New Flavanol Derivatives from Grape (*Vitis vinifera*) Byproducts. Antioxidant Aminoethylthio−Flavan-3-ol Conjugates from a Polymeric Waste Fraction Used as a Source of Flavanols

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A new family of antioxidants has been obtained from a residual fraction of polymeric polyphenols of grape origin. The integral exploitation of resources is important in any sustainable production scheme. Many byproducts and residues generated by the agroindustries contain polyphenols with potential application as preventative agents against cancer and cardiovascular diseases. Among these polyphenols oligomeric proanthocyanidins are particularly significant. The polymeric forms, considered of less interest because of their astringent properties, constitute the largest portion of the biologically active plant proanthocyanidins. The new compounds described here result from the breakdown of polymeric flavanols in the presence of cysteamine and bear an amino function, which facilitates their isolation from complex mixtures by cation-exchange gels or resins. In this way, valuable antioxidant molecules can be efficiently obtained from otherwise wasted polymers. The new molecules appear to be as effective as their underivatized counterparts (flavan-3-ols) as antioxidants.

Keywords: *Grape; Vitis vinifera; polyphenols; proanthocyanidins; catechins; flavan-3-ols; cysteamine; amines; cation exchange; isolation; antioxidants; antiradical activity*

INTRODUCTION

There is increasing public awareness of the fact that natural resources are limited and of the need to rationalize their exploitation. To lessen the current strong dependence on fossil reserves, efforts are being focused on the use of renewable sources of raw materials for the production of new chemicals and alternative fuels. Because even renewable sources might become insufficient in the future, another important trend related to the establishment of a sustainable production model is the integral exploitation of the raw materials. The recovery of high added-value chemicals from residues and byproducts is one such approach.

In agricultural areas, crops such as grapes and olives generate huge amounts of byproducts. In Europe alone ∼112 million tons of grape were processed by the wine industry in 1998. An estimated 13% (14.5 million tons) of this amount corresponded to the byproduct after pressing, consisting mainly of skins and seeds. Grape skins and seeds are rich sources of health-promoting polyphenols, including flavan-3-ols of different degrees of polymerization known as proanthocyanidins. Oligomeric proanthocyanidins, as well as other polyphenols, are potent free radical scavengers useful as preventative agents against cancer, cardiovascular diseases, and premature aging (*1*−*5*). Currently, a variety of health-promoting products obtained from grape byproducts are on the market, and a great deal of research effort is being devoted to testing the putative beneficial effects of tea, grape, and pine bark polyphenols (*2*, *4*, *6*). The products of grape origin include ground dried skins and extracts obtained from skins and/or seeds.

Both grape skins and seeds contain monomeric, oligomeric, and polymeric proanthocyanidins (Figure 1), the mean degree of polymerization being higher for skin flavanols (*7*, *8*). Grape seeds appear to contain only procyanidins (*8*), whereas the skins contain both procyanidins and prodelphinidins (*7*). Oligomeric (roughly two to seven residues) proanthocyanidins are generally considered to be more efficient than the monomers as antioxidants (*6*, *9*, *10*). Materials of higher degrees of polymerization are also active but may be mucosal irritants and show other undesired effects related to their ability to precipitate proteins (astringency) (*11*, *12*), which depends on the polymer kind and size (*13*). Highly polymerized flavanols are present in higher quantities than oligomers in vine (*7*, *8*, *14*, *15*) and other plants (*16*, *17*).

The degree of polymerization of flavanoid extracts is usually estimated by thiolysis using toluene-α-thiol (*18*). The depolymerized mixtures are analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC) (*7*, *8*, *19*, *20*). Using this method the mean degree of polymerization and the percentage of galloylation of proanthocyanidins from grape skins and seeds have been estimated (*7*, *8*). To the best of our knowledge, no other use has been described for the benzylthio derivatives generated. Some interesting amphiphilic derivatives of flavanols have been reported (*21*, *22*). The source of thiols was, in this case, 2-mercaptoethanol.

The present study has determined the recovery of new antioxidant products obtained by acidolysis of a poly-

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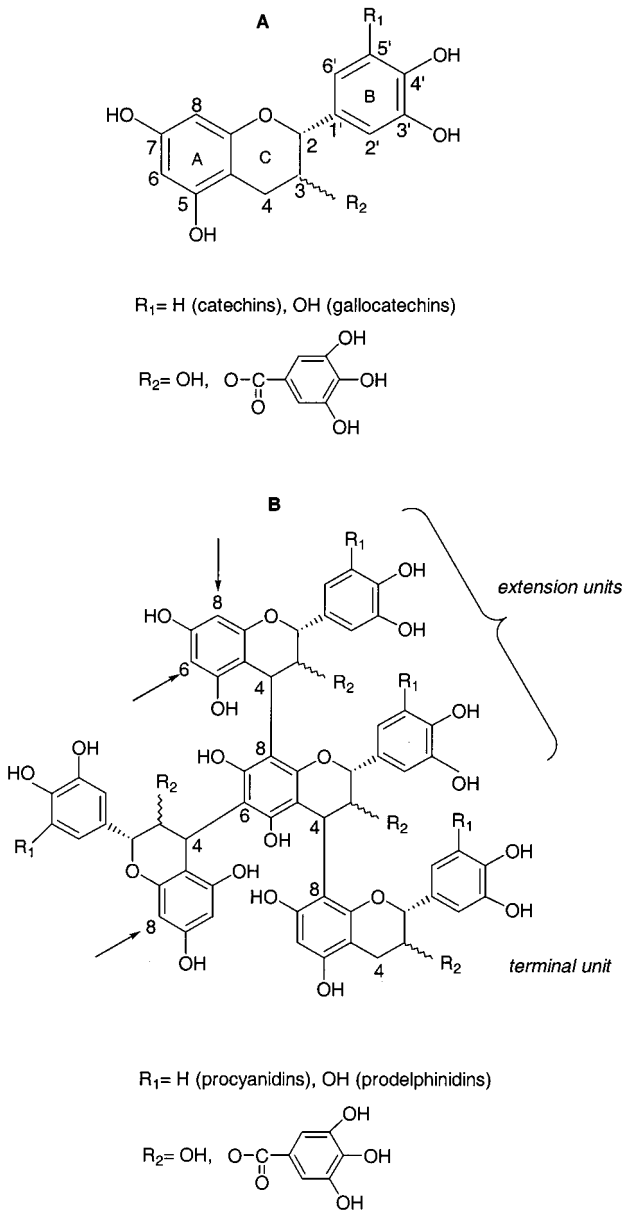


Figure 1. Structures of monomeric and polymeric flavan-3-ols: (A) monomeric flavan-3-ols; (B) oligomeric and polymeric proanthocyanidins. The arrows indicate putative polymerization positions.

meric proanthocyanidin fraction of white grape pomace in the presence of cysteamine. The use of cysteamine was considered to be attractive for two main reasons: (a) the derivatization introduced by an amino group would facilitate the isolation of the new molecules by cation-exchange chromatography, and (b) the reagent and putative degradation product (cysteamine) would not present the toxic and irritant effects of other thiol sources such as toluene-α-thiol or 2-mercaptoethanol.

