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Valorization of Grape (*Vitis vinifera*) Byproducts. Antioxidant and Biological Properties of Polyphenolic Fractions Differing in Procyanidin Composition and Flavonol Content

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Many byproducts and wastes generated by agroindustries contain polyphenols with potential application as food antioxidants and preventive agents against skin cancer and other diseases. The performance of polyphenolic fractions from Parellada grape (*Vitis vinifera*) pomace as antioxidants in different physicochemical environments was tested. Fractions containing oligomers with mean degree of polymerization between 3 and 4 and percentage galloylation ca. 30% were the most potent free radical scavengers and efficient antioxidants in an oil-in-water emulsion. A fraction including glycosylated flavonols was also efficient in the emulsion. All the fractions showed low aquatic toxicity and weak influence on proliferation of human melanoma cells.

KEYWORDS: ***Vitis vinifera;*** **polyphenols; procyanidins; catechins; flavonols; antioxidants; free radical scavenging activity; emulsions; ecotoxicity; cell proliferation**

INTRODUCTION

Agricultural byproducts contain a variety of biologically active species which mostly go to waste. Particularly, most plant-derived materials are rich in antioxidant polyphenols. An estimated 13% by weight of the grapes processed by the wine industry ends up as byproduct after pressing. This grape pomace, consisting of skins, seeds, and stems, is a rich source of polyphenols. These include catechins, namely monomeric and oligomeric flavan-3-ols (proanthocyanidins), and glycosylated flavonols (**Figure 1**). Catechins, as well as other polyphenols, are potent free radical scavengers (*1*, *2*). Since many undesired oxidation processes, including food decay and a variety of diseases, involve free radicals, polyphenols from agricultural byproducts are potential added value products with application as natural food additives and disease chemopreventive agents.

The widely used food antioxidants BHT, BHA, TBHQ, and alkyl gallates are synthetic compounds of phenolic nature (*3*, *4*). Consumer concerns and government regulations are fostering the research on alternative ingredients, mostly natural polyphenols, as presumably safer food antioxidants with added beneficial functional properties (*5*). Since being natural is not necessarily equivalent to being safe, much more research on the biological effects of plant extracts and fractions is needed.

Apart from their free radical scavenging capacity, some catechins are inhibitors of key enzymes involved in the cell cycle (*6*−*8*). They also induce apoptosis in different cell lines (*9*−*11*) and inhibit the expression of certain tumor-related genes (*12*, *13*). All these activities make catechins candidates for preventive agents against cancer, cardiovascular diseases, and premature aging (*5*, *14*−*17*).

The size and composition of oligomeric proanthocyanidins appear to be related to their antioxidant activity, through differences in both the intrinsic scavenging capacity and the physicochemical properties governing their partition behavior within biological environments (*18*, *19*). Moreover, structural features such as the presence of gallate esters appear to be crucial for the cell-cycle-related enzyme inhibition activity of catechins (*6*, *20*). In food systems, partition phenomena also play an important part in the antioxidant preservative action of phenolic compounds (*21*, *22*).

We are primarily interested in possible applications of plant proanthocyanidin fractions in the fields of food preservation and skin protection. Polyphenolic mixtures have already been proposed as food antioxidants (*4*, *23*, *24*) and preventive agents against skin irritation and cancer (*25*, *26*). From an original white

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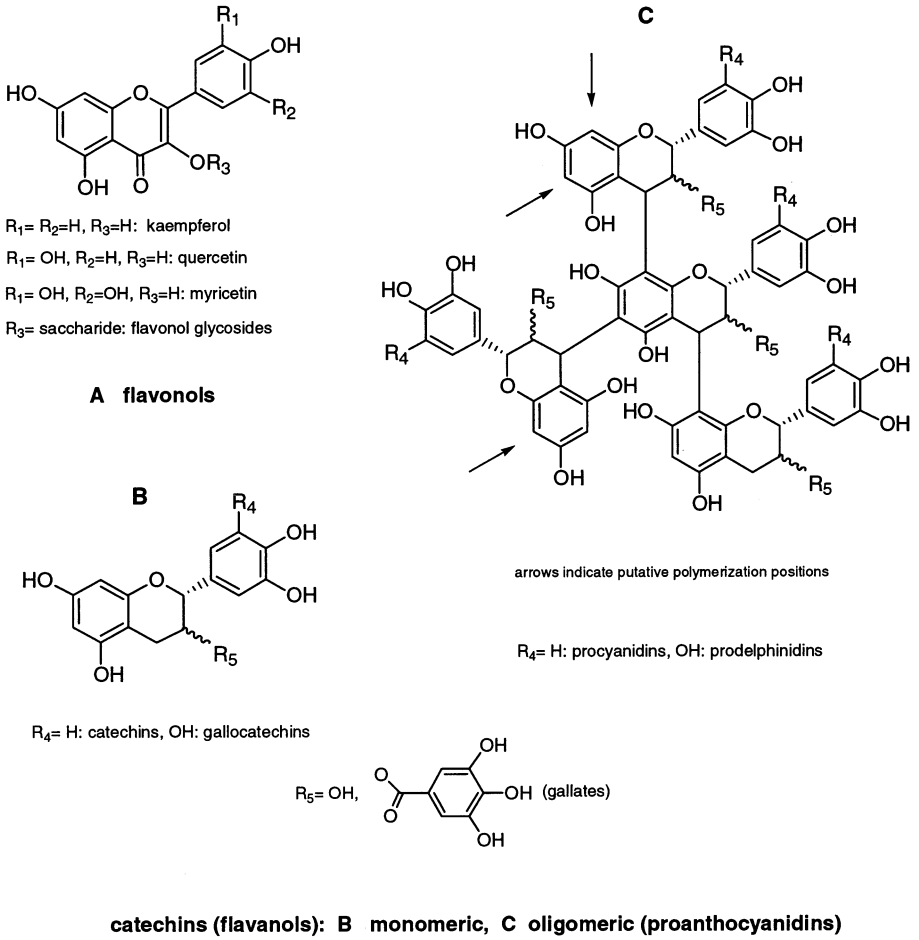

