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Efficient One Pot Extraction and Depolymerization of Grape (*Vitis vinifera*) Pomace Procyanidins for the Preparation of Antioxidant Thio-Conjugates

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Antioxidant thio-conjugates were obtained from white grape pomace by a clean and efficient one pot extraction and depolymerization method using water and cysteamine hydrochloride. To evaluate the potential of grape pomaces of different origins as sources of proanthocyanidins and conjugates, we conducted varietal comparative studies of polyphenol content, antioxidant power, and procyanidin composition. Xarel·lo proved to be the richest source of polyphenols. The total conversion into the conjugates was as high as 8 g/kg of Xarel·lo grape pomace, with a 50-fold excess of cysteamine, and 3 g/kg, with a 5-fold excess of cysteamine. After purification by preparative cation exchange and reversed phase high-performance liquid chromatography, 17 g of 63% pure 4β-(2-aminoethylthio)-epicatechin (acetate salt) was obtained from 35 kg of moist pomace. The procedure described here will make the antioxidant thio-derivatives efficiently available directly from raw plant byproducts such as grape pomace.

KEYWORDS: Grape; *Vitis vinifera*; polyphenols; procyanidins; catechins; flavanols; antioxidants; free radical scavenging

INTRODUCTION

Polyphenols are ubiquitous compounds in plants. Thus, most byproducts generated by agricultural activities, forestry, and related industries contain polyphenols, which have putative applications as food antioxidants and preventative agents against numerous diseases (*1*, *2*). Moreover, there is increasing public awareness of the need to minimize wastes by fostering the integral exploitation of natural resources (e.g., by recovering ubiquitous antioxidants) and to design sustainable processes based on renewable materials. Ideally, sustainability must be economically viable, apart from being environmentally advantageous. The efficient recovery of bioactive species (e.g., antioxidant polyphenols) converted into high value-added products is a challenge for scientists and technologists alike. Currently, a variety of health-promoting products obtained from plant byproducts are on the market and a great deal of research efforts is being devoted to testing the putative beneficial effects of grape, tea, and pine bark polyphenols (*3*−*14*). We have already proposed a method for the recovery of valuable antioxidant polyphenols by means of the formation of new bio-based conjugates from polymeric material (procyanidins) and thiol-containing molecules (e.g., cysteamine) (**Figure 1**), which can be separated from the crude mixtures by cation exchange (*15*, *16*). The new conjugates are promising products since they are more potent than their underivatized counterparts and they include ionic groups, which may be used to modulate their action within different physicochemical and biological environments. We have prepared the conjugates from different plant byproducts including white grape (*Vitis vinifera*) pomace (skin, seeds, and stems), pine bark, or wastewater from almond peeling, by depolymerization in methanol or water (*17*). Grape pomace has yielded three major conjugates, namely, 4β-(2-aminoethylthio)-epicatechin (**1**), 4β-(2-aminoethylthio)catechin (**2**), and 4β-(2-aminoethylthio)epicatechin-3-*O*-gallate (**3**) (**Figure 1**) from Parellada grapes (*15*, *17*). Pine bark was richer in polymeric procyanidins but was devoid of gallate esters (*17*). So far, the conjugates have been prepared in methanol from purified extracts and fractions. We have now investigated the depolymerization of grape procyanidins in water directly from the pomace of Xarel·lo grapes, a variety selected after comparative studies.

MATERIALS AND METHODS

Materials. The raw plant materials, provided by Miguel Torres, S. A. (Vilafranca del Penedès, Spain) consisted of grape pomace, the byproduct from pressing destemmed grapes (*V. vinifera*), and included skins, seeds, and a small amount of stems. The byproduct from different varieties was collected within the boundaries of the Denomination of Origin Penedès in the month of October during the 2000 harvest, cooled immediately after pressing, and frozen at −20 °C. Pomace from Xarel·lo grapes collected during the 2001 harvest was used for the one pot extraction and depolymerization optimization studies and the scale-up procedure. The polyphenolic fraction **OW**, soluble in both ethyl acetate