MATERIALS AND METHODS

Materials. The starting material, provided by Bodegas 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. Water and solvents used were as follows: deionized water and bulk EtOH (Momplet y Esteban, Barcelona, Spain) for polyphenol extraction, bulk hexane (Quimivita, Sant Adrià del Besòs, Spain) for defatting, bulk EtOAc (Quimivita) distilled in-house for polyphenol fractionation, analytical grade MeOH (Panreac, Montcada i Reixac, Spain) for the acidolysis reaction, Milli-Q water and HPLC grade CH_3CN (E. Merck, Darmstadt, Germany) for analytical RP-HPLC, and preparative grade CH_3CN (Scharlau, Barcelona, Spain) for semipreparative cation-exchange and preparative RP-HPLC. Deuterated solvents for nuclear magnetic resonance (NMR) were from SDS (Peypin, France). Cysteamine (E. Merck) was of synthesis grade. Acetic acid, 37% HCl (E. Merck), and NaCl (Carlo Erba, Milano, Italy) were of analytical grade. Triethylamine (E. Merck) was of buffer grade. Trifluoroacetic acid (TFA, Fluorochem, Derbyshire, U.K.) of biotech grade was distilled in-house. Raney nickel in EtOH (1:1) was prepared immediately before use by treatment of Ni/Al alloy with 10% (w/v) aqueous NaOH (Carlo Erba, analytical grade) and washing with water and EtOH. 1,1-Diphenyl-2-picrylhydrazyl free radical (DPPH) (95%) and gallic acid (97%) were from Aldrich (Gillingham-Dorset, U.K.), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (97%) was from Aldrich (Milwaukee, WI), and (−)-epicatechin and the Folin−Ciocalteu phenol reagent were from Sigma-Aldrich (St. Louis, MO).

Chromatographic Equipment and Columns. Semipreparative cation-exchange chromatography was performed on an FPLC system (Amersham-Pharmacia Biotech, Uppsala, Sweden) fitted with a HiLoad 16/10 (10 × 1.6 cm i.d., 20 mL bed volume) SP Sepharose high-performance column (Amersham-Pharmacia Biotech). Preparative RP-HPLC chromatography was performed on a Waters (Milford, MA) Prep LC 4000 pumping system with a Waters PrepPack 1000 module fitted with a PrepPack Waters cartridge (300 × 47 mm i.d.) filled with VYDAC (The Separations Group, Hesperia, CA) C_{18}, 300 Å pore size, 15−20 μm particle size stationary phase. A flow splitter was placed after the column, and detection was done by an analytical Merck-Hitachi (Darmstadt, Germany) L-4000 UV detector. Analytical RP-HPLC was performed on either a Kontron Analytical system (Kontron Instruments, Basel, Switzerland) fitted with a VYDAC C_{18}, 300 Å pore size, 5 μm particle size, 250 × 4.6 mm i.d. column or a Smart System (Amersham-Pharmacia Biotech) equipped with a μ Peak Monitor (Amersham-Pharmacia Biotech) and fitted with a μRPC C2/C18 SC 2.1/10 (100 × 2.1 mm i.d.) column (Amersham-Pharmacia Biotech).

Mass Spectrometry and NMR Analyses. Mass spectrometry analyses were performed at the Servei d'Espectrometria de Masses of the University of Barcelona (ES-MS) and the Unidad de Espectrometría de Masas of the University of Córdoba (HR-FAB-MS). Electrospray mass spectrometry (ES-MS) analyses were recorded on a VG-Quattro system from Fisons Instruments (Altricham, U.K.). The carrier solution was water/CH_3CN (1:1) containing 1% (v/v) formic acid. High-resolution fast atom bombardment mass spectrometry (HR-FAB-MS) analyses were recorded on a VG Autospec system from Micromass Instruments, (Altricham, U.K.). The matrix was thioglycerol in the presence of 1% PEG-300 PEG-600. Nuclear magnetic resonance (NMR) analyses were carried out at the Department of Biological Organic Chemistry, IIQAB-CSIC. ^1H NMR spectra were observed with a Unity-300 spectrometer from Varian (Palo Alto, CA) for $(CD_3)_2CO$ and D_2O solutions.

Extraction and Fractionation of Flavanols. The moist byproduct (16 kg) was thawed and washed with deionized water (5 × 10 L). The residue was macerated with water/EtOH (3:7) (9 L) for 16 h with occasional shaking. The resulting solution was decanted and filtered through glass wool, and the operation was repeated twice. The filtrates (24 L) were pooled, and the EtOH was evaporated under vacuum. The resulting aqueous suspension was lyophilized to yield the crude extract C (587 g). The lyophilized solid was defatted with hexane (3 × 2 L) and the solvent decanted. The residue was dried and suspended in water (2 L). After the addition of acetic acid (10 mL), the monomeric and oligomeric components were extracted

with EtOAc (5 × 500 mL) to yield an organic fraction O (2 L), an aqueous fraction A (2 L), and an interface I (200 mL). Then the solvent was evaporated from fraction O under vacuum and the pellet suspended in water (0.5 L). The remaining organic solvent was eliminated under vacuum, and the resulting suspension was filtered through a porous plate. The pellet (fraction E soluble in EtOH) was washed with water, and the filtrates were pooled (1 L), centrifuged, decanted, and lyophilized to yield fraction OW (15.6 g), which contained species soluble in both AcOEt and water.

The total phenolic content of the fractions was estimated according to the Folin−Ciocalteu method (*23*) and was expressed as gallic acid equivalents (GAE). The antiradical activity of the fractions was calculated as described below. Fractions were also analyzed by RP-HPLC on a μRPC C2/C18, 3 μm column, elution with (A) 0.10% (v/v) aqueous TFA and (B) 0.08% (v/v) TFA in water/CH_3CN (1:4), gradient 0−50% B over 38 min, at a flow rate of 200 μL/min.