Figure 1. Structures of the major polyphenols found in white grape pomace.

grape pomace extract, we have generated fractions with different contents of monomeric catechins, oligomeric procyanidins, and glycosylated flavonols. The fractions have been evaluated using different assays pertinent to their putative applications: free radical scavenging, inhibition of lipid peroxidation of oil and oil-in-water emulsion, ecotoxicity, and antiproliferation on a melanoma cell line.

MATERIALS AND METHODS

Materials. The starting material, provided by Miguel Torres S.A. (Vilafranca del Penedès, Spain), was the byproduct from pressing destemmed Parellada grapes (*Vitis vinifera*) and consisted of skins, seeds, and a small amount of stems. This byproduct was collected in the month of October during the 1998 harvest, cooled immediately after pressing, and frozen. The polyphenolic fraction **OW**, soluble in both ethyl acetate and water, as well as preparative RP-HPLC-derived fractions **I** and **II** were obtained as described before (*27*, *28*). The following water and solvents were used: deionized water, analytical-grade MeOH (Panreac, Montcada i Reixac, Spain), and analytical-grade acetone (Carlo Erba, Milano, Italy) for semipreparative chromatography; Milli-Q water and HPLC-grade CH_3CN (E. Merck, Darmstadt, Germany) for analytical RP-HPLC; and analytical-grade MeOH (Panreac) for the DPPH assay. Trifluoroacetic acid (TFA, Fluorochem, Derbyshire, UK), biotech grade, was distilled in-house. 1,1-Diphenyl-2-picrylhydrazyl free radical (DPPH) (95%) was from Aldrich (Gillingham-Dorset, UK), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) (97%) from Aldrich (Milwaukee, WI), and (−)-epicatechin from Sigma Chemical (St. Louis, MO). Grapeseed oil was obtained from Borges Pont (Tàrrega, Spain) and corn oil stripped of tocopherols from Acros Organics (Fair Lawn, NJ). Soybean lecithin (L-α-phosphatidylcholine) was from Sigma Chemical. Dulbecco modified Eagle's medium (DMEM) and Dulbecco's phosphate-buffered saline were from Gibco-BRL (Eggenstein, Germany), fetal calf serum (FCS) from Invitrogen (Carlsbad, CA), and trypsin−EDTA solution C (0.05% trypsin−0.02% EDTA) from Biological Industries (Kibbutz Beit Haemet, Israel). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was from Sigma Chemical.

