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Efficient Preparation of Catechin Thio Conjugates by One Step Extraction/Depolymerization of Pine (*Pinus pinaster*) Bark Procyanidins

ARIADNA SELGA AND JOSEP LLUÍS TORRES*

Institute for Chemical and Environmental Research (IIQAB-CSIC), Jordi Girona 18-26, 08034 Barcelona, Spain

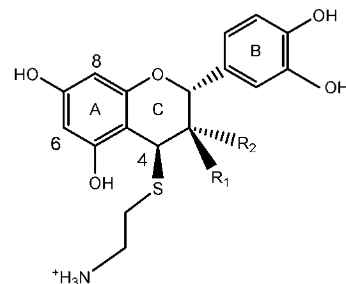
The skin penetrating antioxidant cysteamine derivative of (−)-epicatechin as well as other thio conjugates were efficiently obtained with high yields from pine (*Pinus pinaster*) bark by simultaneous one pot extraction and depolymerization using water and cysteamine hydrochloride. The influence of the concentration of bark, acid, and cysteamine, as well as the reaction time on the total conversion, was studied. The total conversion into the epicatechin and catechin conjugates was as high as 47 g/kg pine bark with 1666 g cysteamine/kg bark and 28 g/kg with 166 g cysteamine/kg bark. A fast cleanup step by absorption/desorption on XAD-16 greatly facilitated further purification of the active major component. At a pilot scale, 4β-(2-aminoethylthio)epicatechin (**1**) (conversion 263 g, purity 35% by reversed phase high-performance liquid chromatography/weight) was obtained from 17 kg of pine bark after simultaneous extraction/depolymerization followed by cleanup with the polymeric resin in ~10 h. The results show that pine (*P. pinaster*) bark is a suitable source of flavanols for the preparation of active thio derivatives. Conditions are given for the fast and efficient preparation of the conjugates.

KEYWORDS: Pinebark; polyphenols; procyanidins; catechins; flavanols; extraction; adsorption resins; antioxidants; free radical scavenging

INTRODUCTION

Flavonoids are plant secondary metabolites of polyphenolic nature with chemopreventive activity against cancer and cardiovascular diseases (*1*−*4*). The health promoting properties of flavonoids are believed to be mainly due to their antioxidant effect, which results from the combination of their free radical scavenging power (*5*, *6*), their metabolism and bioavailability (*7*, *8*), and their capacity to modulate the activity of endogenous defense mechanisms such as the superoxide dismutase and glutathion systems (*9*−*11*). Moreover, flavonoids are increasingly recognized as important players in the regulation of cell functions by mechanisms not necessarily related to their scavenging capacity (*12*). For instance, some flavonoids show antiproliferative activity through the inhibition of key enzymes (kinases, proteases) of the cell cycle (*13*−*16*).

Among the diseases induced, at least in part, by oxidative stress, disorders of the skin (UV light-induced damage), gut (colon cancer), and nervous system (neurodegeneration) are primarily addressed in our laboratory. We have recently shown that the nonphenolic part of epicatechin thio derivatives (**Figure 1**) modulates its penetration into the deep layers of the skin (*17*). Concretely, the cysteamine conjugate 4β-(2-aminoethylthio)epicatechin (**1**) shows an enhanced capacity to penetrate into



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

Figure 1. Structure of the major resulting conjugates after depolymerization of procyanidins in the presence of cysteamine.

the dermis. This is important for dermoprotection against sunlight-induced melanoma since the penetrating UVA radiation appears to trigger melanoma-related DNA mutations (*18*, *19*). Other authors have described that derivatization of flavanols with hydrophobic thiols generates amphypatic molecules active against lipid peroxidation (*20*). More recently, it has been shown that cysteine-based thio derivatives of (−)-epicatechin efficiently protect neural cells from the oxidative glutamate-mediated programmed cell death while the parent flavanol (−)-epicatechin was inactive (*21*).