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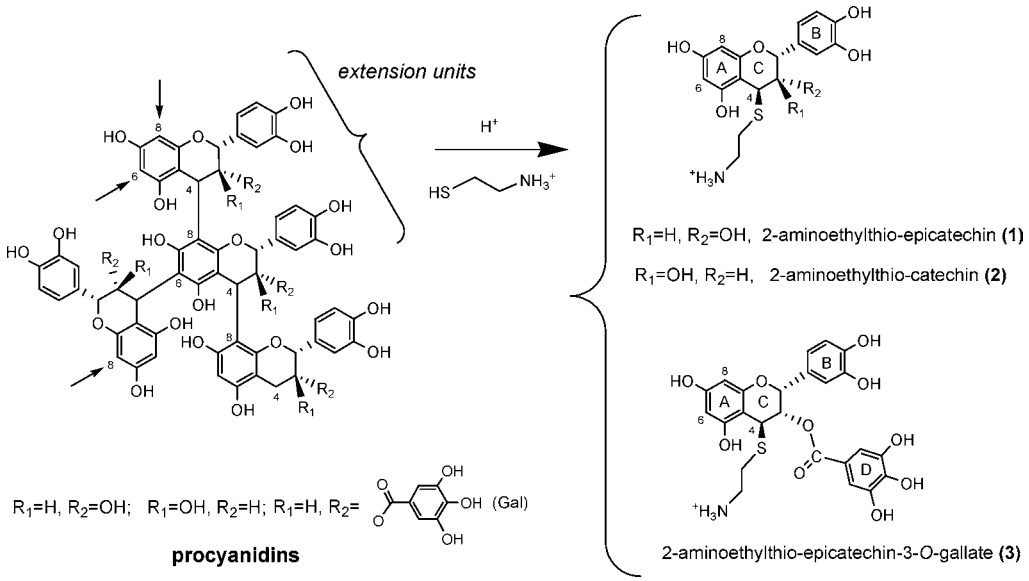

Figure 1. Depolymerization of procyanidins in the presence of cysteamine. Structure of the starting polymers and the resulting cleaved monomers and conjugates.

and water, was obtained from Parellada pomace as described (*15*). Water and solvents used were as follows: analytical grade MeOH, EtOH (Panreac, Montcada i Reixac, Spain), and deionized water for analytical and preparative extractions and depolymerizations; deionized water and bulk EtOH (Montplet, Barcelona, Spain) for cation exchange chromatography; deionized water and preparative grade CH_3CN (Scharlau, Barcelona, Spain) for preparative RP-HPLC (reversed-phase high-performance liquid 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, U.K.) biotech grade was distilled in-house. DPPH (95%) was obtained from Aldrich (Gillingham-Dorset, U.K.), and Trolox (97%) was obtained from Aldrich (Milwaukee, WI). Cysteamine hydrochloride (98%) was from Sigma-Aldrich Chemie Gmbh (Steinhem, Germany).

Extraction of Polyphenols from Pomaces of Different Varieties. Grape pomace (10 g) from the different white grape varieties was thawed, homogenized in a blender, and extracted with water/EtOH (3:7) (20 mL) on a shaker for 12 h (*14*). The mixture was then centrifuged, and the supernatant was decanted and filtered through a 0.45 μm membrane.

Polyphenol Content and Procyanidin Composition. The polyphenolic content of the crude extracts was roughly estimated by measuring the absorbance at 280 nm. The size and composition of the procyanidins within the pomaces of different varieties of white grapes were estimated from the RP-HPLC analysis of the depolymerized extracts, essentially as described (*18*, *19*), except that cysteamine was used instead of toluene-α-thiol (*20*). Briefly, the terminal flavan-3-ols units were released as such by acid cleavage in the presence of cysteamine whereas the extension moieties were released as the C-4 cysteamine derivatives. The cleaved mixtures 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 100 mm × 2.1 mm i.d. μRPC C2/C18 SC 2.1/10 column. Elution: [A] 0.10% (v/v) aqueous TFA, [B] 0.08% (v/v) TFA in water/CH_3CN (1:4), gradient 8−23% [B] over 45 min. The flow rate was 200 μL/min. The detection was done by triple wavelength at 214, 280, and 320 nm. Phenolic compounds were usually detected at 280 nm. At 214 nm, many possible impurities (e.g., peptides) were detected with high sensitivity. Detection at 320 nm allowed the identification of the gallate containing flavan-3-ols. Quantitative determination of the major components was done from calibration curves of the corresponding peak areas, made up with pure standards prepared as described (*15*). The parameters calculated were as follows: mean degree of polymerization (mDP) = total nmol/nmol of terminal units; percentage of galloylation (% G) = 100 × ([nmol of (−)-epicatechin-gallate + nmol of cysteamine-epicatechin-3-*O*-gallate]/total nmol). The procedure was essentially the same used for monitoring the depolymerization performance under different conditions and the preparation of 4β-(2-aminoethylthio)-epicatechin.