Chromatography. Toyopearl TSK HW-40F (TosoHass, Tokyo, Japan), 32−63 μm particle size stationary phase, was packed into a flash chromatography type 240-mm × 25-mm-i.d. glass column and equilibrated with MeOH. Fractions **OW**, **I**, and **II** (6 mL of MeOH solution, 600 mg, two runs × 3 mL) were separately fractionated in two steps, MeOH (250 mL) and water/acetone (2:3) (250 mL) following elution protocols described in the literature (*29*, *30*). The flow rate was 12 mL/min (MeOH) and 3 mL/min (water/acetone). The solvent was then evaporated under vacuum, and the residues were dissolved in Milli-Q water (100 mL) and lyophilized to give slightly colored fluffy solids (from fraction **OW**, 293 mg **III** and 242 mg **IV**; from fraction **I**, 218 mg **V** and 178 mg **VI**; and from fraction **II**, 85 mg **VII** and 305 mg **VIII**). Alternatively, **OW** (four runs × 300 mg) was more extensively fractionated with MeOH and water/acetone (1:1) to generate a fraction of monomers, essentially equal to **III**, 100 mg fraction **IX**, 28 mg fraction **X**, and 243 mg fraction **XI**. The fractions were analyzed by RP-HPLC on a Smart System (Amersham-Pharmacia Biotech, Uppsala, Sweden) equipped with a μ Peak Monitor (Amersham-Pharmacia Biotech) and fitted with a μRPC C2/C18 SC 2.1/10 (100 mm × 2.1 mm i.d.) column (Amersham-Pharmacia Biotech). Elution: [A] 0.10% (v/v) aqueous TFA, [B] 0.08% (v/v) TFA in water/CH_3CN 1:4, gradient 0 to 50% [B] over 38 min; flow rate, 200 μL/min; detection by triple-wavelength 214, 280, and 320 nm. Analytical runs were also performed at 365 nm to detect glycosylated flavonols.

Thiolysis with Cysteamine and RP-HPLC. The size and composition of the procyanidins within the fractions were estimated from the HPLC analysis of the depolymerized fractions, essentially as described (*31*, *32*), except that cysteamine was used instead of toluene-α-thiol

(*33*). Briefly, the terminal flavan-3-ol units were released as such by acid cleavage in the presence of cysteamine, whereas the extension moieties were released as the cysteamine derivatives on the fourth position of the flavanoid system. The resulting mixtures were submitted to analytical RP-HPLC (μRPC column, gradient 8−18% [B] over 30 min), and the molar amount (nanomoles) of all the released moieties was calculated from the peak areas and calibration curves obtained with pure samples:

$$\text{mean degree of polymerization (mDP)} = \frac{\text{total nmol}}{\text{nmol terminal units}}$$

$$\text{mean molecular weight (mMW)} = \text{total mass/nmol terminal units}$$

$$\%\ \text{galloylation} = 100 \times \frac{[\text{nmol of (}-\text{)-epicatechin-gallate (EcG)} + \text{nmol of cysteamine-EcG}]}{\text{total nmol}}$$

Free Radical Scavenging Activity. The antiradical activity of the fractions was evaluated by the DPPH method (*34*, *35*). The samples (0.1 mL) were added to aliquots (3.9 mL) of a solution made up with DPPH (4.8 mg) in MeOH (200 mL), and the mixture was incubated for 1 h at room temperature. The initial concentration of DPPH, approximately 60 μM, was calculated for every experiment from a calibration curve made by measuring the absorbance at 515 nm of standard samples of DPPH at different concentrations. The equation of the curve was $Abs_{517\,nm} = 11345C_{DPPH}$, as determined by linear regression. The results were plotted as the degree of absorbance disappearance at 515 nm ($(1 - A/A_0) \times 100$) against the amount of sample divided by the initial concentration of DPPH. Each point was acquired in triplicate. A dose−response curve was obtained for every fraction. ED_{50} corresponds to either micrograms or micromoles of product able to consume half the amount of free radical divided by micromoles of initial DPPH. The molecular amounts (micromoles) of procyanidin mixtures were calculated with the mean molecular weights (mMW) estimated by thiolysis with cysteamine. The results are expressed as antiradical power (ARP), or $1/ED_{50}$.

Inhibition of Lipid Peroxidation in Oil by Differential Scanning Calorimetry (DSC). A Mettler Toledo (Greifensee, Switzerland) DSC 20 differential scanning calorimeter, calibrated with indium, was used. The samples were prepared essentially as described (*36*). Briefly, controls or fractions (ca. 1 mg) were dissolved in acetone (100 μL), and grapeseed oil (Borges Pont S.A., Tàrrega, Spain) (500 μL) was added. Acetone was then eliminated under vacuum, and the samples were immediately subjected to DSC analysis. The oil samples were weighed on an aluminum pan which was placed on the DSC cell without sealing. The measurements were performed isothermically at 150 °C with an oxygen flow rate of 200 mL/min. The onset point was calculated as the time when the exothermic peroxidation process was started, as recorded from a sudden enthalpy variation.