The results obtained thus far make the thio derivatives of catechins promising chemopreventative agents of application

* To whom correspondence should be addressed. Tel: 34 93 400 61 12. Fax: 34 93 204 59 04. E-mail: jltqbp@iiqab.csic.es.

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in fields as diverse as dermatology and neuroprotection. To complete the biological studies and to run toxicology trials, high amounts of the active molecules are required. In a previous work, conditions have been found for the preparation of gram amounts of the conjugates from white grape pomace in a one pot, two step (extraction and depolymerization) procedure followed by ion exchange chromatography and reversed phase high-performance liquid chromatography (RP-HPLC) (*22*).

The goals of this work were to simplify the extraction/depolymerization process and to improve the final yield. Here, pine (*Pinus pinaster*) bark is used as an alternative source of polymers, and a one pot/one step treatment followed by a cleanup step by absorption/desorption on a polymeric resin is proposed. In this way, yields may be improved 5-fold with respect to the previously described methods at virtually the same cost.

MATERIALS AND METHODS

Materials. Pine (*P. pinaster*) bark (byproduct of a sawmill) was provided by Manuel Bouzas SA (Vedra, A Coruña, Spain). After the bark was air-dried, the solid was ground (GR-250 Mill with 3 mm pore size from Oliver Batlle SA, Badalona, Spain) immediately before extraction/depolymerization.

Water and solvents used were analytical grade MeOH (Panreac, Montcada i Reixac, Spain) and deionized water for analytical and preparative extractions and depolymerizations; deionized water and bulk EtOH (Montplet, Barcelona, Spain) for separation with polymeric resins; deionized water and preparative grade CH_3CN (Scharlau, Barcelona, Spain) for preparative RP-HPLC; milli-Q water; HPLC grade CH_3CN (E. Merck, Darmstadt, Germany) for analytical RP-HPLC; and analytical grade MeOH (Panreac) for the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. Acetic acid was from Panreac, and 37% HCl was from Merck. Cysteamine hydrochloride and cysteine were from Sigma-Aldrich Chemie Gmbh (Steinhem, Germany). Trifluoroacetic acid (TFA) (Fluorochem, Derbyshire, United Kingdom) biotech grade was distilled in-house. Triethylamine (TEA) was from Calbiochem (La Jolla, CA). DPPH free radical (95%) was obtained from Aldrich (Gillingham-Dorset, United Kingdom).

Analysis of the Samples by RP-HPLC. The polyphenolic mixtures were analyzed by RP-HPLC on a Smart System (Amersham-Pharmacia Biotech, Uppsala, Sweden) equipped with a μPeak Monitor (Amersham-Pharmacia Biotech) and fitted with a 100 mm × 2.1 mm i.d. μRPC C2/C18 SC 2.1/10 column. Elution was performed using the following solvent systems: [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 and 18−50% [B] over 20 min. The flow rate was 200 μL/min. The detection was done by triple wavelength at 214, 280, and 320 nm.

Extraction, Depolymerization, and Scale-up of Procyanidins from Pine Bark. Simultaneous extraction and depolymerization in MeOH was considered the positive control (i.e., maximum depolymerization and conversion). Briefly, pine (*P. pinaster*) bark was mixed in MeOH (240 g/L), cysteamine (1666 mg/g bark) and HCl (20 μL/g bark) were added, and the mixture was stirred at 65 °C for 15 min. In water, different conditions were tested and the results were compared with those obtained with MeOH. For one pot extraction/depolymerization in two steps, pine bark was treated with increasing amounts of cysteamine hydrochloride (40, 60, 166, and 1666 g/kg of pine bark) under the following conditions: 30 g pine bark/L water, 90 °C, 2 h, and an extra 2 h after the addition of cysteamine. For the simultaneous one pot extraction/depolymerization, first, the initial conditions were set up at 30 g bark/L, 166 g cysteamine/kg bark, and 20 mL/L HCl at 90 °C and the reaction was allowed to proceed for 2, 3, and 4 h. The second variable tested was the bark concentration. Reactions were set up at 30, 60, 120, 180, and 240 g of bark per liter of water. The third variable was acid concentration. Different concentrations (20, 10, 5, and 2.5 mL/L) were tested. Once the conditions for the simultaneous extraction/depolymerization of pine bark were set up (water, 240 g bark/L, and 5 mL/L HCl, 90 °C, 2 h), the variation of conversion with the excess of cysteamine hydrochloride was tested again at ratios cysteamine/bark of 166, 333, 666, and 1666 g/kg.

