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Anti-oxidant activity of isolates from acid hydrolysates of *Eucalyptus globulus* wood

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Abstract

The ethyl acetate-soluble fraction of *Eucalyptus globulus* wood hydrolysates was fractionated on a Sephadex LH-20 column, using methanol as the mobile phase to give four fractions, and both the DPPH (α,α-diphenyl-β-picrylhydrazyl) radical-scavenging activity and composition of raw extracts and isolates were determined. One of the fractions isolated (denoted F4) showed a remarkable anti-oxidant activity (EC_{50} of 0.15 g/l, in comparison with 0.55 g/l for crude extracts), presenting a comparatively high phenolic content, with ellagic acid as the main component.

Keywords: *Eucalyptus globulus* wood; Acid hydrolysates; Ethyl acetate extract; Anti-oxidant activity

1. Introduction

During the production of hemicellulosic sugar solutions by mild acid hydrolysis of lignocellulosic materials, different inhibitors of microbial metabolism are also formed (Ando, Arai, Kiyoto, & Hanai, 1986; Boussaid et al., 2001; Martín, Galbe, Nilvebrant, & Jönsson, 2002). Extractives, aliphatic fatty acids, phenolic substances derived from lignin and sugar dehydration products were the major compounds identified in hydrolysates (Klinke, Schmidt, & Thomsen, 1998; Martín et al., 2002) and should ideally be removed in order to efficiently utilise sugars as carbon source for bioconversion processes (Clark & Mackie, 1984; Cruz, Domínguez, Domínguez, & Parajó, 1999; Parajo, Domínguez, & Domínquez, 1998). For this purpose, extraction with ethyl acetate (enabling the selective removal of phenolic compounds from sugar solutions) has been proposed (Clark & Mackie, 1984; Cruz et al., 1999; Martín et al., 2002). The search for practical applications of these residual fractions would benefit the integral use of the lignocellulosic materials.

Phenolic compounds in biomass hydrolysates come from the partial depolymerisation of lignin, a part of which is linked to hemicelluloses. The anti-oxidant activity of lignin-derived fractions and phenolic acids has been reported (Kasprzycka-Guttman & Odzeniak, 1994). Under acid hydrolysis conditions, compatible with the production of fermentable sugars, *Eucalyptus globulus* wood yields higher anti-oxidant and antimicrobial activities than other lignocellulosic materials (Cruz, Domínguez, Domínguez, & Parajó, 2001).

In a previous work (González, Cruz, Domínguez, & Parajó, 2004), the operational conditions leading to maximal phenolics recovery by hydrolysis-extraction of *Eucalyptus* wood were established. The phenolic content of crude extracts was in the range reported for extracts from hydrothermal treatments (Felizón, Fernández-Bolaños, Heredia, & Guillén, 2000) or from different plant materials proposed as "natural" anti-oxidants (Exarchou et al., 2002; Rodríguez de Sotillo, Hadley, & Holm, 1994). Benzoic and cinnamic acids and aldehydes were detected in liquors obtained in the mild-acidic processing of hardwoods (Garrote, Cruz, Moure, Domínguez, & Parajó, 2004), whereas tannins (proanthocyanidins and ellagitannins) (Cadahía, Conde, Fernández de Simón, & García-Vallejo, 1997), acids

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(ferulic, ellagic, gallic, syringic and vanillic), aldehydes (syringaldehyde and sinapaldehyde) and flavonoids (naringenin and quercetin) have been detected in *Eucalyptus globulus* wood (Freire, Silvestre, & Nieto, 2002).

Ethyl acetate extracts of *Eucalyptus wood* acid hydrolysates are of dark colour and oily aspect, features that could hinder their direct application. A suitable purification process leading to the removal of fractions with limited anti-oxidant activity would overcome these inconveniences. In this context, it can be noted that phenolic fractions or purified extracts from plants can show higher anti-oxidant capacity than crude extracts (Watanabe, 1999; Watanabe, Ohshita, & Tsushida, 1997). Fractionation of plant extracts containing phenolic compounds has been successfully done with Sephadex LH-20 columns (Watanabe, 1999; Wettasinghe, Shahidi, & Amarowicz, 2002). The aim of the present work is to evaluate the phenolic content and the radical-scavenging capacity of fractions from the ethyl acetate-soluble compounds present in *Eucalyptus* wood hydrolysates.