Inhibition of Lipid Peroxidation in an Oil-in-Water Emulsion. Lipid peroxidation in corn oil emulsion was monitored by measuring UV absorbance at 234 nm, corresponding to the formation of conjugated dienes upon air oxidation (*21*, *37*−*39*). The emulsion was formed essentially as described (*40*). A mixture of corn oil stripped of natural antioxidants (10% w/w) and soybean lecithin (1% w/w) in 25 mM potassium phosphate pH 5 buffer was mixed with an Ultra-turrax T25 (Ika-Labortechnik, Staufen, Germany) at 9000 rpm for 2−3 min until the complete emulsification. The emulsion was then homogenized with a Microfluidics Corp. (Newton, MA) 110L high-pressure homogenizer in six cycles at 300 bar. Polyphenolic fractions dissolved in buffer (100 μL) were added to the emulsion (10 mL) to obtain initial concentrations ranging from 1 to 20 μg/mL. The samples (2.5 mL) were placed into 10-mL screw-capped test tubes and oxidized at 50 °C in a shaker bath for 4 days. Every day, aliquots (50 μL) were taken and dissolved in MeOH (10 mL) to obtain absorbance values in the linear range, and UV absorbance at 234 nm was recorded as a measure of the formation of conjugated diene hydroperoxides. The lipid peroxidation was calculated as millimoles of hydroperoxydes per kilogram of oil using an absorptivity of 26 000 for linoleate hydroperoxydes (*41*) and plotted against oxidation time. The experiments were done in triplicate.

Aquatic Toxicity Assay. A biosensor-based measurement system, Microtox test system (Azur Environmental, Carlsbad, CA), was used for determining the potential toxicity of the different fractions in aqueous solution. Microtox (DIN 38412-34) (*42*) employs a marine luminescent bacteria, specifically the strain *Vibrio fischeri* NRRL B-11177 (Azur Environmental), to measure toxicity from aqueous samples. These bacteria liberate energy in the form of visible light (intensity maximum at 490 nm) as a consequence of the series of metabolic reactions. On exposure to toxic substances, the light output is reduced, and this reduction is proportional to the toxicity of the sample. The concentration of an aqueous solution of a chemical that causes a 50% reduction of the light emitted by the bacteria (EC_{50}) is calculated from a concentration−response curve by regression analysis. Toxicity data obtained in the present work were based on a 30-min exposure of bacteria to the sample solution at 15 °C.

Proliferation Assay on SK-Mel-28 Human Melanoma Cell Line. SK-MEL-28 adherent cells (ATCC HTB-72) were grown in DMEM supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), in the presence of 0.1% (v/v) antibiotics (10 000 U/mL penicillin, 10 000 μg/mL streptomycin), at 37 °C in a humidified environment with 5% CO_2. The cells were split (ratio 1:2 to 1:5) by mild trypsinization every 4−5 days, and the medium was changed every 2−3 days. The cell culture used in this study was free of mycoplasm infection, as shown by the EZ-PCR Mycoplasm test kit (Biological Industries) prior to the treatment with the samples.

Cell growth was determined using the Mosmann assay (*43*) with some modifications. Cells were seeded into 96-well plates at 2×10^4 cells/mL density, 200 μL/well, and incubated for 24 h in the culture medium prior to addition of the samples dissolved in DMEM. Control wells were treated with an equal volume of DMEM as the test cultures. After 72 h of culture, the supernatant was aspirated, and 100 μL of sterile filtered MTT (0.5 mg/mL in DMEM) was added to each well. The plates were incubated at 37 °C, 5% CO_2 for 1 h. The supernatant was removed, and the blue MTT formazan that precipitated was dissolved in DMSO (100 μL) and its optical density (OD) measured at 550 nm on a multiwell reader (Merck ELISA System MIOS).

The inhibitory effect of the fractions at each concentration was expressed as a percentage [(mean OD of treated cells after 72 h of incubation with the product/mean OD of control cells after 72 h of incubation with extra medium instead of product) × 100]. The IC_{50}, or sample concentration causing a 50% reduction in the mean OD value relative to the control at 72 h of incubation, was estimated using GraFit 3.00 (Data Analysis and Graphics Program, Erithacus Software Ltd., Microsoft Corp., Surrey, UK), curve option IC_{50} curve start at 0.

RESULTS AND DISCUSSION

Characterization of the Fractions and Free Radical Scavenging Activity. The total extract **OW** contained mainly catechins, both monomeric and oligomeric, and glycosylated flavonols. From this mixture, a set of fractions differing in composition and procyanidin structure has been generated using a combination of chromatographic techniques (**Scheme 1**). RP-HPLC discriminates among solutes by hydrophobicity, while Toyopearl HW-40 has been shown to separate flavonoids in order of increasing size (*29*, *44*) by the combined action of gel permeation and adsorption phenomena. The gallate esters of the procyanidin dimers elute together with bulkier oligomers (*29*). From a total extract **OW**, soluble in both ethyl acetate and water, which was obtained by liquid−liquid extraction (*27*, *28*), we have generated fractions containing only catechin or glycosylated flavonol monomers (**V** and **VII**, respectively), procyanidin dimers with low gallate content (**IX**), procyanidin oligomers with variable galloylation (**IV**, **VI**, **VII**, **X**, **XI**), and different combinations of monomers and oligomers (**I**, **II**, **III**). **Table 1** summarizes the qualitative composition of the fractions and their corresponding antiradical power by the DPPH assay. Procyanidin size and composition were estimated by thiolysis with cysteamine, and glycosylated flavonols were detected by analytical RP-HPLC at 365 nm.