The operations at pilot scale were carried out at the Centre de Química Fina (A+/LGAI, Bellaterra, Spain). Ground pine bark (17 kg) was placed in a 250 L stainless steel reactor (RE-2012 enameled reactor from De Dietrich, Niederbronn, France), previously loaded with filtered deionized water (80 L). Cysteamine hydrochloride (3 kg) and hydrochloric acid (500 mL) were added, and the mixture was stirred at 80−85 °C for 2 h under continuous nitrogen flow (8 L/min). The reactor was then cooled, an additional 60 L of deionized water was added, and the mixture was pumped out through a grid. After filtration on a centrifugal system (Centrifugal Filter Riera Nadeu, SA, Motcada i Reixac, Catalonia, Spain) and bubbling with nitrogen, the resulting mixture S (crude thio conjugate mixture, ca. 140 L) was stored at 4 °C.

Partial Purification of Thio Derivatives and Scale-up. Trial runs were performed on a semipreparative FPLC system (Amersham-Pharmacia Biotech) with detection at 254 nm at a flow rate of 1−2 mL/min. Solvents used were as follows: [C], 0.2% acetic acid in deionized water; [D], 0.2% acetic acid in EtOH/water (1:4); and [E], acetone/water (3:2). The fractions obtained were analyzed by RP-HPLC on the Smart system as described above. The polymeric resins tested were SephadexLH-20 (12 g) washed with water/MeOH (1:1) for 2 h, packed into a glass 50 cm × 0.78 cm i.d. column (Omnifit, Cambridge, United Kingdom), 32 mL bed volume; XAD adsorbent resins, Amberlite XAD-1180, XAD-16, XAD-4, and XAD-761 (Rohm & Haas, Chauny, France) washed with EtOH (30 min) and water (30 min), and packed into a glass 100 cm × 0.78 cm i.d. column (Omnifit); 67 mL bed volume.

For scaled-up partial purification, Amberlite XAD-16 (Rohm & Haas) was used as follows: Amberlite XAD-16 (80 kg) was mixed with deionized water, and the resulting slurry was bubbled with nitrogen, stirred, loaded onto a stainless steel column (Maivisa, Vilassar de Dalt, Spain), 2.73 dm diameter, 14.8 dm height, 170 dm^3 column volume, and allowed to compact overnight (final volume ca. 120 dm^3). The resin was equilibrated with [C] (125 L) and loaded with mixture S (140 L), and the crude thio conjugate mixture was eluted with solvent [D] (175 L, ca. 1.5 bed volumes) to give mixture X (partially purified thio conjugate mixture containing compounds **1** and **2**). Then, the column was washed with solvent [E] (125 L). Mixture X was finally obtained as a solid by concentration and lyophilization.

