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Influence of extraction conditions on phenolic yields from pine bark: assessment of procyanidins polymerization degree by thiolysis

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Abstract

Pinus pinaster extract is a polyphenol-rich food supplement containing diverse types of flavonoids. The latter are known to have strong antiradical activity and thus different beneficial effects on human health. A 2^3 experimental design was performed to study the effect of temperature, contact time and liquid–solid ratio on the ethanol extraction efficiency. High values of phenol yields (17.0 ± 0.00 eq. g gallic acid/l) and antiradical activity (85.6 ± 3.94 DPPH inhibition percentage) were detected at 50 °C (values varied between 25 and 50 °C), at 90 min (between 30 and 90 min) and at a 5:1 liquid–solid ratio (between 5:1 and 10:1). A subsequent HPLC analysis allowed identification of the phenols occurring in the extracts. Previously, an aqueous extract (**A**), and another one soluble in both water and ethyl acetate (**OW**), were obtained after fractionation and subjection to thiolysis. The main monophenols detected were catechin > epicatechin in the **A** fraction and dihydroquercetin > catechin > epicatechin in the **OW** one. In the former fraction, the average degree of polymerization ($\overline{DPn}$) of procyanidins was 7.0, while only oligomer phenols with $\overline{DPn} = 3.0$ were found in the **OW** fraction.

Keywords: *Pinus pinaster*; Thiolysis; Procyanidins; Fractionation; HPLC

1. Introduction

It is increasingly recognized that free radicals are responsible for the progression of a great number of human diseases, including arthritis, atherosclerosis, Alzheimer's and Parkinson's diseases, tumors, and carcinogenesis (Gordon, 1996; Rohdewald, 2002). The potential capacity of antioxidant phenols to scavenge free radicals are well-known and these compounds are reported to be widely present in a large variety of fruits, vegetables and plant materials (Barzana et al., 2002; Heim, Tagliaferro, & Bobilya, 2002; Núñez, Sineiro, & Pinelo, 2003). For this reason, recent antioxidant studies have focussed on the improvement of phenol extraction techniques from some natural products. Bark pine is a good example, being an important source of polyphenolic compounds. Among other applications, the latter were reported to be used as adhesives in the wood agglomerate industry and have favourable pharmacologic properties (Fitzpatrick, Bing, & Rohdewald, 1998; Pepino et al., 1999). In fact, *Pinus pinaster* bark extract (Pycnogenol) showed good results in the treatment of chronic venous insufficiency and cardiovascular diseases. In addition, oral intake of this extract was reported to be effective against oxidative stress in several cell systems and to protect against UV-radiation-induced erythema (Packer, Rimbach, & Virgili, 1999).

Procyanidins (or condensed tannins) were identified as the main phenolic components in pine bark. These compounds consist of mixtures of oligomers and polymers containing (+)-catechin and/or (−)-epicatechin

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units linked mainly through C4 → C8 and/or C4 → C6 bonds. These flavan-3-ol units can be doubly linked by a C4 → C8 bond and an additional ether bond from O7 → C2 (Porter, Ma, & Chan, 1991). Pine bark is reported to contain oligomeric proanthocyanidins, such as dimers and trimers, together with tannins with higher degrees of polymerization. Likewise, the presence of catechols, benzoic acids and monomeric flavonols, in small quantities, was also confirmed in some previous findings (Porter, 1974; Rohr, Meier, & Sticher, 2000).

Procyanidin properties were found to strongly depend on the degree of polymerization (DP) of the tannin molecule. In fact, Lea and Arnold (1978) pointed out the influence of the DP of procyanidins in relation to bitterness and astringency of drinks in which these compounds were added. On the whole, the DP and the nature of the constitutive units are important structural features that are related to the ability of proanthocyanidins to associate with proteins and polysaccharides (Cheynier, Rigaud, & Da Silva, 1992). This explains the quite different characteristics between oligomeric and polymeric procyanidins and the availability of several methods to fractionate this type of compounds. In fact, many chromatographic methods to separate these fractions can be found in the literature: chromatography on Sephadex LH-20, fractionation on C_{18} Sep-Pack cartridges and Fractogel (Toyopearl) are some examples (Hammerstone, Lazarus, Mitchell, Rucker, & Schmitz, 1999; Yanagida, Kanda, Shoji, Ohnishi-Kameyama, & Nagata, 1999). Recently, Labarbe, Cheynier, Brossaud, Souquet, and Moutonet (1999) proposed a method of quantitative fractionation that corresponded to a sequential dissolution of proanthocyanidins on an inert glass powder column. Likewise, chemical methods were also developed to carry out this separation; these are based on the different solubilities of these compounds in several solvents.