2. Materials and methods

2.1. Manufacture of crude extracts

Milled *Eucalyptus globulus* wood samples were treated with 5% H_2SO_4 at 130 °C at a liquid:solid ratio (LSR) of 8:1 g/g for 60 min (González et al., 2004). The liquid phase was separated by filtration and neutralised with $CaCO_3$. The precipitate was removed by filtration before extraction with ethyl acetate at a hydrolysate:ethyl acetate volume ratio of 1:3 (v:v) in a single extraction stage (Cruz et al., 1999; Cruz et al., 2001). Ethyl acetate was removed by vacuum evaporation and reutilised, and the solid extract was freeze-dried and used for further characterisation. The average yield in ethyl acetate soluble compounds was 3.86 g/100 g dry wood.

2.2. Chromatographic fractionation

Sephadex LH-20 gel (Amersham Biosciences, Uppsala, Sweden) was used for fractionation by column chromatography. The ethyl acetate-soluble fraction of *Eucalyptus* wood hydrolysates (0.198 g) was redissolved in methanol (5 ml) and loaded on the column, which was eluted with methanol at a flow rate of 3 ml/min. Sampling was carried out using a Gilson FC 203B fraction collector with 3 ml test tubes. The samples were combined to obtain the fractions selected. Absorbance (280 nm) was measured on-line in a flow cell using an Agilent 8453 spectrophotometer.

2.3. Spectrophotometric determination of phenols

Total phenols were determined by two spectrophotometric methods (Folin–Denis and Folin–Ciocalteu). Absorbance readings were made at 745 nm in the case of the Folin–Denis method (AOAC, 1997) and 765 nm in the Folin–Ciocalteu assay (Singleton & Rossi, 1965). A standard curve made with gallic acid (Sigma Chem. Co.) was used for quantification in both cases.

2.4. HPLC determination of phenols

Ethyl acetate extracts (obtained from 25 ml hydrolysates) were dissolved in methanol (10 ml) and analysed by HPLC in a Hewlett–Packard 1050 instrument fitted with a 1050 DA detector using a Supelcosil LC-18 column (5 μm, 4.6 mm × 25 cm) operating at room temperature. Gradient elution was carried out at a flow rate of 1.0 ml/min using 0.01 M sodium citrate buffer (pH 5.4, adjusted with 50% acetic acid) (solution A) and methanol (solution B). The best separation was obtained using the following gradient: from 0 to 12 min, B increases from 2% to 4%; from 12 to 20 min, B increases from 4% to 13%; from 20 to 22 min, B is kept constant in 13% B; from 22 to 26 min, B decreases from 13% to 2%; from 26 to end, B is kept constant at 2%.

2.5. Qualitative GC–MS analysis

Samples were derivatised as reported by Quesada, Rubio, and Gomez (1997): around 50 mg of standard reagents or extracts were weighed into a 25 ml round bottom flask and trimethylsilylated by adding 200 μl of pyridine, 1 ml of BSTFA and 50 μl of TMCS. The round bottom flask was sealed, shaken vigorously, kept at 60 °C under stirring for 30 min in a water bath and cooled to room temperature before GC–MS analysis (injection volume, 1.5 μl). This procedure was successfully employed in the analysis of low molecular weight compounds (phenolics and carboxylic acids) from hydrolysates obtained by wet oxidation of wheat straw (Klinke, Ahring, Schmidt, & Thomsen, 2002), steam explosion and acid impregnation of sugarcane bagasse (Martín et al., 2002) and acidic processing of pinewood (Clark & Mackie, 1984). The identity of the compounds was confirmed by comparing both the retention time and the mass spectral data with those of pure compounds (similarity percentage higher than 85%).