Purification of 4β-(2-Aminoethylthio)epicatechin (1). Preparative (gram scale) RP-HPLC was performed on a Waters (Millipore Corporation, Milford, MA) Prep LC 4000 pumping system equipped with a Waters PrepPack 1000 module fitted with a 300 mm × 47 mm i.d. PrepPack cartridge filled with 300 Å pore size, 15−20 μm particle size C_{18} Vydac (The Separations Group, Hesperia, CA) stationary phase. Solid mixture X (2 g) was loaded onto the cartridge, and the elution was performed with solvents [F], triethylamine phosphate buffer (TEAP), pH 2.4, and [G], TEAP, pH 2.4/CH_3CN (2:3). The pure fractions containing **1** were pooled, loaded onto the same cartridge, and eluted with solvents [A] and [H], 0.08% (v/v) TFA in water/CH_3CN (2:3), under gradient mode from 3 to 23% [H] over 60 min at a flow rate of 100 mL/min with detection at 230 nm. The fractions were analyzed using a RP-HPLC system consisting of a LaChrom Merck-Hitachi L-7100 pump (E. Merck) fitted with a 250 mm × 4.6 mm i.d., 300 Å pore size, 5 μm particle size Vydac peptide and protein C_{18} column (The Separations Group, Hesperia, United States) and equipped with a Rheodyne (Cotati, CA) injector, a LC-75 spectrophotometer detector (Perkin-Elmer, Norwalk, CT), and a Merck-Hitachi (E. Merck) D-2000 integrator, eluted with solvents [A] and [B] described above under isocratic conditions at 15% [B] at a flow rate of 1 mL/min and detection at 214 nm. The fractions containing **1** were pooled and lyophilized to obtain 195 mg of pure product. The identity and purity of the final product were established by comparison with an authentic sample (*23*) using RP-HPLC, proton nuclear magnetic resonance (^{1}H NMR), and ion spray mass spectrometry.

Free Radical Scavenging Activity. The antiradical activity of solid mixture X was evaluated by the DPPH method (*24*, *25*). The samples (0.1 mL) of increasing concentrations ranging from 0.05 to 0.3 mg/mL were added to aliquots (3.9 mL) of a solution made up with DPPH

(4.8 mg) in MeOH (200 mL), and the mixtures were 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 517 nm of standard samples of DPPH at different concentrations. The equation for the curve was $Abs_{517nm} = 11345 \times C_{DPPH}$ as determined by linear regression. Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid) was used as a positive control. The results were plotted as the degree of absorbance decline at 517 nm $[(1 - A/A_0) \times 100]$ against the amount (micromoles) of sample divided by the initial amount (micromoles) of DPPH. Each point was repeated in triplicate. ED_{50} corresponds to micrograms of the mixture able to consume half the amount of free radical divided by micromoles of initial DPPH.

RESULTS AND DISCUSSION

Because the thio conjugates of epicatechin are new antioxidants with possible pharmaceutical applications and to obtain amounts appropriate for toxicological and preclinical studies, rapid and efficient preparation methods are of great interest. Efficient strategies must include both fast chemical reaction and simple purification procedures. In a previous work, we found that suitable reaction conditions were procyanidin extraction from grape pomace in water at 90 °C for 2 h followed by depolymerization in the same pot by addition of an excess of cysteamine hydrochloride (22 g/kg byproduct) and HCl (20 mL/L) and reaction at the same temperature for an extra 2 h (90 °C, 2 h of extraction + 2 h of depolymerization) with a conversion into the thio conjugates of 4 g/kg pomace (*22*).

In contrast to grape pomace, pine bark is available all year long, cheaper, more stable or less perishable, and easier to handle. To obtain the catechin thio conjugates in high yields and low costs, we first set out to evaluate the suitability of pine bark as an alternative source of polymeric procyanidins and second to look for fast and efficient purification strategies. The starting point were the conditions for the one pot extraction and depolymerization found for grape pomace. To monitor the reaction performance, three significant parameters, namely, the percentage of depolymerization (%DEP), the percentage of conversion (%CON), and total conversion (CON) were used. $\%DEP = [(mDP_n - 1)/(mDP - 1)] \times 100$, where mDP_n is the partial degree of polymerization calculated for a given sample (reaction incomplete) and mDP is the degree of polymerization obtained in methanol at 65 °C for 15 min (reaction considered complete). $\%CON = (nmol_n/nmol) \times 100$, where $nmol_n$ are the total nanomoles of depolymerized extension units (cysteamine conjugates) calculated for a given sample (reaction incomplete) and nmols are total nanomoles of depolymerized extension units in methanol with a large excess of cysteamine at 65 °C for 15 min (reaction considered complete). CON was calculated as the ratio (g/kg) between the total amount of cysteamine derivatives estimated by RP-HPLC and the total amount of byproduct.