Chemical methods are also generally used to determine the structural characterization of the proanthocyanidin fraction. They consist of the acid-catalyzed cleavage of the interflavanyl linkages of proanthocyanidins in the presence of a nucleophile reagent such as phloroglucinol or toluene-α-thiol (Matsuo, Tamaru, & Saburo, 1984). Thiolysis allows distinction between extension and terminal units when coupled to reversed-phased HPLC (Rigaud, Perez-Ilzarbe, da Silva, & Cheymeir, 1991). The method was used to calculate the average DP and to determine the proportions of the constitutive units in the proanthocyanidins fraction.

In this work, a study of the influence of some critical extraction variables on the phenolic yield and antiradical activity of resultant extracts is carried out. An experimental design was performed in order to find the relevant optimal values of these variables. Then, a chemical separation was used to separate oligomeric and polymeric procyanidins from the extracts obtained. Their DP and the identification of monomeric units were evaluated by a thiolysis method using benzyl-α-thiol.

2. Materials and methods

2.1. Sample preparation

The bark pine (*Pinus pinaster*) was supplied by MANUEL BOUZAS GARRIDO, S.A. (Vedra, A Coruña, Spain) and dried at room temperature for one week. It was ground in a knife mill and the powdered bark was sieved to select particles smaller than 1 mm.

2.2. Experimental design

A full factorial 2^3 experimental design was developed to evaluate the effect of the temperature (**T**), time of contact (**t**) and liquid–solid ratio (**L/S**) (Box, Hunter, & Hunter, 2000). Temperature values varied between 25 and 50 °C, time contact between 60 and 90 min and liquid–solid ratio between 5:1 and 10:1 (v/w in wet basis). Variables were codified in the way that their value ranged between +1 and −1, taking, as central point, the zero value. So

$$t = (\mathbf{t} - 60)/30,$$

$$T = (\mathbf{T} - 37.5)/12.5,$$

$$L/S = (\mathbf{L}/\mathbf{S} - 7.5)/2.5.$$

Table 1 shows the factorial design matrix, with variables in both forms coded/non-coded, for better comprehension. Data were adjusted to a response surface R

$$R = a_0 + a_1 t + a_2 T + a_3 L/S + a_{12} tT + a_{13} tL/S + a_{23} TL/S + a_{123} tTL/S,$$

where a_0 is the value of the objective function (phenol yields and antioxidant capacity) in the central point conditions, a_1, a_2, a_3 represent the principal effects associated with each variable and the others represent the crossed effects among variables.

2.3. Analytical methods

2.3.1. Total polyphenolic compounds

The total phenolics were assayed colorimetrically by means of the Folin–Ciocalteu method, as modified by Singleton and Rossi (1965). 2.5 ml of tenfold diluted Folin–Ciocalteu reagent, 2 ml of 7.5% sodium carbonate, and 0.5 ml of phenolic extract were mixed well. The absorbance was measured at 765 nm (Shimadzu UV-160A) after 15 min of heating at 45 °C. A mixture of water and reagents was used as a blank. Since most of the works dealing with phenolic content in natural

Table 1
Extraction conditions of the experimental design

Experiment	**t** (min)	**T** (°C)	**L/S**	*t*	*T*	*L/S*	Total phenols (g/l)	Inhibition percentage
1	30	25	10	−1	−1	+1	6.58 ± 0.29	36.6 ± 0.81
2	30	50	10	−1	+1	+1	7.15 ± 0.8	35.6 ± 2.22
3	30	25	5	−1	−1	−1	13.44 ± 1.1	62.8 ± 4.12
4	30	50	5	−1	+1	−1	15.11 ± 0.3	69.9 ± 4.03
5	90	25	10	+1	−1	+1	6.68 ± 0.53	32.1 ± 1.31
6	90	50	10	+1	+1	+1	7.43 ± 1.31	43.0 ± 4.35
7	90	25	5	+1	−1	−1	15.8 ± 0.41	76.4 ± 5.15
8	90	50	5	+1	+1	−1	17 ± 0.07	85.6 ± 3.94
9	60	37.5	7.5	0	0	0	11.25	52.0
10	60	37.5	7.5	0	0	0	12.51	50.7
11	60	37.5	7.5	0	0	0	11.72	53.1
12	60	37.5	7.5	0	0	0	11.42	57.0

Not coded/coded variables. Polyphenol concentration and antiradical activity of extracts, expressed as inhibition percentage, obtained under the conditions of the experimental design.

products use gallic acid as a standard (independently of phenolic species detected), the content of phenolics in this work was expressed as gallic acid equivalents to facilitate comparative tasks.