Free Radical Scavenging Activity. The antiradical activity of the crude extracts was evaluated by the DPPH method (*21*, *22*). 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 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 $Abs_{517nm} = 11\,345 \times C_{DPPH}$ as determined by linear regression. Trolox 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 volume (μL) or amount (μmol) of sample divided by the initial amount (μmol) of DPPH. Each point was repeated in triplicate. A dose−response curve was obtained for every crude mixture. ED_{50} corresponds to microliters of crude or micromoles of pure product able to consume half the amount of free radical divided by micromoles of initial DPPH. The results are also expressed as antiradical power (ARP) or $(1/ED_{50}) \times 10^2$.

Preparative Depolymerization of Procyanidins. To set up depolymerization conditions in water, the model fraction **OW** (1 mg) was dissolved in deionized water (1 mL) and the depolymerization mixture (950 μL) was added. The depolymerization mixtures were solutions of HCl (200 μL) and variable amounts of cysteamine hydrochloride in water (10 mL).

To set up conditions for the one pot extraction/depolymerization, pomace from Xarel·lo grapes (3 g) was suspended in deionized water (10 mL) and the mixture was kept at 90 °C for 2 h. Then, HCl (200 μL) and the appropriate amount of cysteamine hydrochloride were added and the mixture was kept at the same temperature for 2 h.

For the final scaled-up process, frozen moist pomace (35 kg) from Xarel·lo grapes was placed in a stainless steel reactor (200 L) built at Miguel Torres SA, previously loaded with filtered deionized water (123 L) and dry ice (0.5 kg). The mixture was heated and kept at 85 °C for 2 h under vigorous stirring. Then, a solution of cysteamine hydrochloride (760 g) in water (1.5 L) and hydrochloric acid (1.3 L) were added and the mixture was stirred at 80−85 °C for an additional 2 h. The reactor was then cooled, and the mixture was pumped out through a grid. After bubbling with nitrogen, filtered mixture **S** (125 L) was immediately frozen (−20 °C).

Preparative Cation Exchange Chromatography. Semipreparative cation exchange chromatography runs were performed on an FPLC system (Amersham-Pharmacia Biotech) fitted with an Omnifit (Cam-

bridge, U.K.) 8 cm × 1 cm i.d., 6 mL bed volume column filled with Macro Prep High S (Bio-Rad Laboratories, Hercules, CA). Mixture **S** was loaded after centrifugation, and the solutes were eluted with a binary system: [C] water/EtOH (7:3) 0.5% acetic acid; [D] water/EtOH (3:2), 0.5% acetic acid, 0.2 M NaCl. Elution conditions: 30 mL (5 bed volumes) of [C] to wash out the monomeric flavan-3-ol units and 30 mL (5 bed volumes) of [D] to obtain mixture **X**, which contained the 2-aminoethylthio-flavan-3-ols. The flow rate was 2 mL/min, and the detection wavelength was 254 nm.

Preparative cation exchange chromatography was performed on a flash chromatography type glass column (8 cm i.d.) filled with Macro Prep High S (26.5 cm bed height, 1 L bed volume). Mixture **S** was chromatographed in multiple runs (5 L load per run) after filtering through glass wool. Elution was carried out with 5 L (5 bed volumes) of [C] followed by 5 L of [D]. After each run, the resin was regenerated with a mixture of 0.5% acetic acid in water/EtOH (3:2), 1 M NaCl. The operation was repeated 17 times until the whole mixture was consumed (85 L). The fractions eluted with [D] were pooled (85 L of mixture **X**), and the volume was reduced to 7 L by evaporation at 35 °C under vacuum in a pilot plant at the Centre de Química Fina (A+/LGAI, Bellaterra, Spain).

Preparative Reversed Phase Chromatography. Preparative 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. The elution was performed with solvents [A] and [B] described above under gradient mode from 1 to 16% [B] over 45 min at a flow rate of 90 mL/min with detection at 225 nm. Repetitive runs (150 mL load each) were made until the whole mixture **X** (7 L) was processed. The pure fractions (elution between 12 and 16% [B]) were pooled (45 L), and the volume was reduced up to 22 L by evaporation under vacuum. To exchange the counterion from trifluoroacetate to acetate, aliquots (2 L) of the purified fraction containing 4β-(2-aminoethylthio)epicatechin (**1**) were loaded onto the same cartridge and washed with 0.5% (v/v) aqueous acetic acid (0.9 L), and compound **1** was eluted with 0.5% (v/v) acetic acid in water/acetonitrile (17:8). The operation was repeated 11 times in order to process the whole 22 L. The fractions from the 4β-(2-aminoethylthio)epicatechin purification process were analyzed using a RP-HPLC system consisting of a LaChrom Merck-Hitachi L-7100 pump (E. Merck) equipped with a Rheodyne (Cotati, CA) injector, a LC-75 spectrophotometer detector (Perkin-Elmer, Norwalk, CT), a Merck-Hitachi (E. Merck) D-2000 integrator, and 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, U.S.A.). The compounds were eluted with solvents [A] and [B] described above, under isocratic conditions at 12% [B] and a flow rate of 1.5 mL/min with detection at 214 nm. The 4β-(2-aminoethylthio)epicatechin (**1**)-containing fractions were pooled and lyophilized.