Treatment of the Aqueous Phase A with Acid in the Presence of Cysteamine. The solvent (water saturated with EtOAc) was eliminated from an aliquot (80 mL, 0.8 g of polyphenols as GAE, 1.2 g estimated of polyphenols by weight, coming from 0.64 kg of grape byproduct) of the aqueous suspension A. The pellet was then dissolved in MeOH (80 mL) and dried. This operation was repeated three times to eliminate moisture. The resulting syrupy residue was dissolved in MeOH (80 mL), and a solution of cysteamine (4 g) and 37% HCl (1.7 mL) in MeOH (80 mL) was added. The mixture was kept at 65 °C for 20 min under agitation. The reaction was then quenched with cold water (800 mL).

Cation-Exchange Fractionation of the Depolymerized Mixture Generated from the Aqueous Phase A. The new compounds were isolated and fractionated on a HiLoad 16/10 (10 × 1.6 cm i.d., 20 mL bed volume) SP Sepharose high-performance column (Amersham-Pharmacia Biotech). The eluents were (A) 20 mM sodium acetate, pH 4.75, buffer/CH_3CN (9:1) and (B) 20 mM sodium acetate, pH 4.75, buffer/CH_3CN (4:1), 0.8 M NaCl. The column was equilibrated with eluent A, loaded with the quenched depolymerized mixture (120 mL), and washed with equilibrating eluent A (200 mL, 10 bed volumes). The retained flavan-3-ol derivatives were eluted with a gradient of 0−100% B over 20 bed volumes (400 mL) at a flow rate of 10 mL/min with detection at 254 nm. Three fractions (1−3) were collected. Fraction 1 (centered around 8 bed volumes) contained cysteamine and compounds **1** and **3**. Fraction 3 (centered around 16 bed volumes) contained compound **2**. The operation was repeated (seven times total) until the whole mixture was consumed. After each run, the column was regenerated with 1 M NaOH and then 1 M HCl, both in the presence of 20% CH_3CN. After the seven repetitive runs, some colored material remained on the upper part of the column even after regeneration. The fractionation was monitored by analytical RP-HPLC on a VYDAC C_{18} column eluted with a binary system, (A) 0.10% (v/v) aqueous TFA, (B) 0.09% (v/v) TFA in water/CH_3CN (2:3) under isocratic conditions 16% B (compounds **1** and **3**), 21% B (compound **2**), at a flow rate of 1.5 mL/min and with detection at 215 nm, 0.016 absorbance unit full scale (aufs). The purity of the fractions was also assessed on a μRPC C2/C18 column also eluted with a binary system, (A) 0.10% (v/v) aqueous TFA, (B) 0.08% (v/v) TFA in water/CH_3CN (1:4), gradient 8−18% B over 30 min at a flow rate of 200 μL/min with simultaneous detection at 214, 280, and 320 nm.

Purification of the 2-Aminoethylthio Derivatives. The 4β-(2-aminoethylthio)flavan-3-ols were purified from the cation-exchange fractions by preparative RP-HPLC and identified by mass spectrometry and nuclear magnetic resonance.

*4β-(2-Aminoethylthio)epicatechin (**1**).* Fraction 1 from cation exchange was diluted with water (1:3) up to a total volume of 2.2 L, loaded onto a cartridge (300 × 47 mm i.d.) filled with VYDAC C_{18}, 300 Å pore size, 15−20 μm particle size, and eluted using a CH_3CN gradient (0.5−12.5% over 60 min) in triethylamine phosphate, pH 2.25, buffer, at a flow rate of 100 mL/min, with detection at 230 nm. Analysis of the fractions was accomplished under isocratic conditions in 0.10% (v/v) aqueous TFA/CH_3CN using the VYDAC C_{18} column, solvent system, flow rate, and detection described above with isocratic elution at 16% B. The pure fractions were pooled, diluted (1:1), and rechromatographed on the same cartridge by a CH_3CN gradient (5.5−17.5% over 30 min) in 0.10% (v/v) aqueous TFA. After combining the eluates and lyophilization, pure 4β-(2-aminoethylthio)epicatechin (**1**) (203 mg) was obtained as the trifluoroacetate: ES-MS, positive ions, *m*/*z* 366.3 $[M + H]^+$; HR-FAB-MS, positive ions, found 366.1029, calculated for $C_{17}H_{20}N_1O_6S_1$ $[M + H]^+$ 366.1011, 365.0943 (69% intensity) calculated for $C_{17}H_{19}N_1O_6S_1$ $[M]^+$ 365.0933; ^1H NMR [$(CD_3)_2$-CO, 300 MHz] δ 2.9−3.8 (4H, m, S−$\underline{C}H_2$−$\underline{C}H_2$−N), 4.02−4.07 (1H, 2m, 3-H), 4.08−4.18 (2H, m, S−$\underline{C}H_2$−$\underline{C}H_2$−$N\underline{H}_2$), 4.10−4.22 (1H, m, d, *J* = 2.1 Hz, 4-H), 5.12−5.23 (1H, 2s, 2-H), 5.89 (1H, d, *J* = 2.4 Hz, 6-H), 6.07−6.09 (1H, 2d, *J* = 2.4 Hz each, 8-H), 6.83 (2H, m, 5′-H, 6′-H), 7.10 (1H, d, *J* = 2.1 Hz, 2′H); ^1H NMR (D_2O, 300 MHz) δ 2.64−3.26 (4H, 3m, S−$\underline{C}H_2$−$\underline{C}H_2$−N), 3.77 (1H, bs, 3-H), 3.91 (1H, bs, 4-H), 5.11 (1H, bs, 2-H), 5.84 (1H, bd, 6-H), 5.89 (1H, bd, 8-H), 6.72 (2H, bs, 5′-H, 6′-H), 6.82 (1H, bs, 2′-H). Purity (>99.5%) was ascertained by RP-HPLC on (a) a VYDAC C_{18}, 300 Å, 5 μm, 250 × 4.6 mm i.d. column [elution, (A) 0.10% (v/v) aqueous TFA, (B) 0.09% (v/v) TFA in water/CH_3CN (2:3), gradient 8−23% B over 45 min at a flow rate of 1.5 mL/min with detection at 215 nm, 0.016 aufs] and (b) a μRPC C2/C18, 3 μm column [elution, (A) 0.10% (v/v) aqueous TFA, (B) 0.08% (v/v) TFA in water/CH_3CN (1:4), gradient 8−18% B over 30 min at a flow rate of 200 μL/min with simultaneous detection at 214, 280, and 320 nm]. Desulfuration with Raney nickel yielded (−)-epicatechin, detected by analytical RP-HPLC.