Scheme 1

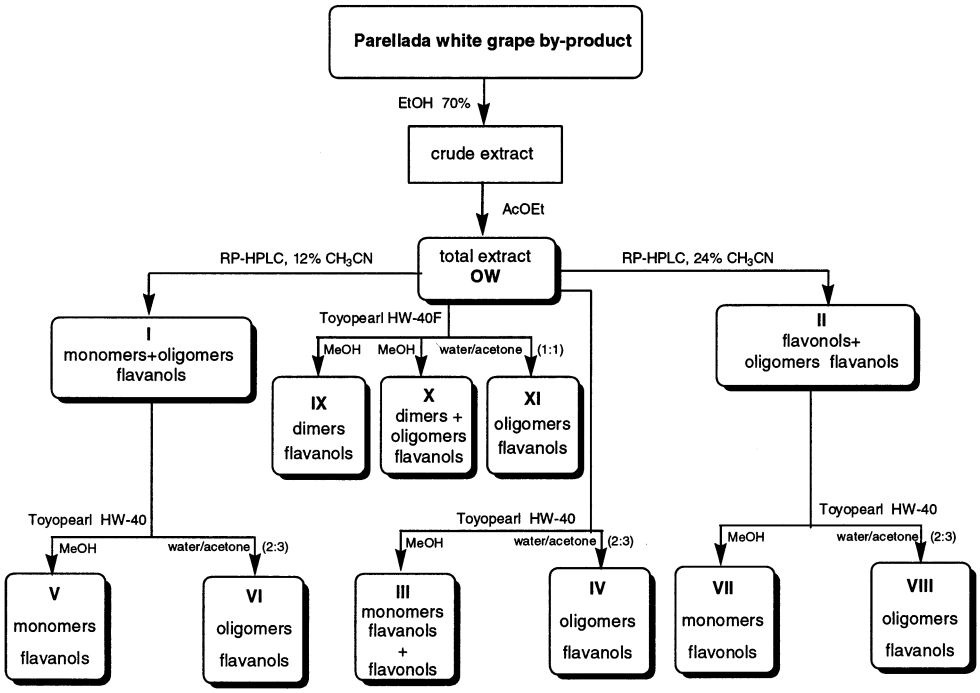

Table 1. Size, Composition,[a] and Antiradical Power[b] of Polyphenolic Fractions from Parellada White Grape Pomace

fractions	mDP	mMW	galloylation (%)	ARP[c] ($1/ED_{50}$) $\times 10^3$	molar ARP[d] ($1/ED_{50}$)
OW	1.7	552	15	19	–
I	1.4	422	7	26	11
II	3.0	1005	31	22	–
III	1.0[e]	–	<1	10	–
IV	2.7	880	25	27	24
V	1.0[e]	290	<1	19	6
VI	2.4	751	16	24	18
VII	1.0[e]	–	0	7	–
VIII	3.4	1160	34	27	31
IX	2.0	624	12	23	14
X	2.2	759	30	30	22
XI	3.7	1232	31	28	35

controls	DP	MW	galloylation	ARP[c] ($1/ED_{50}$) $\times 10^3$	molar ARP ($1/ED_{50}$)
(–)-epicatechin	1.0	290	0	21	6
Trolox	1.0	250	–	16	4

[a] Mean of two independent thiolysis experiments with RP-HPLC replicate injections. [b] Mean of three experiments. [c] ED_{50} in microgram fraction or control/micromoles DPPH. [d] ED_{50} in estimated micromoles fraction/micromoles DPPH only for procyanidin fractions devoid of flavonols. [e] Not calculated.

The DPPH assay provides information about the intrinsic free radical scavenging power in solution irrespective of the physicochemical environments encountered in biological systems (*35*, *45*, *46*). The antiradical power (ARP) is expressed in relation to both mass (micrograms) and molecular amount (micromoles). The former provides an idea of the weighed amount of fraction needed to exert a given antiradical effect, and the latter carries information on the efficiency of the procyanidin components of that fraction. There was a correlation between procyanidin mean degree of polymerization and antiradical power for the fractions devoid of flavonols. At equal galloylation (**VIII**, **X**, **XI**), molar ARP was roughly proportional to mDP. The results also indicate that the more galloylated a fraction, the higher its ARP was. This is consistent with the fact that the pyrogallol moiety provides more hydrogen atoms or electrons than the catechol group, as proven under different experimental setups (*47*−*49*). Glycosylated flavonols (fraction **VII**) were less efficient than the non-galloylated monomeric flavanols (**V**, mostly (+)-catechin). The presence of glycosylated flavonols, which are less efficient scavengers than the aglycons (*1*, *50*), lowered the overall antiradical power of fractions **OW**, **II**, and **III**. All the fractions, except those consisting of glycosylated flavonols (**VII**) or a mixture of glycosylated flavonols and flavanol monomers (**III**), were more effective than Trolox. In conclusion, the highest antiradical power corresponded to the mixtures of compounds with the highest degree of polymerization and galloylation and no glycosylated flavonols.