RESULTS AND DISCUSSION

ARP and Composition of White Grape Pomaces from Different Varieties. To choose the richest source of procyanidins for the preparation of thio-conjugates, the pomaces from different varieties of white grapes grown in the Penedès area were extracted with water/EtOH (3:7) and their antiradical activity and procyanidin composition were evaluated. The polyphenolic fraction from grape seeds, skins, and stems is composed mainly of catechins, proanthocyanidins (mostly procyanidins), and glycosylated flavonols (*19*, *23*−*25*). **Figure 2** compares the capacity of the extracts as scavengers of the DPPH radical (**Figure 2A**) with parameters related to their polyphenolic content and composition (**Figure 2B**−**D**). These include UV absorbance at 280 nm, an approximation to the total polyphenolic content, the mDP, and the % G of the procyanidins within the fractions. These parameters may be related to the antiradical capacity of the mixtures (*25*−*27*). Here, the ARP

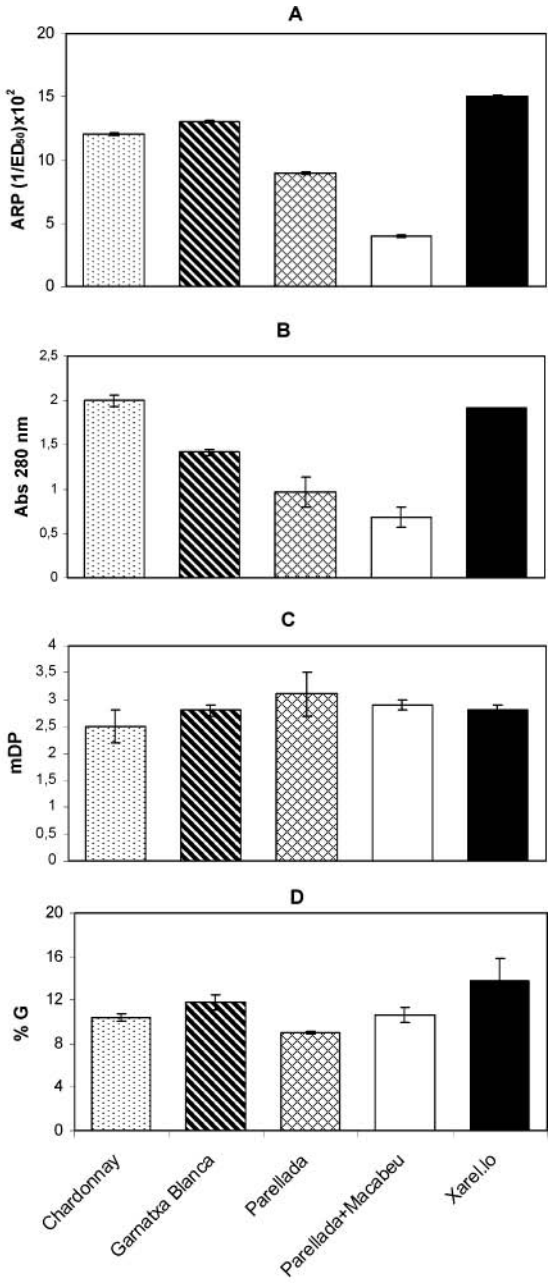

Figure 2. Varietal comparison of grape pomaces. (**A**) ARP, antiradical power; (**B**) absorbance at 218 nm; (**C**) mDP, mean degree of polymerization; (**D**) % G, percentage of galloylation. Mean of 2–3 samples. Bars indicate standard error of the mean (SEM) intervals.