*4β-(2-Aminoethylthio)epicatechin 3-O-Gallate (**2**).* Fraction 3 from cation exchange was diluted with water (1:3) up to a total volume of 2.1 L, loaded onto a cartridge (300 × 47 mm i.d.) filled with VYDAC C_{18}, 300 Å pore size, 15−20 μm particle size, and eluted using the same system as described for compound **1**. In this case the first acetonitrile gradient in triethylamine phosphate, pH 2.25, buffer was 3−15% CH_3CN over 60 min. Then second and third acetonitrile gradients in 0.1% aqueous TFA, 6−18% CH_3CN over 20 min and 9−15% CH_3CN over 20 min, respectively, were run. Analysis of the fractions was accomplished under isocratic conditions in 0.10% aqueous TFA/CH_3CN using the column, solvent system, flow rate, and detection described above with elution at 21% B. After pooling of the best fractions and lyophilization, 4β-(2-aminoethylthio)epicatechin 3-*O*-gallate (**2**; 30 mg) was obtained as the trifluoroacetate: ES-MS, positive ions, *m*/*z* 518.2 $[M + H]^+$; HR-FAB-MS, positive ions, found 518.1129, calculated for $C_{24}H_{24}N_1O_{10}S_1$ $[M + H]^+$ 518.1121; ^1H NMR [$(CD_3)_2CO$, 300 MHz] δ 3.03−3.86 (4H, m, S−$\underline{C}H_2$−$\underline{C}H_2$−N), 4.15−4.28 (1H, 2d, *J* = 2 Hz each, 4-H), 4.17−4.37 (2H, m, S−CH_2−CH_2−$N\underline{H}_2$), 5.21−5.31 (1H, 2m, 3-H), 5.36−5.47 (1H, 2bs, 2-H), 6.01−6.02 (1H, 2d, *J* = 2.4 Hz each, 6-H), 6.08−6.11 (1H, 2d, *J* = 2.4 Hz each, 8-H), 6.75−6.80 (1H, 2d, *J* = 8 Hz each, 5′-H), 6.84−6.90 (1H, m, *J* = 8, 2 Hz, 6′-H), 6.98−7.01 (2H, m, galloyl H), 7.14−7.17 (1H, 2d *J* = 2 Hz each, 2′-H); ^1H NMR (D_2O, 300 MHz) δ 2.71−3.27 (4H, 3m, S−$\underline{C}H_2$−$\underline{C}H_2$−N), 3.96 (1H, bd, 4-H), 5.04−5.11 (1H, 2bs, 3-H), 5.30 (1H, bs, 2-H), 5.90 (2H, bs, 6-H, 8-H), 6.60−6.68 (2H, m, 5′-H, 6′-H), 6.70−6.73 (2H, m, galloyl H), 6.82 (1H, bs, 2′-H). Purity (>99.5%) was ascertained by RP-HPLC on the two systems described for compound **1**. Desulfuration with Raney nickel yielded (−)-epicatechin 3-*O*-gallate, detected by analytical RP-HPLC (small peak).

*4β-(2-Aminoethylthio)catechin (**3**).* Compound **3** was purified from a hydrophilic fraction generated during the purification (gradient of acetonitrile in triethylamine phosphate, pH 2.25, buffer) of compound **1**. This fraction (2.4 L after dilution with 0.1% aqueous TFA, 1:2) was reloaded onto the preparative cartridge and eluted with CH_3CN gradient (0−12% over 30 min) in 0.1% aqueous TFA. Fractions were analyzed under isocratic conditions in 0.10% aqueous TFA/CH_3CN using the column, solvent system, flow rate, and detection described above with elution at 16% B. After pooling of the best fractions and lyophilization, 4β-(2-aminoethylthio)catechin (**3**; 2.5 mg) was obtained as the trifuoroacetate: ES-MS, positive ions, *m*/*z*

366.3 [M + H]$^+$; HR-FAB-MS, positive ions, found 365.0935, calculated for $C_{17}H_{19}N_1O_6S_1$ [M]$^+$ 365.0933; ^{1}H NMR [$(CD_3)_2CO$, 300 MHz] δ 2.92−3.70 (4H, m, S−C$\underline{H}_2$−C$\underline{H}_2$−N), 4.01−4.21 (1H, 2d, J = 9.6 Hz each, 3-H; 2H, m, S−C$\underline{H}_2$−CH$_2$−N$\underline{H}_2$), 4.29−4.51 (1H, 2d, J = 4.2 Hz each, 4-H), 4.82−5.02 (1H, 2d, J = 9 Hz each, 2-H), 5.80 (1H, m, 6-H), 6.09 (1H, m, 8-H), 6.80 (2H, m, 5′-H, 6′-H), 6.94−6.96 (1H, m, 2′-H); ^{1}H NMR (D_2O, 300 MHz) δ 2.7−4.2 (5H, several multiplets, S−C$\underline{H}_2$−C$\underline{H}_2$−N, 3-H), 4.73−4.76 (1H, d, J = 9.3 Hz, 2-H), 5.70−5.71 (1H, bd, 6-H), 5.91−5.92 (1H, bd, 8-H), 6.75 (2H, bs, 5′-H and 6′-H), 6.82 (1H, bs, 2′-H). The signals corresponding to the 4-H proton, not detected, may fall under the band of water. Purity (98.5%) was ascertained by RP-HPLC on the two systems described for compound **1**. Desulfuration with Raney nickel yielded (+)-catechin, detected by analytical RP-HPLC (small peak).

Evaluation of Antiradical Activity. The antiradical activity of the crude extract and fractions and the antiradical efficiency of the final derivatives were evaluated by using the DPPH method (*24*, *25*). The samples (0.1 mL) were added to aliquots (3.9 mL) of a solution made up with 4.8 mg of DPPH in 200 mL of solvent, and the mixture was incubated for 1 h at room temperature. The initial concentration of DPPH, ~60 μM, was calculated for every experiment from a calibration curve made by measuring the absorbance at 517 nm of standard samples of DPPH at different concentrations. The equation of the curve was $Abs_{517nm} = 11526C_{DPPH}$ as determined by linear regression. The results were plotted as the degree of absorbance disappearance at 517 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 product. For the crude and purification fractions, the activity unit was defined as the amount, either weight or volume, of sample able to consume half the amount of free radical. For the final derivatives of known molecular weight, the results are expressed as the efficient dose ED_{50}. ED_{50} is given as micromoles of product able to consume half the amount of free radical divided by micromoles of initial DPPH.