2.6. Anti-oxidant activity (DPPH radical-scavenging activity)

Two ml of a 6×10^{-5} M methanolic solution of DPPH (α,α-diphenyl-β-picrylhydrazyl) were added to 50 μl of a methanolic solution of the anti-oxidant, and the decrease in absorbance at 515 nm was recorded in an

Agilent 8453 spectrophotometer for 16 min (von Gadow, Joubert, & Hansmann, 1997). The inhibition percentage (IP) of the DPPH radical was calculated as the percentage of reduction in absorbance between 0 and 16 min. EC_{50} was calculated as the concentration of ethyl acetate soluble extracts (redissolved in methanol) causing a 50% inhibition of the DPPH radical.

3. Results and discussion

3.1. Spectrophotometric determination of phenols in crude extracts

The phenolic content of crude extracts was measured by the Folin–Denis and the Folin–Ciocalteau assays, which led to percentages of 0.39 and 1.04 g equivalents of gallic acid/100 g oven-dry wood, respectively.

3.2. Chromatographic fractionation of crude extracts

Fig. 1 shows the Sephadex LH-20 elution profile determined for the crude extracts and the fractions (1–4, here denoted F1–F4) selected for further study. Eluates between F1 and F2 and between F3 and F4 were rejected. The crude extract was dark coloured with oily appearance, but the various fractions were clearer, with granular texture and manageable.

Table 1 shows the recovery yields of the fractions, as well as the results determined for crude extracts and fractions concerning both phenolic content (expressed as gallic acid equivalents) and DPPH radical-scavenging activity (expressed as EC_{50}). The anti-oxidant power of the fractions increases with the elution time. The highest recovery yield (2.17 g extract/100 g wood, accounting for 56% of the crude extract weight) was determined for F3, which also had the highest peak area in Fig. 1. F3 showed both phenolic content and anti-oxidant activity closely related to those of the crude extracts. The next major fraction in terms of recovery yield was F2, which showed both decreased phenolic content and lower anti-oxidant activity with respect to the crude extracts. F1 was obtained at a yield accounting for just 1% of the crude extract weight, and presented an anti-oxidant activity ($EC_{50} = 2.15$ g/l) comparable to that of BHT ($EC_{50} = 2.79$ g/l). F4 was obtained at a yield of 5.3 wt% of the crude extracts, and showed a strong anti-oxidant activity ($EC_{50} = 0.15$ g/l), even better than that of BHA ($EC_{50} = 0.24$ g/l).

Table 1
Characterisation of crude extract (CE) and fractions F1–F4

	CE	F1	F2	F3	F4
Recovery yield (% weight crude extract)	–	1.06	29.06	56.15	5.29
Phenolic content, Folin–Denis method (g gallic acid equivalents/ 100 g crude extract or fraction)	10.1	7.9	4.1	10.2	14.9
EC_{50} (g/l)	0.55	2.15	1.21	0.52	0.15

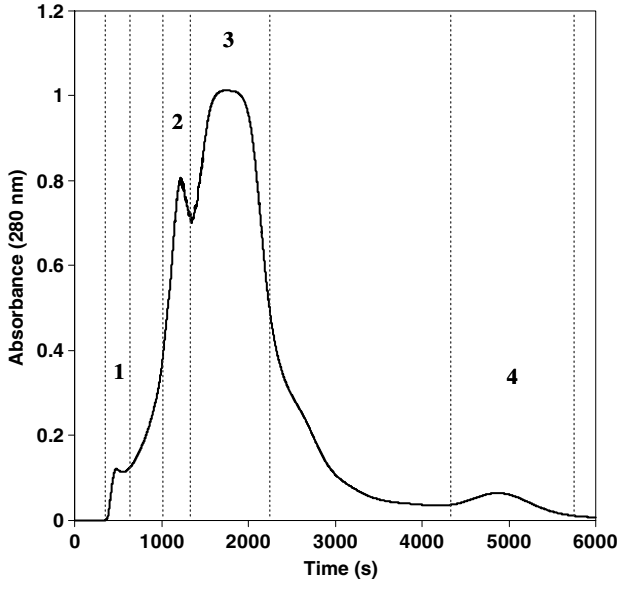

Fig. 1. Chromatographic profile of crude extracts.