2.3.2. Antiradical activity

A DPPH radical-scavening assay was performed, using the method described by Brand-Williams, Cuvelier, and Berset (1995) to determine the hydrogen-donating ability of the crude extract. A volume of 980 μl of 6.1×10^{-5} M. DPPH· methanol solution was used. The reaction was started by the addition of 20 μl of crude extract. The bleaching of DPPH· was followed at 515 nm at 25 °C for 16 min (until the steady state is reached). The antiradical activity was expressed as the inhibition percentage (IP) of the DPPH· radical and was calculated as follows:

$$\mathrm{IP} = \frac{(\mathrm{absorbance}_{t=0\ \mathrm{min}} - \mathrm{absorbance}_{t=16\ \mathrm{min}})}{(\mathrm{absorbance}_{t=0\ \mathrm{min}})} \times 100.$$

2.4. Extraction

250 ml capped flasks were used to extract 4 g of ground pine bark (w.b.) in a rotatory shaker G24 New Brunswick Scientific Co. Inc. (NJ, USA) at constant stirring rate of 140 rpm. 98° Ethanol (Carlo Erba, Milano, Italy) was used as a solvent. Solids were separated by filtration and the crude extracts were analyzed for polyphenols and antiradical activity.

2.5. Fractionation of extracts

Conditions that maximize the values of antiradical activity (exp. 8) were used to carry out the extraction process; 40 g of ground pine bark were extracted using EtOH as a solvent in order to obtain a suitable quantity of liquid. The latter was filtered and ethanol was subsequently evaporated under vacuum. The resulting solid was resuspended in water and freeze-dried to yield the crude extract, which was Soxhlet-defatted during 2 h with petroleum ether. The residue was dried and suspended in water (40 ml). After the addition of acetic acid (200 μl), the monomeric and oligomeric components were 3-times extracted with EtOAc (20 ml) to yield an organic fraction **O** (55 ml) and, an aqueous fraction **A** (35 ml), this latter containing mainly polymers. Then the solvent was evaporated from fraction **O** under vacuum, and the pellet suspended in water, which was filtered through a porous plate. The filtrate was lyophilized to yield fraction **OW** (74.3 mg), containing species soluble in both AcOEt and water (mainly oligomers) (Torres & Bobet, 2001).

2.6. Thiolysis

Thiolysis was carried out on both aqueous phase **A** and fraction **OW,** redissolved in methanol up to 10 mg/ml. Aqueous phase **A** was subjected to a previous treatment: the solvent (water saturated with EtOAc) was eliminated. The pellet was then dissolved in methanol to remove moisture. The resulting syrupy residue was dissolved in MeOH.

A method based on that of Guyot, Marnet, and Drilleau (2001) was adopted to perform the thiolysis. 100 μl of the corresponding fraction were mixed with 100 μl of methanol acidified by concentrated HCl (3.3% v/v), and 200 μl of toluene-α-thiol (5% v/v in methanol) were added. The reaction mixture was placed in a sealed Pyrex glass test tube, heated at 40 °C for 30 min and finally cooled with running water.

2.7. High-pressure liquid chromatography

Thiolysis reaction media (20 μl) were directly injected into the HPLC system. The HPLC apparatus, with a pump PU-980 connected to a quaternary gradient unit LG-1580-04, a JASCO UV-1575 UV–Vis detector and

a Rheodyne model 7725 loading sample injector with a 20 μl sample loop were used to determine the phenolic compositions of the different fractions. The column (250 mm × 4.6 mm) was a C_{18} Hypersil ODS (5 μm particle size) (Supelco).

The two solvents used to make the gradient were (A) 0.5% acetic acid Milli-Q water solution and (B) methanol. The solvent gradient in volumetric ratios of solvents A and B was as follows: 0–10 min, 95A/5B; 10–60 min, 50A/50B; 60–80 min, 30A/70B; 80–90 min, 95A/5B. Detection was carried out using 280 nm as preferred wavelength.

Flow rate was set to 0.7 ml/min. Three determinations were done on each extract obtained. Standards of gallic acid, catechin, epicatechin, quercetin and resveratrol were purchased from Sigma (St. Louis, MO).