RESULTS AND DISCUSSION

Extraction and Fractionation. Antioxidant polyphenols were obtained from the grape (*Vitis vinifera*) pomace resulting from the pressing operation. This residue contained skins, seeds, and a small amount of stems. First, a crude preparation (C) was obtained by maceration with water/EtOH (3:7). The crude C contained monomeric flavan-3-ols [mainly (+)-catechin and (−)-epicatechin], oligomeric, and polymeric proanthocyanidins and glycosylated flavonols. This crude was fractionated as described under Materials and Methods to yield a fraction OW that contained species (flavan-3-ol monomers, flavonols, and proanthocyanidin oligomers) soluble in both AcOEt and water. The procedure also yielded a fraction A that contained species soluble in water but not in the organic solvent. Figure 2 shows the RP-HPLC profiles of both the crude extract (C) and the aqueous layer (A). The profile corresponding to the aqueous fraction A consisted mainly of a broad hump. This chromatographic behavior is expected from polymerized proanthocyanidins (*17*, *26*) which, owing to their astringent properties, are considered of less interest than the oligomers. Moreover, fraction A was contaminated by other water-soluble materials such as polymerized sugars that would hamper any recovery process. For these reasons we first classified fraction A as a residue.

For all of the fractions, the total amount of polyphenols and the antiradical activity were measured (Table 1). Interestingly, half of the amount of total

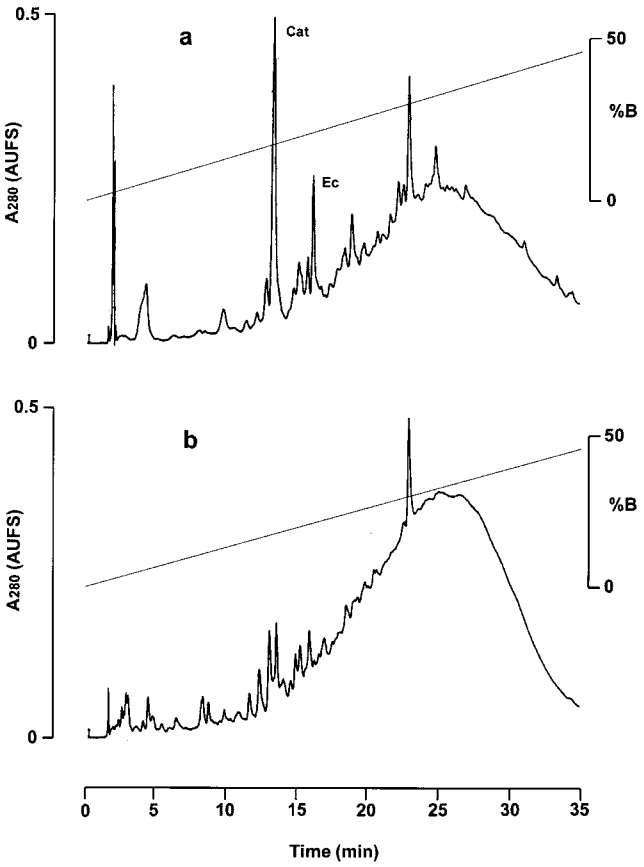


Figure 2. RP-HPLC profiles of proanthocyanidin fractions: (a) crude extract C; (b) aqueous phase A. Conditions: column, μRPC C2/C18, 3 μm; load, 3 μg of GAE of (a) crude C and (b) fraction A. Cat, (+)-catechin; Ec, (−)-epicatechin.

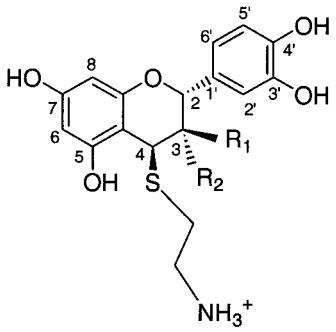
Table 1. Total Phenolics and Antiradical Activities of the Fractions Obtained during the Fractionation Process

fraction	total wt/vol	total phenols[a] (g)	total AR activity units[b] ($\times 10^{-6}$)
crude C	587 g	70.5	11.0
fraction P	2000 mL	40.3	8.6
interface I	200 mL	4.2	0.5
fraction A	2000 mL	20.5	6.1
fraction E	350 mL	4.1	0.2
fraction OW	15.6 g	10.1	1.3

[a] Values expressed as gallic acid equivalents obtained by using the Folin−Ciocalteu method (*23*) modified. [b] Values obtained by using the DPPH method The antiradical (AR) activity unit is defined as the amount of sample able to consume 50% of the initial amount of free radical.

phenols remained in the residual aqueous fraction A, in agreement with the results of other authors (*16*, *26*). More strikingly, fraction A contained 71% of the activity of the defatted fraction P, whereas the monomeric/oligomeric fraction OW contained only 15%.

Depolymerization in the Presence of Cysteamine. The treatment of polymeric proanthocyanidins with an acid results in the cleavage of carbon−carbon bonds between position 4 of one flavan-3-ol unit and either position 6 or 8 of another. The terminal flavan-3-ols units are released as such, whereas in the presence of a thiol-containing reagent the extension moieties are released as the thio derivatives at position 4 of the flavanoid unit. There are examples of cleavage of proanthocyanidins in the presence of different sources of thiols, including thioglycolic acid (*27*, *28*), toluene-α-thiol (*7*, *8*, *18*−*20*), and 2-mercaptoethanol (*21*, *22*).



1 R_1=H, R_2=OH, 4β-(2-aminoethylthio)epicatechin

2 R_1=H, R_2=Gal, 4β-(2-aminoethylthio)epicatechin 3-*O*-gallate

3 R_1=OH, R_2=H, 4β-(2-aminoethylthio)catechin

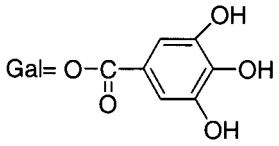


Figure 3. Structures of the 4*β*-(2-aminoethylthio)flavan-3-ols.

In our search for new uses of residues and byproducts we have been considering the acid-catalyzed cleavage of proanthocyanidins in the presence of different thiols as a way to modulate the properties of polyphenols by introducing new functional groups. In the present work, the main target was to facilitate the recovery of active flavanols. The introduction of an amine group through conjugation with cysteamine appeared to be useful for the isolation of the new molecules using cation-exchange chromatography. Moreover, the new derivatives would give us an opportunity to investigate the effects of the introduction of an amine group on the activity of the flavan-3-ols. We have obtained the aminoethylthio derivatives of flavan-3-ols by treatment of the proanthocyanidin mixtures with cysteamine. In the literature, the acid-catalyzed cleavage is usually carried out with a 25−50-fold excess of thiol with respect to the amount of proanthocyanidins (*7*, *8*, *14*, *19*, *21*). Typically, the reaction is performed in MeOH in the presence of 0.2 M hydrochloric acid and a 50-fold excess (w/w) of toluene-α-thiol for either 10 min at 60 °C (*7*) or 2 min at 90 °C (*14*). For preparative purposes we wanted to reduce the amount of reagents. We found that a 3-fold excess (w/w) of thiol (cysteamine) was enough to consume the polymeric material in 15 min at 60−65 °C, as ascertained by HPLC under the elution conditions stated for the fractions. Using the above-described reaction conditions the cleavage products were readily obtained from different fractions, namely, crude extract C, aqueous fraction A, and fraction OW. The composition of the depolymerized mixtures varied slightly depending on the starting material, the main products being essentially the same in each case. The main conjugates detected after the cleavage were, in all cases, the 2-aminoethylthio derivatives of epicatechin **1**, epicatechin 3-*O*-gallate **2**, and catechin **3** (Figure 3). The reaction was scaled-up for fraction A (components soluble only in water, Figure 2b). Figure 4 shows the RP-HPLC profile of the cleavage products obtained after depolymerization of fraction A.