Inhibition of Grapeseed Oil Peroxidation. The polyphenolic fractions were assayed for their ability to inhibit grapeseed oil peroxidation using differential scanning calorimetry (DSC) in the isothermal mode. DSC measurements on oil peroxidation correlate with the results obtained by other procedures, such as Rancimat (*51*), and have been used to test the antioxidant protection exerted by natural and synthetic compounds (*52*, *53*). α-Tocopherol and Trolox were used as controls. To set up the working conditions for testing the fractions in less than 60 min, peroxidation was triggered at different temperatures between 135 and 175 °C in the presence and in the absence of Trolox. At 150 °C, the onset time for grapeseed oil peroxidation was ca. 6 and ca. 50 min in the presence and in the absence of Trolox (2 mg/mL), respectively. Thermogravimetric analysis showed that the tested polyphenols (fraction **OW**) were stable at this temperature in the presence of oxygen. The protective performance of α-Tocopherol, Trolox, and the polyphenolic fractions was then tested by DSC at 150 °C. α-Tocopherol exerted weak protection against peroxidation, whereas Trolox retarded the process by ca. 50 min (**Figure 2**). These results are in agreement with those obtained by measuring the initiation of oil peroxidation by UV spectrometry at 234 nm (*21*, *38*). Frankel and colleagues suggested that the less lipophilic Trolox might better protect lipids against oxidation by being located at oil−air

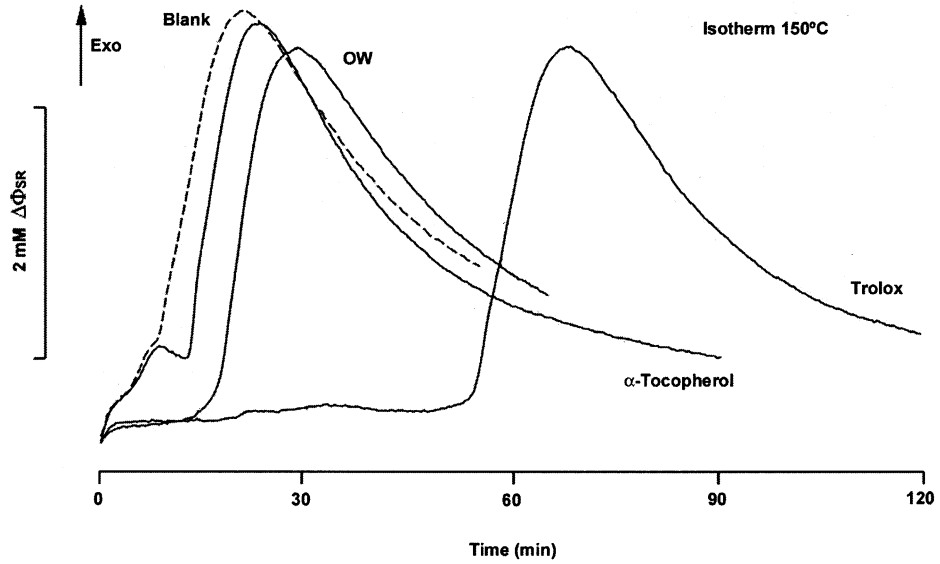

Figure 2. DSC curves of grapeseed oil peroxidation. Φ_{SR}, heat flow rate. Concentrations: Trolox and **OW**, 2 mg/mL; α-tocopherol, 4 mg/mL.

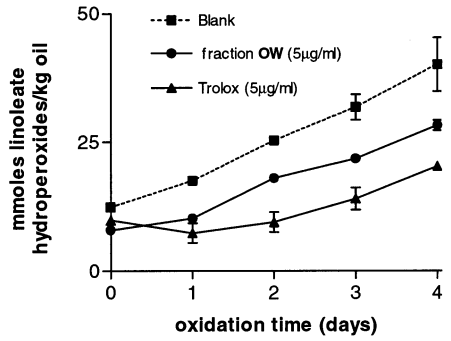

Figure 3. Inhibition of corn oil oxidation in an oil-in-water emulsion. Comparison between extract fraction **OW** and Trolox. Bars represent SEM (standard error of the mean) confidence limits.