Related studies (Amarovicz, Naczk, & Shahidi, 2000) reported similar patterns for the phenolic content of fractions from canola hulls, whereas differences in anti-oxidant activity among fractions separated in a Sephadex LH-20 column have also been pointed out (Balasinska & Troszynska, 1998): the first fractions eluted with acetone:water from evening primrose cake extracts were considerably less effective in protecting phosphatidylcholine oxidation than the next ones, and a related behaviour has been reported for the fractions of beach pea (Shahidi, Chavan, Naczk, & Amarowicz, 2001). In this context, the first and the last fractions separated from ethanolic extracts of japanese barnyard millet grains showed an enhanced anti-oxidant activity (Watanabe, 1999).

The total phenolic content of the separated fractions is higher than those determined in extracts from oilseeds such as rape, sunflower or mustard obtained with different solvents, for which values in the range 2.6–44.7 mg gallic acid equivalents/g (determined by the Folin–Ciocalteau method) have been reported (Matthäus, 2002). Evening primrose cake extracts, eluted with acetone–water in a Sephadex LH-20 column, contained 50.9–180.4 mg catechin equivalents/g (again determined by the Folin–Ciocalteau method) (Balasinska & Troszynska, 1998). Other authors have employed the Folin–Denis method for quantification of low molecular weight phenolics (Wettasinghe et al., 2002): dealing with

this method, Amarovicz et al. (2000) reported 14–112 mg sinapic acid equivalents/g for the fractions isolated from acetone–water extracts of canola hulls.

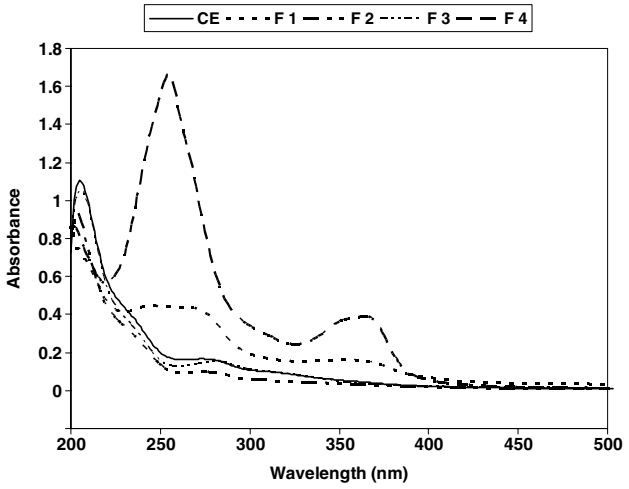

Fig. 2. UV spectra of crude extracts and fractions F1–F4.

3.3. Spectral features of the fractions

UV spectra of fractions F1–F4 are shown in Fig. 2. The maxima observed at 220 and 260 nm also appear in the spectra of protocatechuic acid and vanillic acid (Robbins, 2003). Crude extracts show an absorbance profile similar to that of cinnamate derivatives, with an additional broad band from 270 to 360 nm. The remarkable differences among the spectral maxima F4 and the rest of fractions (particularly F2 and F3) reveal the presence of substances with different chemical natures. All fractions absorb at 210–220 nm, behaviour related to both benzoic and cinnamic acid, which show spectral maxima at 210–215 nm (Sakakibara, Honda, Nakagawa, Ashida, & Kanazawa, 2003). The absorption maxima of F4 at 260–360 nm is in agreement with the high content of this fraction in phenolic compounds, which are reponsible for its enhanced anti-oxidant activity (see Table 1). Oppositely, F1 showed a flat spectrum at these wavelengths, in agreement with its limited content of phenolic compounds and its poor anti-oxidant activity. The crude extract shows lower absorbance values at 240 and 270 nm, this latter being