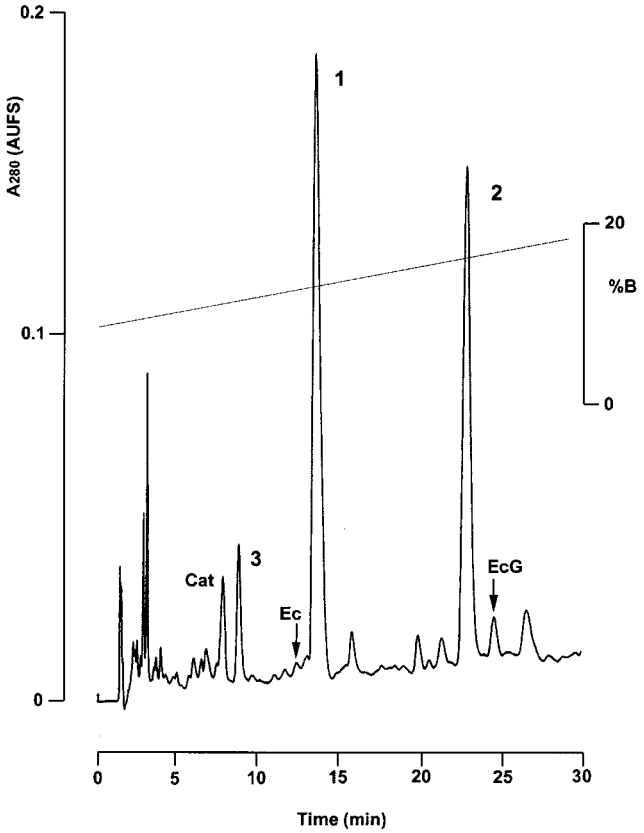


Figure 4. RP-HPLC profile of the depolymerized mixture generated from aqueous phase A and cysteamine. Conditions: column, *μ*RPC C2/C18, 3 *μ*m; load, 5 *μ*L of depolymerized mixture after quenching. Cat, (+)-catechin; Ec, (−)-epicatechin; EcG, (−)-epicatechin 3-*O*-gallate. Structures of compounds **1**−**3** are shown in Figure 3.

Isolation, Purification, and Characterization of the 2-Aminoethylthio Derivatives of Flavan-3-ols. A crucial aspect of the present work is the isolation of the new derivatives. One important advantage of the introduction of an amine function was the simplification of the isolation step. The aminated molecules are electrostatically retained by a cation-exchange stationary phase, and the rest of the material is washed away. This is important because it allows working with crude plant extracts, that is to say, in the presence of great amounts of accompanying materials of different nature, which, as far as they do not contain exchangeable cations, are easily eliminated from the target species. Of course, there is always the problem of non-electrostatic interactions of the impurities with the stationary phase, and the elution conditions have to be adapted for every crude preparation. In any case, the purification of the individual compounds from the cleaved mixtures is much more complicated without this simple cleanup step. The depolymerized mixture generated from fraction A was particularly complex because A contained almost all the water-soluble material of the crude C. In our hands, the cation-exchange isolation step was equally efficient independent of the fraction used as starting material.

The new 2-aminoethylthio derivatives of flavan-3-ols were efficiently isolated from the crude depolymerized residual fraction A by strong cation exchange on a solid support bearing the sulfonic group. To minimize un-

wanted hydrophobic interactions, a hydrophilic polymer of cross-linked agarose was chosen. The isolation of the aminated derivatives was achieved in sodium acetate buffer at pH 4.75 in the presence of an appropriate amount of organic cosolvent. It is well-known that the core polymers of ion-exchange stationary phases retain bioproducts such as peptides through hydrophobic interactions (*29*). Addition of water-miscible organic solvent to the mobile phase, usually 10−20% CH_3CN, is an established method to eliminate such interactions. In this study, conditions have been found that allow both the elimination of underivatized monomers (mainly catechin, epicatechin, and epicatechin 3-*O*-gallate), as well as other small molecules, and the separation of the two major thioconjugates, namely, 4*β*-(2-aminoethylthio)epicatechin (**1**) and 4*β*-(2-aminoethylthio)epicatechin 3-*O*-gallate (**2**). The third major compound, 4*β*-(2-aminoethylthio)catechin (**3**) was isolated together with compound **1**. The crude diluted depolymerized mixture generated from fraction A was loaded onto a column filled with SP Sepharose. The isolation and separation were accomplished by a first washing step in the presence of 10% CH_3CN and subsequent elution with two simultaneous gradients of both NaCl and CH_3CN over 20 bed volumes. Compounds **1** and **3** eluted at 8 bed volumes, and compound **2** eluted at 16 bed volumes. These major derivatives are likely to show identical cation-exchange behavior, so the separation between compounds **1** and **2** must have been caused by hydrophobic interactions with the stationary phase, only partially eliminated with the amounts of cosolvent used. It is interesting to note that only one galloyl moiety exerted a significant influence on the hydrophobic retention behavior of the epicatechin derivatives, even on a fairly hydrophilic polymer such as cross-linked agarose.

The individual 2-aminoethylthio derivatives **1**−**3** were further purified by preparative RP-HPLC on VYDAC C_{18}, using CH_3CN to obtain compounds **1** and **2** with a purity >99.5% by analytical RP-HPLC and compound **3** with a purity of 98.5% by the same technique.

^1H NMR spectra in deuterated acetone of compounds **1** and **2** showed a broad singlet at fields lower than 5 ppm, assigned to position 2 on the C ring. The spectrum corresponding to compound **3** showed a doublet at fields higher than 5 ppm with a coupling constant of 9 Hz. These observations are in agreement with a 2,3-*cis* structure (epicatechin-like) for compounds **1** and **2** and with a 2,3-*trans* structure (catechin-like) for compound **3** (*18*). All three compounds showed multiplicity of signals for some of the protons, mainly 2-H, 3-H, and 4-H on ring C and 8-H on ring A. This multiplicity may be explained, at least in part, by the existence of more than one conformation because no variation in the coupling constants was detected for any of the affected protons. Examples of this are the chemical shifts and coupling constants of protons 3-H and 8-H in compound **1**, most of the protons in compound **2**, and protons 4-H and 2-H in compound **3**, as reported under Materials and Methods. Following Haslam and colleagues (*18*), compounds **1** and **2** showed a 3,4-*trans* configuration ($J_{3,4}$ = 2.1 and 2.0 Hz, respectively) and compound **3** showed a 3,4-*cis* configuration ($J_{3,4}$ = 4.2 Hz). No multiplicity for the flavanoid protons was detected in deuterated water.