Optimum Conditions for Extraction and Depolymerization. First, the excess of cysteamine necessary to obtain a good conversion into the conjugates, namely, **1** and 4β-(2-aminoethylthio)catechin (**2**), was estimated. The previous study with moist white grape pomace concluded that 22 g of cysteamine hydrochloride per kg of byproduct was an appropriate compromise between conversion and amount of reagent (*22*). Because the moisture amounted for approximately half of the pomace mass, this excess was about 44 g of cysteamine hydrochloride per kg of dry pomace. The pine bark was treated with increasing amounts of cysteamine hydrochloride in water (30 g bark/L, 90 °C, 2 h, and an extra 2 h after the addition of cysteamine). The major components (in parentheses relative proportions among them) of the mixtures were as follows: (+)-catechin (8.5 ± 1%), (−)-epicatechin (0.5 ± 0.1%), and their cysteamine conjugates **2** (18 ± 1%) and **1** (73 ± 2%). No gallate esters were detected. The conversions obtained are depicted in **Figure 2**. The partial conclusion was that 166 g/kg of cysteamine appeared adequate since conversion increased only 2-fold upon a 10-fold increase in the excess of cysteamine (up to 1666 g/kg). Pine bark gave conversions much higher than grape pomace at similar excesses of cysteamine (e.g., 16 g **1** + **2**/kg bark vs 4 g **1** + **2**/kg pomace with an excess of cysteamine of 75 g/kg), meaning that pine bark was richer than white grape pomace in depolymerizable procyanidins. It is known that the amount of catechin monomers, oligomers, and other small phenolics (i.e., fraction soluble in ethyl acetate) is similar for both sources except that grape procyanidins are partly galloylated (presence of gallate esters) while pine bark procyanidins are not (*26*, *27*). The fact that the conversion to depolymerized conjugates is significantly higher for pine bark indicates that this source must be richer than white grape pomace in water soluble bulky procyanidin polymers. The results from this study show that pine bark is more suitable than grape pomace as raw material for the preparation of catechin and epicatechin thio conjugates because, apart from being permanently available and easy to handle, it is a richer source of polymeric procyanidins.

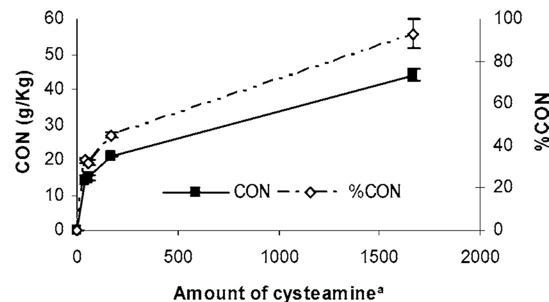

Figure 2. Dependence of procyanidin conversion after one pot extraction (2 h) and depolymerization (2 h) from pine bark with the amount of cysteamine. [a]Milligrams of cysteamine hydrochloride per gram of pine bark. Means of four samples ± SEM.

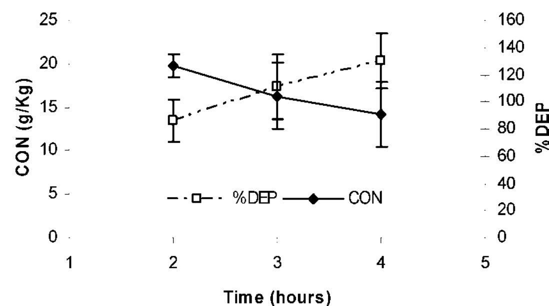

Figure 3. Dependence of procyanidin depolymerization in water with time after simultaneous one pot extraction and depolymerization from pine bark. Reaction conditions were 30 g bark/L, 166 g cysteamine/kg bark, and 20 mL/L HCl at 90 °C. Means of three samples ± SEM.

