R. A review on the electron ionisation and fast atom bombardment mass spectrometry of polyphenols naturally occurring in olive wastes and some of their synthetic derivatives. *Phytochem. Anal.* **1999**, *10*, 299−306.

(191) Aramendía, M. A.; Boráu, V.; García, I.; Jiménez, C.; Lafont, F.; Marinas, J. M.; Urbano, F. J. Qualitative and quantitative analyses of phenolic compounds by high-performance liquid chromatography and detection with atmospheric pressure chemical ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* **1996**, *10*, 1585−1590.

(192) Ryan, D.; Lawrence, H.; Prenzler, P. D.; Antolovich, M.; Robards, K. Recovery of phenolic compounds from *Olea europaea*. *Anal. Chim. Acta* **2001**, *445*, 67−77.

(193) Bicchi, C.; Binello, A.; Rubiolo, P. Determination of phenolic diterpene antioxidants in rosemary (*Rosmarinus officinalis* L.) with different methods of extraction and analysis. *Phytochem. Anal.* **2000**, *11*, 236−242.

(194) Skoog, D. A.; Holler, F. J.; Nieman, T. A. High-performance liquid chromatography. In *Principles of Instrumental Analysis*; Skoog, D. A., Holler, F. J., Nieman, T. A., Eds.; Harcourt Brace: Philadelphia, PA,1998.

(195) Bocchi, C.; Careri, M.; Groppi, F.; Mangia, A.; Manini, P.; Mori, G. Comparative investigation of UV, electrochemical and particle beam mass spectrometric detection for the high-performance liquid chromatographic determination of benzoic and cinnamic acids and of their corresponding phenolic acids. *J. Chromatogr. A* **1996**, *753*, 157−170.

(196) Brenes, M.; Garcia, A.; Garcia, P.; Garrido, A. Rapid and complete extraction of phenols from olive oil and determination by means of a coulometric electrode array system. *J. Agric. Food Chem.* **2000**, *48*, 5178−5183.

(197) Antolovich, M.; Prenzler, P.; Robards, K.; Ryan, D. Sample preparation in the determination of phenolic compounds in fruits. *Analyst* **2000**, *125*, 989−1009.

(198) Skoog, D. A.; Holler, F. J.; Nieman, T. A. Capillary electrophoresis and capillary electrochromatography. In *Principles of Instrumental Snalysis*; Skoog, D. A., Holler, F. J., Nieman, T. A., Eds.; Harcourt Brace: Philadelphia, PA,1998.

(199) Lafont, F.; Aramendia, M. A.; García, I.; Borau, V.; Jiménez, C.; Marinas, J. M.; Urbano, F. J. Analyses of phenolic compounds by capillary electrophoresis electrospray mass spectrometry. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 562−567.

(200) Pomponio, R.; Gotti, R.; Hudaib, M.; Cavrini, V. Analysis of phenolic acids by micellar electrokinetic chromatography: application to *Echinacea purpurea* plant extracts. *J. Chromatogr. A* **2002**, *945*, 239−247.

Received for review August 29, 2004. Revised manuscript received November 21, 2004. Accepted November 23, 2004. RIRDC and Charles Sturt University are acknowledged for funding this work.

JF048569X