The molecular ions were detected by ES-MS and HR-FAB-MS. The amino group present in the new molecules

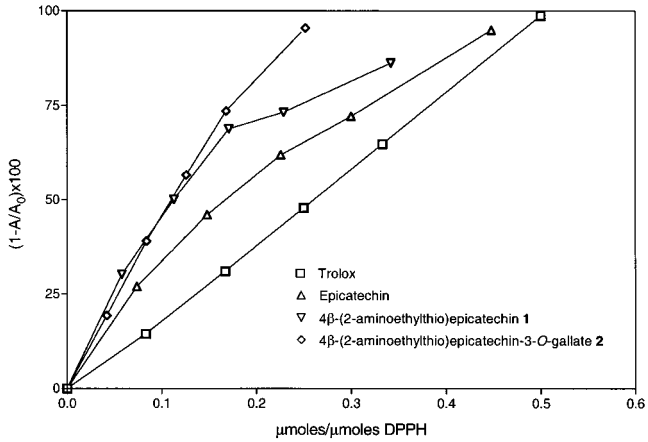


Figure 5. Antiradical activity of the aminoethylthio derivatives of flavan-3-ols on the DPPH assay. Absorbance (A) at 517 nm is a measure of the amount of free radical remaining in solution. $(1 - A/A_0) \times 100$ represents the percentage of DPPH already reacted with the antioxidant. Each point represents the mean of three determinations.

was easily protonated and facilitated the detection of the positive species. The $[M + H]^+$ ions were recorded by ES-MS for compounds **1**−**3** and by HR-FAB-MS for compounds **1** and **2**. We have also detected ions at $[M]^+$ for compounds **1** (69% intensity) and **3** (main peak) by HR-FAB-MS. The scavenging properties of polyphenols are often associated with their ability to form relatively stable semiquinone free radicals detectable by techniques such as EPR spectroscopy (*30*). Radicals of this kind would give $[M]^+$ ions such as those recorded for **1** and **3**. They would not be detected by mass spectrometry in the negative mode, commonly used for underivatized polyphenols (*31*, *32*).

Antiradical Activity in the DPPH Assay. The pure new molecules proved to be potent free radical scavengers. Figure 5 presents the results of the DPPH assay from which curves, the ED_{50} values, were calculated. The antiradical efficacies of compounds **1** and **2** were compared with those of Trolox and (−)-epicatechin. The ED_{50} values obtained were as follows: Trolox, 0.26; (−)-epicatechin, 0.19; 4*β*-(2-aminoethylthio)epicatechin (**1**), 0.11; 4*β*-(2-aminoethylthio)epicatechin 3-*O*-gallate (**2**), 0.11. The introduction of a 2-aminomethylthio group onto position 4 of epicatechin and epicatechin gallate yielded compounds with improved antiradical efficacy in solution. The antiradical activity of epicatechins is exerted through the formation of semiquinone free radicals and quinones, triggered by the abstraction of one proton and one electron from some of the phenolic hydroxyl groups (*30*, *33*). The amino group and/or the sulfur atom of the new derivatives might modulate the reactivity of the molecules. The oxidation of the thioether to sulfoxide and/or sulfone might contribute to this antioxidant effect.

In conclusion, new products with potential application as antioxidants have been obtained from otherwise useless proanthocyanidin polymers and cysteamine. The new molecules can be easily isolated from complex mixtures of plant material by cation-exchange chromatography through the introduction of an amine function. As an example, the 2-aminoethylthio derivatives of flavan-3-ols have been prepared, isolated, and purified from a residual fraction generated during the process of extraction of oligomeric proanthocyanidins from grape pomace. This formerly useless fraction contained most of the antioxidant activity of the crude extract.

There are potentially interesting applications of the new derivatization of flavan-3-ols described in this work. The antioxidants are cationized, and this property might be used to target the molecules to selected cells, tissues, or organs. Moreover, the amino function might facilitate further derivatization through the formation of amide bonds. For analytical purposes, depolymerization with cysteamine combined with micropreparative cation-exchange chromatography and/or RP-HPLC has been proven to allow the separation of the main cleavage products (*34*). Thus, cysteamine might be tested as an alternative thiol donor for the characterization of polymeric proanthocyanidins.

ABBREVIATIONS USED

DPPH, 1,1-diphenyl-2-picrylhydrazyl free radical; Trolox, 2,5,7,8-tetramethylchroman-2-carboxylic acid; RP-HPLC, reversed-phase high-performance liquid chromatography; NMR, nuclear magnetic resonance; GAE, gallic acid equivalents.

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LITERATURE CITED

(1) Diplock, A. T.; Charleux, J. L.; Crozier-Willi, G.; Kok, F. J.; Rice-Evans, C.; Roberfroid, M.; Stahl, W.; Viña-Ribes, J. Functional food science and defence against reactive oxidative species. *Br. J. Nutr.* **1998**, *80* (Suppl. 1), S77−S112.

(2) Yang, C. S.; Lee, M.-J.; Chen, L.; Yang, G.-Y. Polyphenols as inhibitors of carcinogenesis. *Environ. Health Perspect.* **1997**, *105* (Suppl. 4), 971−976.

(3) Yang, G. Y.; Liao, J.; Kim, K.; Yurkow, E. J.; Yang, C. S. Inhibition of growth and induction of apoptosis in human cancer cell lines by tea polyphenols. *Carcinogenesis* **1998**, *19*, 611−616.

(4) Soleas, G. J.; Diamandis, E. P.; Goldberg, D. M. Wine as a biological fluid: History, production, and role in disease prevention. *J. Clin. Lab. Anal.* **1997**, *11*, 287−313.

(5) Ruf, J. C. Wine and polyphenols related to platelet aggregation and atherothrombosis. *Drug Exp. Clin. Res.* **1999**, *25*, 125−131.

(6) Packer, L.; Rimbach, G.; Virgili, F. Antioxidant activity and biologic properties of a procyanidin-rich extract from pine (*Pinus maritima*) bark, pycnogenol. *Free Radical Biol. Med.* **1999**, *27*, 704−724.