2.8. Statistical analysis

The results reported in this work are the averages of at least three measurements, and the coefficients of variation, expressed as the percentage ratio between standard deviations (SD) and the mean values, were found to be <10 in all cases. Significant variables were calculated, subjecting results to a linear regression, using SPSS statistical programme version 10.0 (SPSS Inc., Chicago, IL). Only variables with a confidence level above 95% ($p < 0.05$) were considered as significant.

3. Results and discussion

Table 1 shows the values of both polyphenol concentration and DPPH inhibition percentage for extracts obtained under conditions of the experimental design. As can be observed, higher values of both variables were obtained when extraction was carried out under the conditions of experiment 8 (higher temperature 50 °C, higher contact time 90 min, and lower liquid–solid ratio 5:1). Applying statistical analysis, the following significant model was obtained for polyphenol concentration:

$$C_{\text{polyphenols}} = 11.3 - 4.1L/S + 0.6T$$

$$F_{\text{model}} = 103.6 \; p = 0.000 \; R^2 = 0.984.$$

A clear dependence on L/S ratio and to a minor extent on temperature was detected, whereas contact time was not found to be significant.

Increasing temperature favours extraction by enhancing solubility of solute and increasing the diffusion coefficient. As a consequence, an increase of extraction yields is always observed at higher values of this variable. However, despite the positive effects of enhanced temperature, this cannot be increased indefinitely; the decrease of phenolic compounds and the denaturation of membranes could occur at high temperatures (Cacace & Mazza, 2003). Likewise, the use of low liquid–solid ratios was found to be positive, allowing more concentrated extracts (usual when saturation effects are not present). This seems obvious since, the lower the amount of solvent used, the higher was the concentration of extract obtained. Only the contact time was found not to be a significant variable in the interval of variation assayed. Values found are in accordance with previous findings. Kähkönen et al. (1999), for instance, subjected scottish pine bark to methanolic extraction and reported values of 3.8 g eq. gallic acid/l at a L/S ratio of 20:1. Likewise, Pinelo, Rubilar, Sineiro, and Núñez (2004) obtained values between 4 and 8.5 g/l for pine sawdust ethanol extracts under the same conditions as in our experimental design.

With regard to antiradical activity values, expressed as inhibition percentage, the response function model showed dependence on all variables studied:

$$\text{IP} = 54.5 - 18.4L/S + 4.0t + 3.3T - 3.3L/St$$

$$F_{\text{model}} = 70.2 \; p = 0.000 \; R^2 = 0.974.$$

The influence of these extraction variables on the inhibition percentage of extracts from other natural materials has previously been reported. Joubert (1988) studied the effect of batch extraction conditions on yields from rooibos tea – containing some phenols in common with pine, such as like catechin and epicatechin, – finding a significant increase of their antioxidant activity when temperature was increased from 23 to 90 °C. Likewise, Rubilar, Pinelo, Franco, Sineiro, and Núñez (2003) studied the effect of time contact and liquid–solid ratio, in the extraction process, on the value of this variable in extracts from grape pomace. In accordance with our results, both variables were reported to have a considerable effect on the antiradical activity of extracts obtained. In Figs. 1 and 2, the response surfaces for the antiradical activity, expressed as DPPH inhibition percentage, are plotted. No noticeable changes of

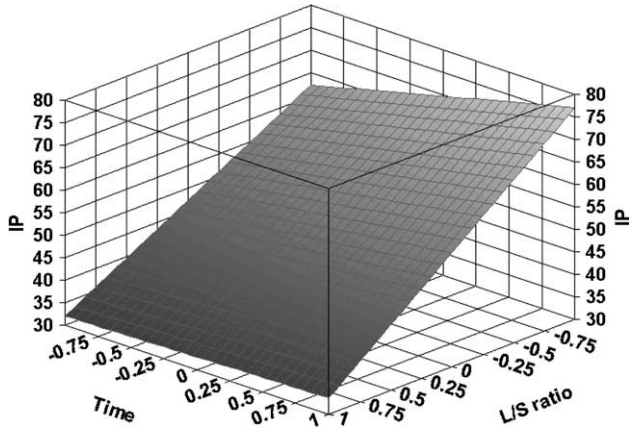

Fig. 1. Response surface plot for antiradical activity in extracts of pine bark in ethanol at 25 °C.