To simplify the preparation of the cysteamine conjugates, we performed the procyanidin extraction and depolymerization in a single step by adding the depolymerizing reagents from the beginning. Moreover, to minimize costs, the reaction times and concentrations of bark and acid were optimized. **Figure 3** summarizes the outcome of the reaction (%DEP and CON) at three different times. After 2 h, the conversion and depolymerization were similar to those obtained using the 2 h extraction + 2 h depolymerization strategy. Then, conversion decreased with time while depolymerization increased. As suggested

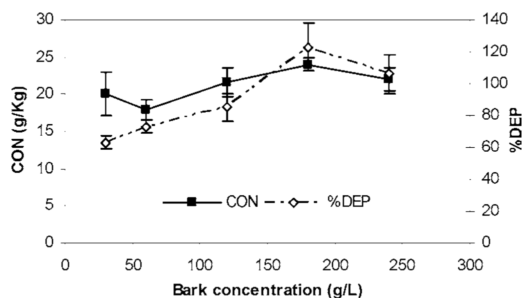

Figure 4. Dependence of procyanidin depolymerization with pine bark concentration after simultaneous one pot extraction and depolymerization in water. Means of 3–5 samples ± SEM.

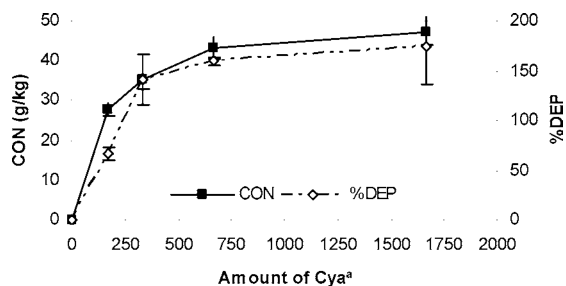

Figure 5. Dependence of procyanidin depolymerization with the amount of cysteamine upon simultaneous one pot procyanidin extraction/depolymerization in water. [a]Milligrams of cysteamine hydrochloride per gram of pine bark. Means of 3–6 samples ± SEM.

before, longer times allow for more extensive depolymerization but conversion decreases due to degradation of the products at high temperatures (*22*). In consequence, a reaction time as short as 2 h was chosen. The second variable tested was the bark concentration. Reactions were set up at different concentrations, and results are shown in **Figure 4**. Higher concentrations were not tested because 240 g/L was found to be the maximum concentration that allowed wetting the whole bark. Depolymerization and conversion increased up to a concentration of 180 g/L. The reaction performance was slightly poorer at 240 g/L probably due to a lower accessibility of the reagents. The third variable was acid concentration. We found that the amount of acid could be lowered down to 5 mL/L with no loss of conversion.

Once the conditions for the simultaneous extraction−depolymerization of pine bark were set up (water, 240 g bark/L, and 5 mL/L HCl, 90 °C, 2 h), the variation of conversion with the excess of cysteamine hydrochloride was tested again at different ratios cysteamine/bark. The results are shown in **Figure 5**. As compared to **Figure 2**, a higher amount of reagent was needed to reach the plateau. Again, the conversion was only doubled upon a 10-fold increase in the excess of cysteamine. An amount of cysteamine hydrochloride between 166 and 333 g per kg of pine bark was considered adequate for preparative purposes.