(**Figure 2A**) and the procyanidin size expressed as mean degree of polymerization (**Figure 2C**) did not seem to be related. There was some coincidence in the order of DPPH scavenging power and the order of both total phenolic content expressed as absorbance at 280 nm (**Figure 2B**) and galloylation (**Figure 2D**). Particularly, the order of ARP and % G was the same: Xarel·lo > Garnatxa blanca (White Grenache) > Chardonnay > Parellada, with the exception of the crude from Parellada + Macabeu. The results are in agreement with the fact that the pyrogallol group (three contiguous aromatic hydroxyls) present in the gallate esters is a more efficient radical scavenger than the catechol group (two contiguous aromatic hydroxyls) present in catechin and epicatechin (*28*, *29*). Although pomace from the combined varieties Parellada + Macabeu showed a high percentage of galloylation, its ARP was low probably due to its very low total phenolic content (**Figure 2B**). Most of the

galloylation (ca. three-fourths) corresponded to (−)-epicatechin-3-*O*-gallate coming from free monomers and/or proanthocyanidin terminal units. 4β-(2-Aminoethylthio)epicatechin-3-*O*-gallate from the extension units accounted for ca. one-quarter of the galloylation (~3−4% of the total cleaved units).

In view of the results from the varietal study, Xarel·lo appeared to be the richest source of procyanidins, with the highest percentage of galloylation, among the varieties from the Penedès 2000 harvest compared in this study.

Procyanidin Depolymerization in Water. In the literature, the acid-catalyzed cleavage of proanthocyanidins is usually carried out in methanol with a 25−50-fold excess of thiol with respect to the amount of proanthocyanidins both for analytical applications (*18*, *19*, *23*, *24*) and on a preparative scale (*30*). 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 or 2 min at 90 °C (*24*). We have described the procedure of acid-catalyzed cleavage of procyanidins in the presence of cysteamine as a rapid, efficient, and convenient alternative to thiolysis with toluene-α-thiol (*31*) and demonstrated that a 3−5-fold excess of thiol (cysteamine) was enough to consume most of the polymeric material in 15 min at 60−65 °C (*15*) on a preparative scale, using MeOH as solvent.

Because the conjugates appear to be interesting new antioxidants with possible industrial application and to obtain amounts appropriate for toxicological and preclinical studies, we set out to devise a protocol more suitable for the preparative scale. Initially, we investigated replacing MeOH with water. The procyanidin cleavage in water is slower than in MeOH (*32*). To establish suitable conditions in water, the depolymerization reaction was carried out with the model polyphenolic fraction **OW** at three temperatures (30, 60, and 90 °C) for five reaction times (15 and 45 min and 2, 6, and 24 h). The resulting mixtures were analyzed by RP-HPLC. The major components (relative proportions in parentheses) of the mixtures were (+)-catechin (36%), (−)-epicatechin (16%), (−)-epicatechin-3-*O*-gallate (16%), and their cysteamine conjugates 4β-(2-aminoethylthio)catechin (**2**) (8%), 4β-(2-aminoethylthio)epicatechin (**1**) (26%), and 4β-(2-aminoethylthio)epicatechin-3-*O*-gallate (**3**) (7%) (**Figure 1**). To monitor the reaction performance, two significant parameters were defined, namely, the percentage of depolymerization (% DEP) and the percentage of conversion (% CON). % 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). The % CON = $(nmol_n/nmol) \times 100$, where $nmol_n$ is the total nanomoles of depolymerized extension units (cysteamine conjugates) calculated for a given sample (reaction incomplete) and nmol is the total nanomoles of depolymerized extension units in methanol at 65 °C for 15 min (reaction considered complete). **Table 1** summarizes the results of the temperature/time study. At 30 °C, both depolymerization and conversion were poor. At 60 °C, depolymerization progressed whereas conversion increased for 6 h and slowly decreased afterward. At 90 °C, depolymerization progressed also for 6 h, but conversion decreased even after the first hour. After 1 day, the conjugated compounds were hardly detectable by HPLC while a myriad of smaller peaks appeared. A temperature of 90 °C was considered as a good starting point for further studies because it provided high conversions over relatively short periods of time. To evaluate more accurately the reaction time needed to reach a high conversion at 90 °C, we submitted the same fraction **OW** to acid cleavage with a 5-fold (w/w) excess of cysteamine for periods of 1, 2, 4, and 6 h. The results are shown in **Figure 3**. Conversions between 80 and 100% in 1 or 2 h were the best obtained. After 1 h of reaction, variability was too high but after that point results were more reproducible. In agreement with the results of the previous experiment at different temperatures (**Table 1**), depolymerization progressed for 6 h while conversion decreased significantly. In fact, conversion decreased even after 1 h of treatment, probably due to the lability of the monomers and monomeric conjugates at high temperature. The conversion did not improve by exhaustive elimination of oxygen before the reaction. The disappearance of end products might be due to the action of other pro-oxidants or to some other kind of reaction such as polymerization involving nucleophilic and electrophilic positions on rings A and B (*33*). This latter possibility would be supported by the observation that the nucleophilic character of positions 6 and 8 on ring A (**Figure 1**) might be enhanced by thio-derivatization at C-4 (*16*). Another possible side reaction leading to other cysteamine derivatives and/or oligomers is the formation of C-2 adducts with nucleophiles (e.g., the thiol or positions C-6, C-8 on ring A) as suggested in the instance of depolymerization with phloroglucinol (*34*). In any case, the best reaction conditions should result from a compromise between the extent of depolymerization and the formation of byproducts. The partial conclusion was that in water, the best depolymerization conditions were 90 °C for 2 h with a conversion around 83%, corresponding to 0.47 g of monomers per gram of polyphenols.