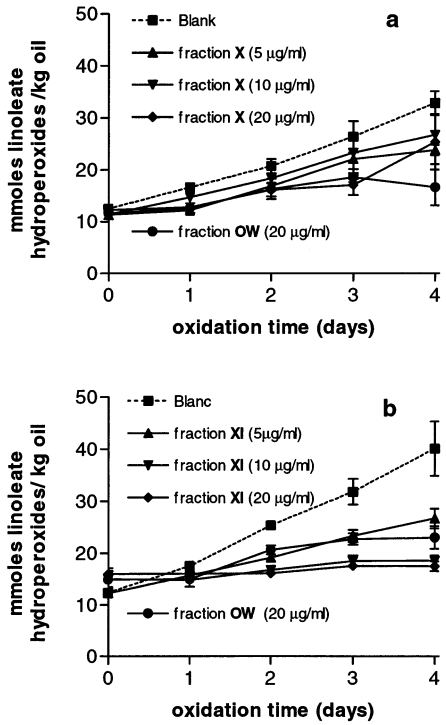

Figure 4. Inhibition of corn oil oxidation in an oil-in-water emulsion. Comparison between fractions with equal galloylation and different degree of polymerization. Bars represent SEM (standard error of the mean) confidence limits.

interfaces or at the oil surface. All the fractions generated in this work (**Scheme 1**) were tested and were less efficient than Trolox. The total extract **OW** as well as the fractions containing monomers and/or dimers showed some protective action (**Figure 2**), whereas fractions with higher degree of polymerization were completely inactive. The reason for these results might be that grape oligomeric procyanidins are poorly soluble in the oil triglycerides. In fact, they formed hazy mixtures. Curiously, trimers and bulkier oligomers are able to establish more hydrophobic interactions than monomers and dimers in certain nonpolar environments, as suggested by their higher retention on reversed-phase HPLC. This does not seem to be the case with oils. In any case, polyphenolic fractions from the grape byproduct showed low capacity to protect oil from oxidation.

Inhibition of Lipid Peroxidation in an Oil-in-Water Emulsion. Since most food and living systems are colloids rather than solutions in solvents of a given polarity, assays in models with interfaces (e.g., lipid−water) must be part of the evaluation of antioxidants (*54*). The antioxidant performance of the fractions and Trolox has been tested in an oil-in-water emulsion made up with corn oil stripped of natural antioxidants under the conditions described in the Materials and Methods. **Figure 3** compares the efficiency of the total extract **OW** with Trolox. **OW**, (−)-epicatechin, and most of the fractions were less efficient than Trolox as antioxidants in the emulsion. Since Trolox is less water soluble than **OW (**soluble in both ethyl acetate and water) and its fractions, the results are in agreement with the so-called polar paradox (*55*, *56*), i.e., the apparent contradiction that oil-soluble antioxidants perform better in emulsions than in oils and water-soluble antioxidants are better in oils than in emulsion systems. Interestingly, fractions **IV**, **VIII** (data not shown), and **XI** (**Figure 4b**), with mean degree of polymerization around 3, were very efficient in this system. Since these fractions are clearly more water soluble than Trolox, the results appear not to be in complete agreement with the polar paradox. If the protection against lipid peroxidation in oil-in-water emulsions depends on the tendency of an antioxidant molecule to be located at water−oil interfaces, as suggested by Frankel and co-workers (*21*, *22*, *54*, *57*), it could very well be that oligomeric polyphenols show such a phase behavior to some extent, regardless of their poor oil solubility. Bulky procyanidins might be able to establish hydrophobic and/or hydrophilic

Table 2. Acute Aquatic Toxicity of Polyphenolic Fractions from Parellada White Grape Pomace and Trolox

	toxicity (Microtox)		
fraction	EC_{50} (mg/mL)	EC_{50} (μM)	log(1/EC_{50} (μM))
OW	52	94	1.03
III	85	293	0.55
VIII	45	39	1.41
IX	65	105	0.98
X	60	81	1.09
Trolox	38	152	0.82

interactions, depending on the environment. They may be a peculiar kind of flexible molecules with hydrophilic hydroxyl groups linked to a hydrophobic core (aromatic rings) with a capacity to expose both or any of the two regions, resulting in surfactant-like oil−water interface active conformations.

Galloylation does not appear to be crucial for the efficacy of procyanidins in the oil emulsion. **Figure 4** shows that fraction **X**, with high galloylation (30%) and relatively low mDP (2.2), was less efficient than fraction **XI** (mDP 3.7), which is equally galloylated (31%).

Interestingly, glycosylated flavonols (fraction **VII**), which were less potent free radical scavengers than monomeric catechins (fraction **V**), were equally efficient in the emulsion. Fractions that included flavonols (**II**, **III**) were more efficient than expected from their ARPs in the DPPH assay. Again, these molecules may be favorably located at the oil−water interface.