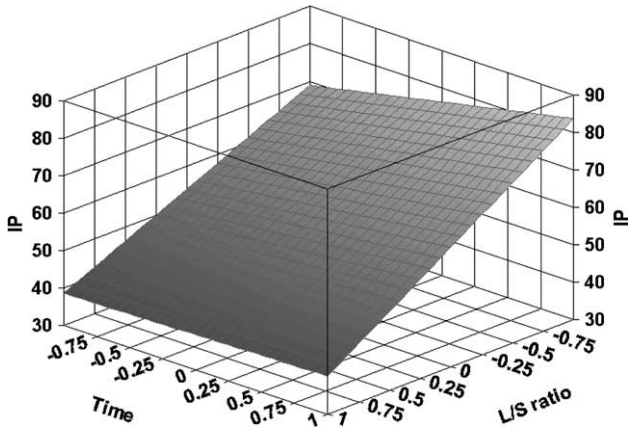

Fig. 2. Response surface plot for antiradical activity in extracts of pine bark in ethanol at 50 °C.

antiradical activity were detected when time contact varied. By contrast, the *L*/*S* ratio was found to have a strong influence on antiradical activity. Variations in the latter variable, at 25 and 50 °C, were plotted and the only difference was the higher values of the antiradical activity reached at the higher temperature.

Fig. 3 shows the chromatogram corresponding to the phenol profile of the crude extract obtained under the conditions of experiment 8. Increasing values of intensity, especially for retention time values, between 20 and 50 min, pointed to the presence of a mixture of compounds detected together. It is possible to find, in the literature similar phenol chromatograms corresponding to other natural product extracts. Reversed-phase HPLC permits a good resolution of compounds with a low DP (from monomers to trimers), whilst higher species are detected as a wide peak (Yang & Chien, 2000). In fact, Saucier, Mirabel, Daviaud, Longieras, and Glories (2001) studied the procyanidin composition of grape seed extract, and the typical wide peak, containing both monomers and polymers, was also detected. A similar result was obtained by Guyot, Doco, Souquet, Moutounet, and Drilleau (1997) in studies on phenol characterization of apple extracts. In both cases, a subsequent fractionation of extracts allowed the separation of oligomeric and polymeric compounds and the determination of the DP of these species.

The chromatogram corresponding to the aqueous fraction (**A**) is shown in Fig. 4. The detection of the great wide peak indicated the presence of polymeric proanthocyanidins again. Thiolysis was therefore applied to this relevant fraction, and the resultant chromatogram is plotted in Fig. 5. Catechin was detected as the main monomeric phenol. With regard to the benzylthioethers, 3 peaks were identified when proanthocyanidins are subjected to thiolysis. A similar result was obtained by Thompson, Jacques, Haslam, and Tanner (2000) when they studied the proanthocyanidin contents of several selected plants; 3,4-*trans*-benzylthyoepicatechin was the resultant product when the internal units of epicatechin contained in proanthocyanidins were subjected to thiolysis. No 3,4-*cis* isomer was detected. By contrast, from internal units of catechin, however, both 3,4-*cis* and 3,4-*trans*-benzylthyocatechin were obtained.

Fig. 6 shows the chromatogram of a 10 mg/ml **OW** fraction, containing monomers of flavan-3-ol and other oligomeric proanthocyanidins. Catechin, epicatechin and dihydroquercetin were detected, whilst other peaks corresponded to oligomers of proanthocyanidins. 100 μl of the **OW** fraction solution were subjected to thiolysis. After adding the thiolysis reagents, the overall