Table 1. Procyanidin Depolymerization in Water[a,b]

	30 °C		60 °C		90 °C	
	% DEP	% CON	% DEP	% CON	% DEP	% CON
15 min		46 ± 7	17 ± 2	49 ± 2	64 ± 1	75 ± 7
45 min		41 ± 4	29 ± 2	52 ± 2	81 ± 2	84 ± 2
2 h	6 ± 1	45 ± 7	46 ± 2	60 ± 2	94 ± 1	61 ± 7
6 h	7 ± 1	46 ± 3	80 ± 8	70 ± 7	116 ± 17	45 ± 7
24 h	24 ± 1	48 ± 2	98 ± 3	64 ± 3		

[a] Experiments carried out with model polyphenolic fraction **OW**. [b] Mean of three samples, one injection per sample ± SEM.

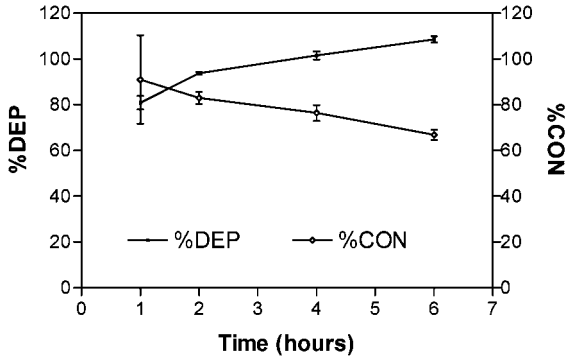

Figure 3. Dependence of procyanidin depolymerization in water with time. Cleavage in methanol at 65 °C for 15 min was considered to give 100% depolymerization and conversion. Mean of four samples ± SEM.

The experiments performed so far were set up with a 5-fold (w/w) excess of cysteamine with respect to **OW**, which had been found to be adequate for depolymerizations in methanol (*15*). To evaluate the efficiency of the depolymerization with the excess of cysteamine in water, fraction **OW** was submitted to cleavage at 90 °C for 2 h in the presence of 2-, 5-, 10-, and 50-fold excess of thiol. The results are presented in **Figure 4**. In water, % DEP increased progressively from samples treated

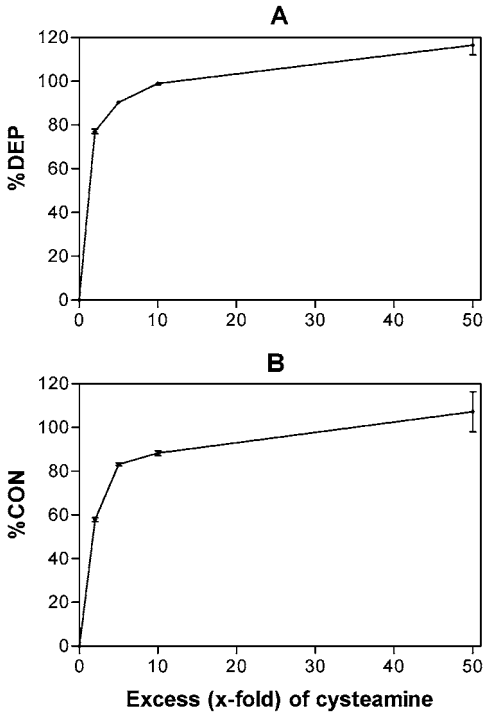

Figure 4. Dependence of procyanidin depolymerization in water with the excess of cysteamine. Cleavage in methanol at 65 °C for 15 min was considered to give 100% depolymerization and conversion. Mean of three samples ± SEM.