Partial Purification of 4*β*-(2-Aminoethylthio)epicatechin with Polymeric Resins. Cation exchange separation of the aminoethylthio derivatives on a strong cationic exchanger (Macro Prep High S) eluted with sodium phosphate pH 2.27 buffer or unbuffered 0.5% acetic acid in the presence of ethanol followed by preparative RP-HPLC had been previously proposed (*22*, *28*). This is a very convenient procedure at analytical scale, particularly when using simple devices such as functionalized centrifugal membranes (*29*). On the other hand, at preparative scale, a considerable amount of material is nonspecifically retained in the columns causing mechanical problems and shortening the column useful life. To simplify the preparation of the thio conjugates, particularly the most abundant, **1**, a simple cleanup step with adsorption supports such as Sephadex LH-20 and Amberlite XAD resins was considered. Low-pressure liquid chromatography using Sephadex LH-20 has been used in various studies to separate phenolic compounds that occur in wine, strawberry, rapeseeds, and other plant sources (*30*−*32*). Adsorbent XAD resins have been used for the fractionation of phenolic compounds from olive mill wastewater (*33*) or apple extracts (*34*). The resins (Sephadex LH-20, Amberlite XAD-4, XAD-16, XAD-761, and XAD-1180) were packed into semipreparative columns and loaded with mixture S. The conjugates were eluted with stepwise increasing concentrations of ethanol. The aims were to maximize load and minimize dilution using the lowest possible amount of ethanol. **Table 1** summarizes the results with the different adsorbents. The conjugate was initially retained and eluted with no need of ethanol on LH-20 and XAD-761 but either the load capacity was too low on LH-20 or the resin (XAD-761) was clogged. The latter was the resin with the highest pore diameter (600 Å) and the lowest surface area. Some polymeric material from the mixture may have irreversibly entered the pores and eventually blocked the solvent flow. On XAD-4, the product was eluted over 2.5 bed volumes and up to 80% ethanol was needed. In this case, the pore size (100 Å) was probably too low. The bulkier material would be excluded from the pores and efficiently retained hydrophobicity, but the small target compounds would easily enter the pores causing extended times of residence inside the column. Intermediate situations with pore sizes between 100 and 600 Å appeared to be preferable for this particular case where relatively small molecules must be separated from complex mixtures containing polymeric material. XAD-1180 yielded the conjugate with minimum dilution and a moderate amount (20%) of ethanol, but the load was low (132 mg). The best results were obtained with XAD-16: a maximum load (264 mg of procyanidins) with moderate dilution (1.73 bed volumes) and a moderate amount (20%) of ethanol.

Table 1. Partial Purification of **1** with Different Adsorbent Resins

resin	surface area (m^2/g)[a]	avg pore diameter (Å)[a]	bed volume (mL)	max load (mg)	max concn (g/L)	elution (bed volumes)	% EtOH
LH-20			32	25	2.5	1	0
XAD-1180	700	400	72	132	4.4	1.35	20
XAD-16	800	150	65	264	4.4	1.73	20
XAD-4	750	100	67	220	4.4	2.5	20–80
XAD-761	200	600	68	220	4.4	1.5	0

[a] Data available from Rhom and Haas.

Scaled-up Preparation of 1. To obtain multigram amounts of **1**, the optimum experimental conditions found were scaled-up in a pilot plant for 17 kg of milled dry pine bark. According to the results obtained in this study, the conditions chosen for the simultaneous one pot extraction/depolymerization were 240 g bark/L water, 20 mL of HCl, and 166 g of cysteamine hydrochloride per kg pine bark, 90 °C, 2 h under nitrogen atmosphere. The conversion into **1** was approximately 22 g Cya-Ec/kg of pine bark (374 g total), estimated by analytical RP-HPLC from a standard curve. This was approximately 5-fold higher than the conversion obtained with white grape pomace (*22*). Then, the crude thio conjugate mixture containing **1** was partially purified on XAD-16 by scaling up the conditions found before. Mixture S (ca. 140 L) was loaded onto a stainless steel column fitted with ca. 80 kg of resin (ca. 120 L). Elution with 1.5 bed volumes of 0.2% acetic acid in water/ethanol (4:1) gave