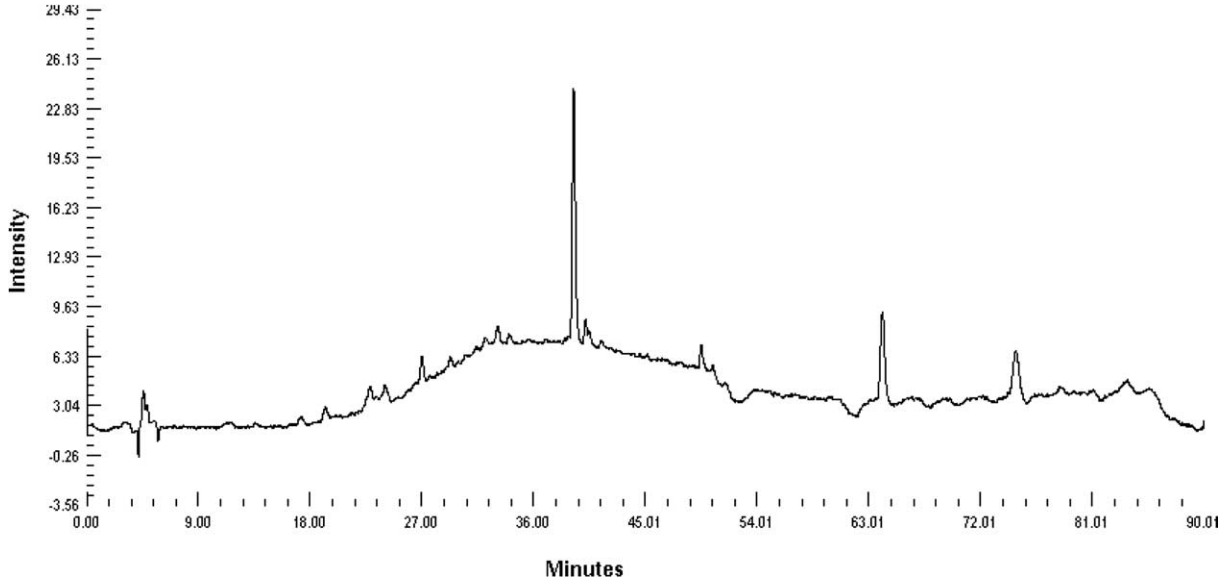

Fig. 3. Chromatogram corresponding to a crude extract of pine bark in ethanol.

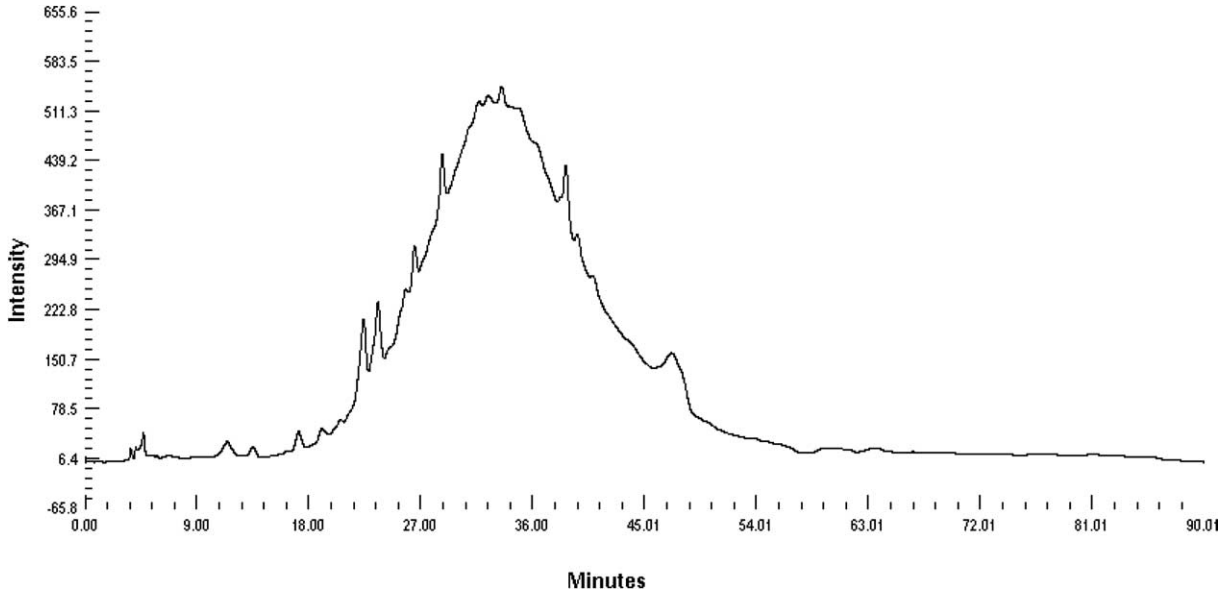

Fig. 4. Chromatogram corresponding to the aqueous fraction **A** of a pine bark extract in ethanol.