with 5-fold excess of cysteamine to those treated with 50-fold excess, a behavior that was not observed in methanol. The most extensive depolymerization was obtained with a 50-fold excess of reagent. However, an order of magnitude increase in excess cysteamine, from 5- to 50-fold, led only to a modest 26% increase in percentage of depolymerization (**Figure 4A**). Similarly, only small differences in percentage of conversion, which estimates the actual amount of endproducts generated, were found between samples treated with excesses of cysteamine from 5- to 50-fold (**Figure 4B**). Samples treated with a 2-fold excess of reagent gave significantly lower % DEP and % CON. This is in agreement with the results obtained with methanol (*15*). The partial conclusion was that a 5-fold excess of cysteamine hydrochloride with respect to the total amount of polyphenols was adequate for the preparative depolymerization of procyanidins in water at 90 °C, with a conversion of 83%.

To summarize the results with the model fraction **OW**, depolymerization was less effective in water than in methanol but this could be counterbalanced with extended reaction times (**Figure 3**) as well as with an appropriate excess of reagent (**Figure 4A**). For preparative purposes though, extended reaction times are not adequate since conversion decreases significantly with time (**Figure 3**) and high reagent excesses are economically unfavorable as well as unnecessary (**Figure 4B**). A treatment at 90 °C for 2 h in the presence of a 5-fold excess of reagent was considered adequate.

One Pot Extraction and Depolymerization of Procyanidins from Xarel·lo Pomace. To simplify the preparation of the cysteamine conjugates, depolymerization was tried directly on the grape pomace. In view of the results of the varietal study, pomace from Xarel·lo was chosen for the one pot treatment. Tests were run with 3 g of Xarel·lo pomace in water (10 mL). First, the polyphenols were extracted at 90 °C for 2 h, and then, the mixtures were treated with increasing amounts of cysteamine hydrochloride (3, 5, 18, and 185 mg/g of pomace) under the

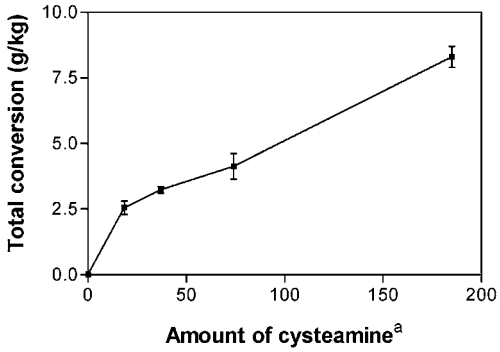

Figure 5. Dependence of the total conversion with the amount of cysteamine upon one pot procyanidin extraction/depolymerization in water. [a]Milligrams of cysteamine hydrochloride per gram of grape pomace. Mean of 3–6 samples ± SEM.

conditions found previously (90 °C, 2 h). The excess of cysteamine per gram of pomace was estimated from the polyphenolic yield obtained by maceration with water/EtOH (3:7) (*15*), where the total amount of polyphenols obtained was about 4 g/kg of byproduct. The highest excess of cysteamine (185 mg/g byproduct) would correspond approximately to a 50-fold excess of cysteamine with respect to the estimated amount of polyphenols. Because the goal was to obtain the conjugates and it was not clear what should be considered 100% conversion for the one pot procedure, we preferred to express the results as total conversion into cysteamine derivatives. The total conversion was calculated as the ratio (g/kg) between the total amount of cysteamine derivatives estimated by RP-HPLC and the total amount of byproduct. The results, shown in **Figure 5**, were roughly consistent with the previous estimate of the excess of cysteamine as seen by comparing **Figures 4B** and **5**. Again, because the conversion was only doubled upon a 10-fold excess increase, an amount of cysteamine between 20 and 30 mg per gram of pomace was considered adequate for preparative purposes.