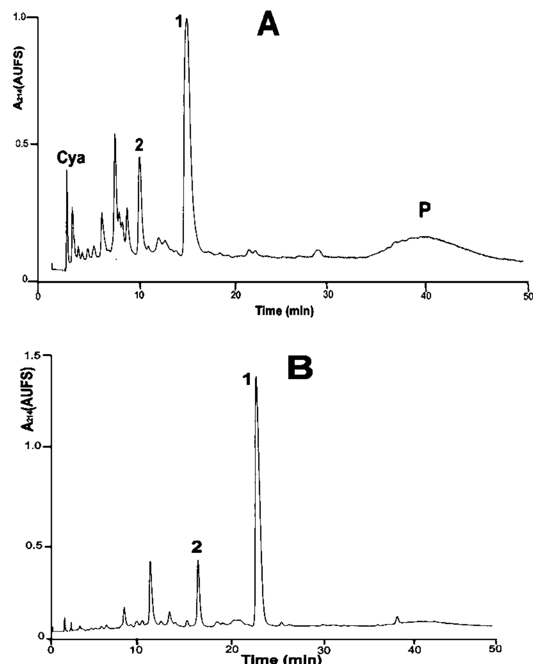

Figure 6. RP-HPLC profile of the mixture obtained after extraction and depolymerization of pine bark. (**A**) Mixture S. Load 5 μL of the mixture diluted 1/16 with 0.10% TFA. (**B**) Mixture X. Load 5 μL of the mixture diluted 1/10 with 0.10% TFA. Column μRPC SC 2.1/10 C2/C18, 3 μm particle size (100 mm × 2.1 mm i.d.). Eluents: [A], 0.10% (v/v) aqueous TFA; [B], 0.08% (v/v) TFA in 1:4 water–CH_3CN. Elution with a gradient of 8–18% [B] over 30 min and 18–50% [B] over 20 min, at a flow rate of 200 μL/min. Detection at 214 nm. Cya, cysteamine; P, polymeric material.

mixture X. After lyophilization of an aliquot, **1** was obtained with 35% purity (0.35 g **1** per g of mixture X) and 70% purity by RP-HPLC profile. The CON calculated by RP-HPLC, after extraction, depolymerization, and partial purification, was ca. 15 g **1**/kg of pine bark. **Figure 6** shows the analytical RP-HPLC profile of the mixtures before and after partial purification on XAD-16. This resin appears to be an adequate option for an efficient cleanup step previous to the high-resolution purification with more expensive and delicate stationary phases. This mixture shows antiradical efficiency (DPPH assay, $ED_{50} = 45$ expressed as μg/μmoles initial DPPH), similar to the 98.5% pure product obtained before ($ED_{50} = 40$) (*22*). This means that accompanying minor components were also active scavengers and the whole mixture may be used as such for some applications.

To prove its structure, compound **1** was then thoroughly purified from mixture X by preparative RP-HPLC to obtain the final product with a purity of 99%. The same procedure was applied to the preparation of the cysteine derivative 4β-cysteinyl-epicatechin with similar results.

In conclusion, we have significantly improved the preparation of biologically active **1** with respect to the methods reported before. Pine (*P. pinaster*) bark is a rich and easy to handle source of polymeric procyanidins, which can be readily depolymerized in a single step in water at 90 °C for 2 h in the presence of cysteamine hydrochloride (166 g/kg bark). Moreover, a simple cleanup step on XAD-16 resulted in multigram amounts (15 g/kg bark) of the potent free radical scavenger and biologically active compound with a purity of 35% (0.35 g/g). We are currently exploring the application of the simultaneous one pot procedure and separation with polymeric resins to other sources of procyanidins.

ABBREVIATIONS USED

DPPH, diphenyl-2-picrylhydrazyl free radical; Trolox, 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid.

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