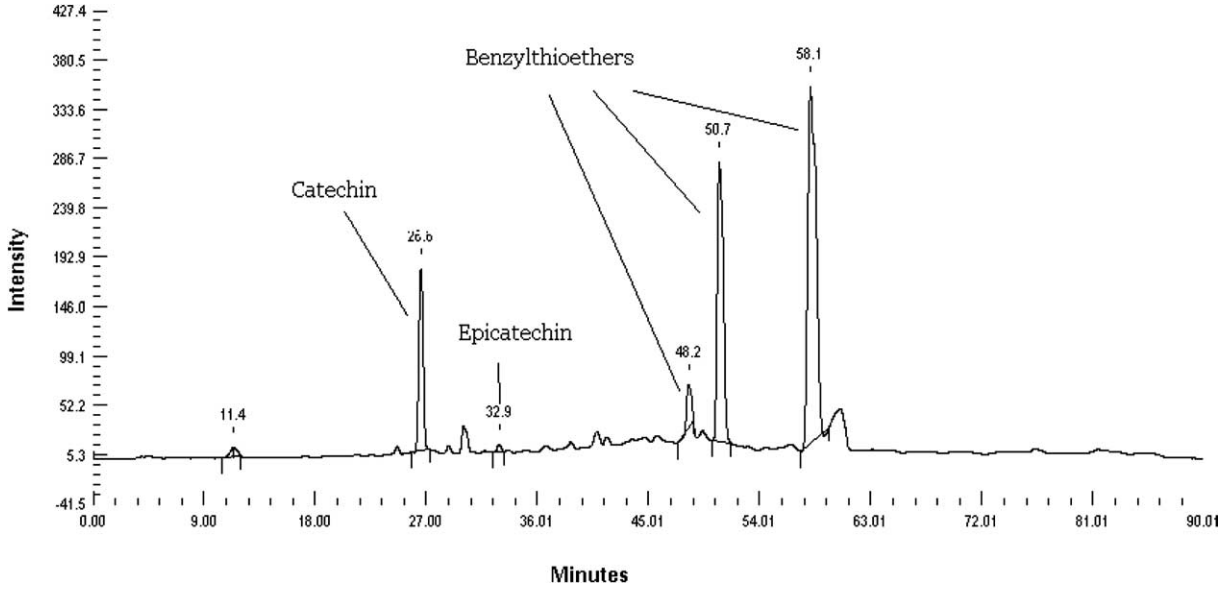

Fig. 5. Chromatogram after thiolysis of the aqueous fraction **A** of a pine bark extract in ethanol.

volume of mixture was 400 μl (four-fold diluted solution). The relevant chromatogram is plotted in Fig. 7. In comparison to that one from the aqueous fraction (**A**) (Fig. 5), the most remarkable difference is the presence of dihydroquercetin in the latter. Since only the terminal units of tannins become free after thiolysis (catechin or epicatechin as such), this chromatogram demonstrates that catechin monomers, as terminal units, were more abundant than those corresponding to epicatechin. Moreover, thiolysis allows distinction between extension and terminal units of proanthocyanidins, so this method can be used to assess the average degree of polymerization ($\overline{DPn}$) (Souquet, Cheynier, Brossaud, & Moutounet, 1996) Response factors, for both catechin and catechinbenzylthioether and epicatechin and epicatechinbenzylthioether, are the same at 280 nm. Therefore, catechin and epicatechin can be used as standards to quantify the benzylthioethers and the $\overline{DPn}$ can be assessed by means of the following equation (Gu et al., 2002):

$$\overline{DPn} = [\text{total area of benzylthioethers} / (\text{total area of catechin and epicatechin})] + 1.$$

The **OW** fraction of *Pinus pinaster* showed a $\overline{DPn}$ of 3.0, which corresponded to oligomeric procyanidins,