Ecotoxicity. We have tested the sensitivity of marine luminescent bacteria toward selected polyphenolic fractions (**OW**, **III**, **VIII**, **IX, X**) as a first approximation to the study of their putative toxicity. The influence of the fractions on the bacterial metabolic activity is recorded as changes in light emission. Microtox is a quick, simple, and very reproducible test that presents good correlations with other bacteria and with fish (*58*). Acute toxicity of the fractions is summarized in **Table 2**. All the fractions tested showed low toxicity, similar to that of Trolox. When the values are expressed in molar concentration, the results suggest that some correlation exists between bacterial sensitivity and structure (mDP, galloylation) of the added procyanidins (**Figure 5**).

Proliferation of SK-Mel 28 Human Melanoma Cells. Selected fractions (**OW**, **III**, **IV**, **V**, **VI**, **VIII**) showing different procyanidin polymerization, galloylation, and flavonol content were assayed for their influence on the proliferation of melanoma cells. The fractions exerted a weak antiproliferative effect on this tumoral cell line. The IC_{50} values obtained ranged from 70 (**OW**) to 213 μg/mL (**VIII**). IC_{50} values expressed in molar concentrations showed that the fractions containing oligomeric procyanidins of different mean sizes were equally potent, with values between 124 and 184 μM, whereas the monomers (**V**) were clearly less efficient (655 μM). The results reported here are in overall agreement with those obtained with SK-MEL-1 and/or SK-MEL-28 cells after treatment with flavonoids of the flavonol and flavone type (*59*, *60*). All the polyphenols tested so far have proved to be less potent than the clinically used drug melphalan, which shows IC_{50} values lower than 10 μM (*59*).

In conclusion, fraction **OW**, which contains components that are soluble in both ethyl acetate and water, was a potent antiradical mixture which was also effective in an oil-in water emulsion and showed some activity in oil. The main components of this fraction are catechin monomers, procyanidin oligomers, and flavonol derivatives. Due to concerns about side effects attributed to high doses of catechin (*61*, *62*), the monomeric species are usually removed from polyphenolic preparations of grape origin (*63*, *64*). Fractions (**VIII**, **XI**) devoid of catechin monomers and containing oligomers of relatively high degree of polymerization (between 3 and 4) and galloylation (ca. 30%) were the most potent free radical scavengers and efficient antioxidants in the oil-in-water emulsion. Flavonol derivatives were also efficient in the emulsion, despite their low antiradical power. Fractions such as **II**, with high mDP and flavonol content, together with **VIII** and **XI,** appear to be efficient lipid protecting agents in emulsions and candidates for natural food antioxidants and skin protecting agents.

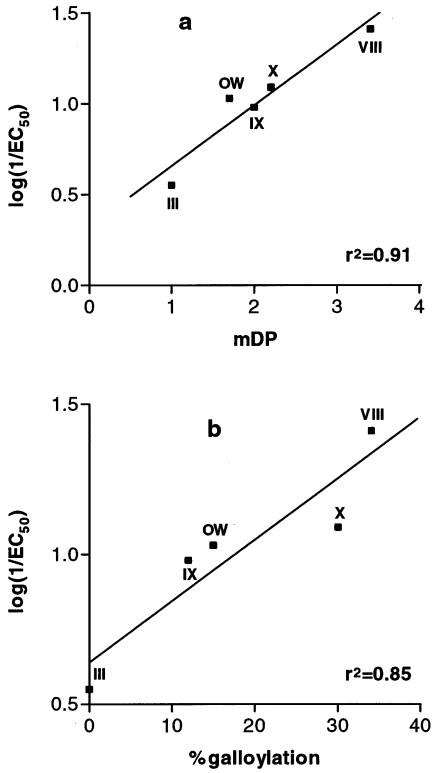

Figure 5. Correlation between sensitivity of luminescent bacteria toward polyphenolic fractions and mean degree of polymerization (a) or percentage galloylation (b).

Since galloylation did not seem to be necessary for lipid protection in the emulsion and gallates have been shown to influence intracellular events (*6*, *65*, *66*), mixtures with high mDP and low galloylation might be even better options than the fractions described here for products designed to offer protection by exclusively scavenging radicals.

ABBREVIATIONS USED

BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; DMEM, Dulbecco modified Eagle's medium; DPPH, 1,1-diphenyl-2-picrylhydrazyl free radical; FCS, fetal calf serum; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; RP-HPLC, reversed-phase high-performance liquid chromatography; TFA, trifluoroacetic acid; THBQ, *tert*-butylhydroquinone; Trolox, 2,5,7,8-tetramethyl-chroman-2-carboxylic acid.

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