(7) Souquet, J.-M.; Cheynier, V.; Brossaud, F.; Moutounet, M. Polymeric proanthocyanidins from grape skins. *Phytochemistry* **1996**, *43*, 509−512.

(8) Prieur, C.; Rigaud, J.; Cheynier, V.; Moutounet, M. Oligomeric and polymeric procyanidins from grape seeds. *Phytochemistry* **1994**, *36*, 781−784.

(9) Blazsó, G.; Gábor, M.; Sibbel, R.; Rohdewald, P. Anti-inflammatory and superoxide radical scavenging activities of procyanidins containing extract from the bark of *Pinus pinaster Sol.* and its fractions. *Pharm. Pharmacol.* **1994**, *3*, 217−220.

(10) Yamaguchi, F.; Yoshimura, Y.; Nakazawa, H.; Ariga, T. Free radical scavenging activity of grape seed extract and antioxidants by electron spin resonance spectrometry in an H_2O_2/NaOH/DMSO system. *J. Agric. Food Chem.* **1999**, *47*, 2544−2548.

(11) Bravo, L. Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance. *Nutr. Rev.* **1998**, *56*, 317−333.

(12) Drewnowski, A.; Gómez-Carneros, C. Bitter taste, phytonutrients, and the consumer: a review. *Am. J. Clin. Nutr.* **2000**, *72*, 1424−1435.

(13) Arnold, R. A.; Noble, A. C.; Singleton, V. L. Bitternes and astringency of phenolic fractions in wine. *J. Agric. Food Chem.* **1980**, *28*, 675−678.

(14) Souquet, J. M.; Labarbe, B.; LeGuerneve, C.; Cheynier, V.; Moutounet, M. Phenolic composition of grape stems. *J. Agric. Food Chem.* **2000**, *48*, 1076−1080.

(15) Labarbe, B.; Cheynier, V.; Brossaud, F.; Souquet, J. M.; Moutounet, M. Quantitative fractionation of grape proanthocyanidins according to their degree of polymerization. *J. Agric. Food Chem.* **1999**, *47*, 2719−2723.

(16) Czochanska, Z.; Foo, L. Y.; Newman, R. H.; Porter, L. J. Polymeric proanthocyanidins. Stereochemistry, structural units and molecular weight. *J. Chem. Soc., Perkin Trans. 1* **1980**, 2278−2286.

(17) Guyot, S.; Marnet, N.; Drilleau, J. F. Thiolysis-HPLC characterization of apple procyanidins covering a large range of polymerization states. *J. Agric. Food Chem.* **2001**, *49*, 14−20.

(18) Thompson, R. S.; Jacques, D.; Haslam, E.; Tanner, R. J. N. Plant proanthocyanidins. Part I. Introduction; the isolation, structure, and distribution in nature of plant procyanidins. *J. Chem. Soc., Perkin Trans. 1* **1972**, 1387−1399.

(19) Rigaud, J.; Pérez-Ilzarbe, J.; Ricardo da Silva, J. M.; Cheynier, V. Micro method for identification of proanthocyanidin using thiolysis monitored by high-performance liquid chromatography. *J. Chromatogr.* **1991**, *540*, 401−405.

(20) Sanoner, P.; Guyot, S.; Marnet, N.; Molle, D.; Drilleau, J. F. Polyphenol profiles of French cider apple varieties (*Malus domestica sp.*). *J. Agric. Food Chem.* **1999**, *47*, 4847−4853.

(21) Tanaka, T.; Takahashi, R.; Kouno, I.; Nonaka, G.-I. Chemical evidence for the de-astringency (insolubilization of tannins) of persimmon fruit. *J. Chem. Soc., Perkin Trans. 1* **1994**, 3013−3022.

(22) Tanaka, T.; Kusano, R.; Kouno, I. Synthesis and antioxidant activity of novel amphipathic derivatives of tea polyphenol. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 1801−1806.

(23) Singleton, V. L.; Rossi, J. A. J. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **1968**, *16*, 144−158.

(24) Blois, M. S. Antioxidant determinations by the use of stable free radical. *Nature* **1958**, *181*, 1199−1200.

(25) Brand-Williams, W.; Cuvelier, M. E.; Berset, C. Use of a free radical method to evaluate antioxidant activity. *Lebensm.-Wiss. -Technol.* **1995**, *28*, 25−30.

(26) Kantz, K.; Singleton, V. L. Isolation and determination of polymeric polyphenols using Sephadex LH-20 and analysis of grape tissue extracts. *Am. J. Enol. Vitic.* **1990**, *41*, 223−228.

(27) Betts, M. J.; Brown, B. R.; Brown, P. E.; Pike, W. T. Degradation of condensed tannins: structure of the tannin from common heather. *Chem. Commun.* **1967**, 1110−1112.

(28) Sears, K. D.; Casebier, R. L. Cleavage of proanthocyanidins with thioglycolic acid. *Chem. Commun.* **1968**, 1437−1438.

(29) Burke, T. W. L.; Mant, C. T.; Black, J. A.; Hodges, R. S. Strong cation-exchange high-performance liquid chromatography of peptides. Effect of non-specific hydrophobic interactions and linearization of peptide retention behaviour. *J. Chromatogr.* **1989**, *476*, 377−389.

(30) Guo, Q. N.; Zhao, B. L.; Li, M. F.; Shen, S. R.; Xin, W. J. Studies on protective mechanisms of four components of green tea polyphenols against lipid peroxidation in synaptosomes. *Biochim. Biophys. Acta-Lipid Lipid Metab.* **1996**, *1304*, 210−222.

(31) Self, R.; Eagles, J.; Galletti, G. C.; Mueller-Harvey, I.; Hartley, R. D.; Lea, A. G. H.; Magnolato, D.; Richli, U.; Gujer, R.; Haslam, E. Fast atom bombardment mass spectrometry of polyphenols (syn. vegetables tannins). *Biomed. Environ. Mass Spectrom.* **1986**, *13*, 449−468.

(32) Cheynier, V.; Doco, T.; Fulcrand, H.; Guyot, S.; Le Roux, E.; Souquet, J. M.; Rigaud, J.; Moutounet, M. ESI-MS analysis of polyphenolic oligomers and polymers. *Analusis* **1997**, *25*, M32−M37.

(33) Kondo, K.; Kurihara, M.; Miyata, N.; Suzuki, T.; Toyoda, M. Mechanistic studies of catechins as antioxidants against radical oxidation. *Arch. Biochem. Biophys.* **1999**, *362*, 79−86.

(34) Torres, J. L.; Lozano, C. Chromatographic characterisation of proanthocyanidins after thiolysis with cysteamine. *Chromatographia* **2001**, in press.

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