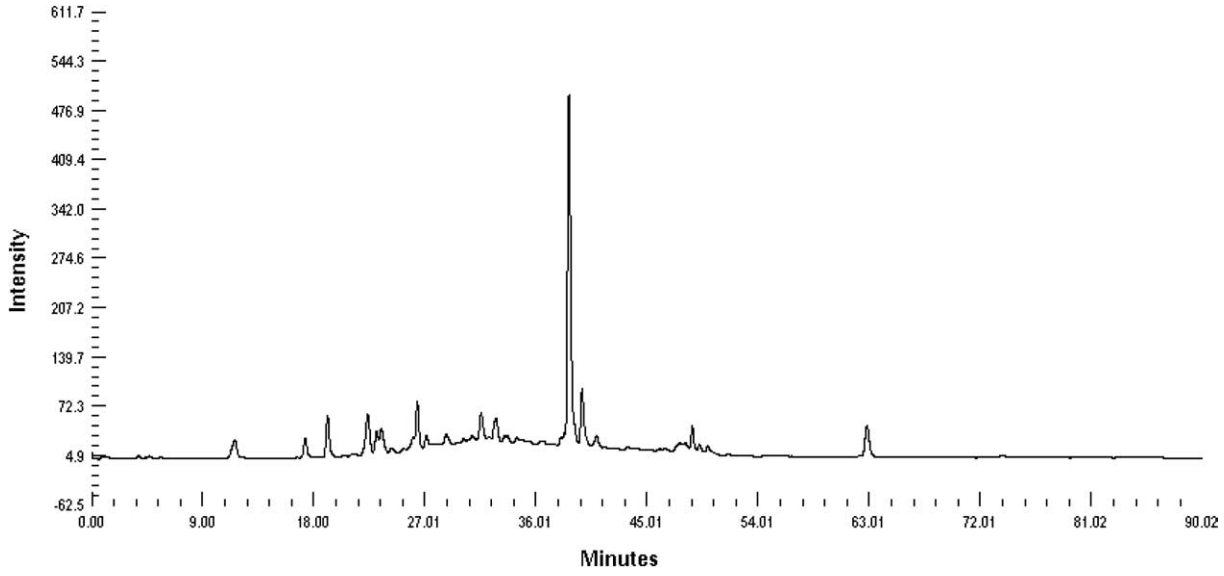

Fig. 6. Chromatogram corresponding to the **OW** fraction of a bark pine extract in ethanol.

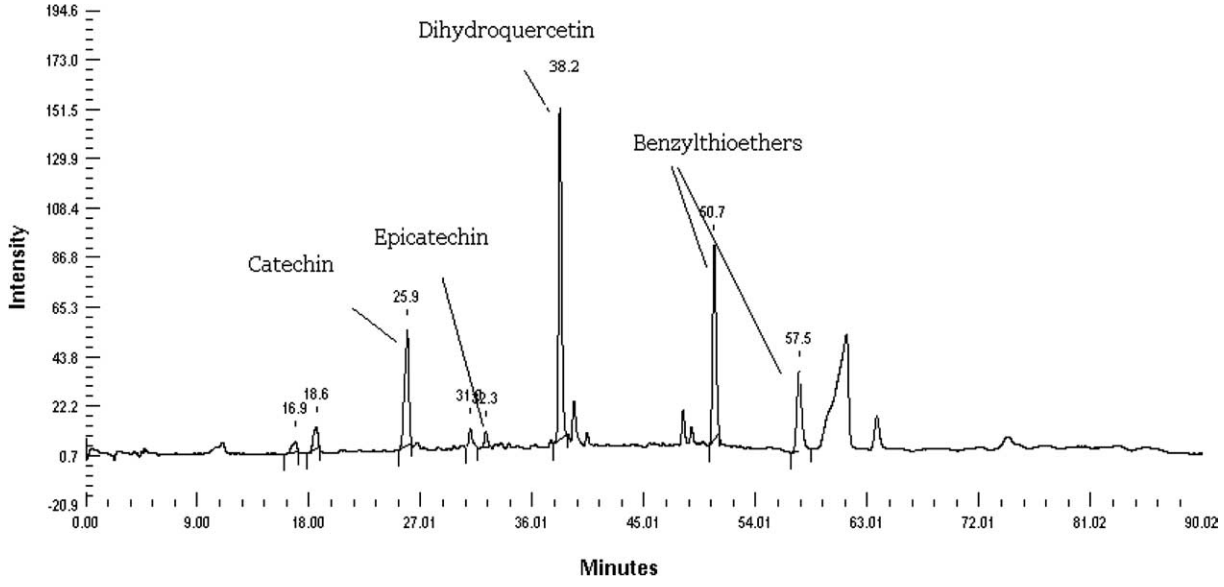

Fig. 7. Chromatogram after thiolysis of the **OW** fraction of a pine bark extract in ethanol.

whereas highly polymerized procyanidins ($\overline{DPn}$ 7.0) were present in the aqueous fraction.

4. Conclusions

Pinus pinaster bark contains considerable quantities of antiradical phenols. This is of great importance for the industry, since the extracts of these byproducts are finding increasing applications as active substances for cosmetic and pharmaceutical compositions. Industrially, however, the economic feasibility of the extraction process involves a search for optimum extraction conditions, in order to maximize process efficiency. In this work, the great influence of temperature on the 'antiradical quality'of extracts is reported, the higher antiradical activity according with the higher extraction temperature assayed. By contrast, the influence of contact time was found to be not so significant, demonstrating that the matrix structure of pine bark allows liberation of phenol species more easily than do other natural matrices and this avoids prolonged extraction times.

Some of the optimal values of the studied variables (temperature, contact time and liquid–solid ratio) were found to be at the lower end of the range chosen. These data are a first step in the optimization of this particular solid–liquid extraction system. A study of the influence

of these variables, on antiradical activity and phenolic yields of extracts could be implemented in further work.

This work reveals that the antiradical activity can be expressed as a function of the mixture effect of some related compounds: monophenols, together with oligomers ($\overline{DPn} = 3.0$) and polymers ($\overline{DPn} = 7.0$) of flavonoids. In the future, a study of the influence of each one on the properties of extracts may help to obtain extracts with specific characteristics.

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