Extraction and Depolymerization Scale-up. The experimental conditions found were applied to scale-up the preparation of gram amounts of 4β-(2-aminoethylthio)epicatechin (**1**), the major component of the depolymerized mixtures, to be used in preliminary toxicological studies. The conditions chosen were as follows: water extraction for 2 h at 90 °C, followed by depolymerization for 2 h at 90 °C in the presence of 37 mL of HCl and 22 g of 98% pure cysteamine hydrochloride per kg Xarel·lo pomace. To reduce the cost of the final product, a less pure cysteamine could be used at industrial scale. The procedure was scaled-up for 35 kg of moist pomace. **Figure 6** shows the RP-HPLC profile of the cleavage mixture **S** obtained after the one pot extraction and depolymerization. The total conversion was 3.6 g of conjugates per kg of pomace. The percentage of galloylated epicatechin derivative in the mixture was 4%. This value coincided roughly with the results obtained in the varietal study after ethanolic extraction of the phenolic fraction from blended pomace and subsequent depolymerization in methanol. In contrast, underivatized (−)-epicatechin-3-*O*-gallate was not detected in the depolymerization mixture obtained by the one pot procedure described here. Probably the first treatment of intact pomace with water at high temperature was not strong enough to extract the hydrophobic (−)-epicatechin-3-*O*-gallate.

Separation, Purification, and Characterization of 4β-(2-Aminoethylthio)epicatechin. The introduction of an amine function onto the extension units simplifies the separation of the amine-containing derivatives from the rest of the components

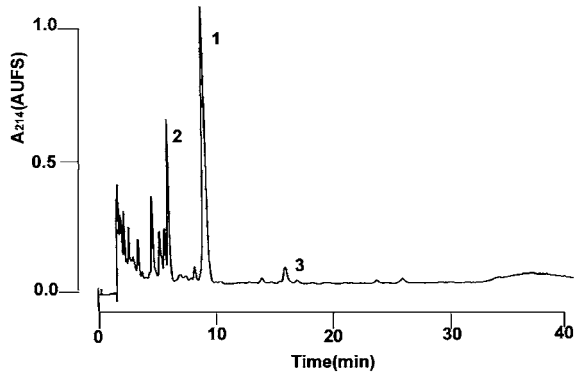

Figure 6. RP-HPLC profile of the mixture obtained after extraction and depolymerization of Xarel·lo pomace. Column μRPC SC 2.1/10 C2/C18. Load 10 μL of filtered mixture **S**, diluted 1/10 with 0.10% TFA.

in the crude plant extraction/depolymerization mixtures (*15*, *31*, *35*). We have previously proposed the cation exchange separation of the aminoethylthio-derivatives on strong cationic exchangers (bearing a sulfonic group) eluted with sodium acetate, pH 4.75, or sodium phosphate, pH 2.27, buffers, and ethanol (*35*). To further simplify the process, we changed to unbuffered 0.5% acetic acid and the separation was equally effective. Solvent [D] contained 40% EtOH to elute all of the cysteamine derivatives in 5 bed volumes. The concentration of NaCl was reduced from 1 to 0.2 M with similar results on the recovery of cysteamine derivatives. To evaluate the column capacity, different volumes of mixture **S** (20, 40, 60, and 80 mL) were loaded onto a 6.6 mL bed volume column filled with Macro Prep High S Support. The column maximum capacity was found to be 40 mL (~40 mg of procyanidins, estimated by RP-HPLC) of mixture **S**.

We used the conditions described above to separate the cysteamine derivatives (mixture **X**) from the whole mixture **S** (85 L) on a preparative column fitted with Macro Prep High S. 4β-(2-Aminoethylthio)epicatechin (**1**) was then purified from mixture **X** by preparative RP-HPLC to obtain the final product with a purity of 63% as the acetate salt, a suitable counterion for toxicity studies. Yield was 17.2 g (0.07%) with respect to the moist byproduct. The identity and purity of the final product was established by comparison with an authentic sample (*15*) using RP-HPLC, proton nuclear magnetic resonance (^{1}H NMR), and ion spray mass spectrometry.

The antiradical efficiency of the 4β-(2-aminoethylthio)-epicatechin (**1**) preparation, evaluated by the DPPH assay (ED_{50} = 0.15, expressed as nmol/nmol initial DPPH) was consistent with the value (ED_{50} = 0.11) obtained before for the 99.5% pure product (*15*).

In conclusion, we have found ecologically sound conditions (water, 90 °C, 2 × 2 h) for the efficient preparation of antioxidant polyphenolic conjugates resulting from the one pot extraction and subsequent acid breakdown of polymeric flavanols from grape pomace in the presence of cysteamine hydrochloride (22 g/kg pomace). Pomace from Xarel·lo was found to be the richest source of flavanols, with the highest percentage of galloylation. A multigram amount of the main component of the cleaved mixture, the potent free radical scavenger 4β-(2-aminoethylthio)epicatechin (**1**), was prepared directly from the pomace (35 kg) in water and purified by cation exchange and RP-HPLC. We are currently exploring the application of the one pot procedure 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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