Table 2
Phenolic compounds identified (GC–MS) in the crude extract (CE) and aliphatic acids, esters and other compounds in the CE and in the fractions F1–F4

Time	Compound	CE or Fraction
Simple phenolic compounds		
29.11	Vanillin (4-hydroxy-3-methoxybenzaldehyde)	CE
33.10	Syringaldehyde (4-hydroxy-3,5-dimethoxybenzaldehyde)	CE
34.34	Vanillic acid (4-hydroxy-3-methoxybenzoic acid)	CE
35.48	Protocatechuic acid (3,4 dihydroxybenzoic acid)	CE
36.81	Syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid)	CE
37.85	Resorcilic acid (2,4 dihydroxybenzoic acid)	CE
Aliphatic acids and esters		
16.12	Pentanoic acid	CE; F3
23.58	Hexanoic acid	CE
35.76	Tetradecanoic acid	CE; F1; F2; F3; F4
38.90	Hexadecanoic acid	CE; F1; F2; F3; F4
42.05	Octadecanoic acid	F1; F2; F3; F4
41.55	9,12-octadecadienoic acid	CE; F1; F2; F3; F4
41.62	Oleic acid	CE; F1; F2; F3; F4
42.03	Heptadecanoic acid	CE; F3
43.80	Nonahexacontanoic acid	CE; F3
45.99	Dehydroabietic acid	F1
Others		
34.54	D-xylose	CE
39.32	Hexadecane	F4
32.70	Heptadecane	CE; F1; F2; F4
34.76	Octadecane	CE; F1; F2; F3; F4
36.60	Nonadecane	CE; F1; F2; F3; F4
34.99	Decane	F1; F2
40.19	Dodecane	CE
48.88	Pentadecane	F3
38.18	Eicosane	F1; F2; F4
39.65	Henicosane	F1
39.65	Docosane	F4

characteristic of soluble lignin. F2 and F3 presented absorption maxima at 290–310 nm, characteristic of benzoic and cinnamic acids (Sakakibara et al., 2003). F4 shows a maximum at 255 nm; benzoic acids also present a maximum at 250–260 nm and another one between 290 and 320 nm. F1 and F4, and particularly this latter, show the ability to absorb UV-B radiation (280–320 nm), and could be of interest in cosmetics and sun protectors. Natural extracts absorbing at wavelengths longer than 280 nm or higher than those of chemical filters such as PABA (*p*-aminobenzoic acid, which absorbs at 320 nm) are of particular utility for skin protection (Yanagida, Kanda, Shiji, & Tanabe, 1997).

3.4. GC–MS and HPLC analyses

Table 2 presents the compounds identified by GC–MS in the crude extracts and in fractions F1–F4. Aliphatic acids, whose formation during mild acid-based processing of lignocellulosics has been reported (Clark & Mackie, 1984; Klinke et al., 2002; Tran & Chambers, 1985; Tran & Chambers, 1986), are some of the major components identified. Fatty acids present in the fractions were tetradecanoic, hexadecanoic, octadecanoic, 9,12 octadecadienoic and oleic. The presence of saccharide compounds coming from hemicellulose hydrolysis, highly susceptible to sylilation, hindered an efficient derivatisation of phenolic compounds, some of which were satisfactorily identified in crude extracts. Because of this, the determination of phenolics was carried out by HPLC with DAD detection. The chromatograms of Fig. 3 show the different concentration profiles obtained for crude extracts and fractions, with the remarkable finding that the highly anti-oxidant fraction F4 is almost exclusively composed of ellagic acid.

Lignin depolymerisation products, obtained during acid hydrolysis or autohydrolysis of different lignocellulosics, are expected to have a closely related chemical nature, since the lignin precursors (guaiacyl, syringyl and *p*-hydroxyphenyl units) are the same. Low molecular weight compounds or simple phenolics (such as benzoic and cinnamic acids) are also naturally present in fruits and vegetables, and show anti-oxidant and antimicrobial capacity. The phenolic compounds identified in the crude extract have also been identified in hydrolysates from the acid processing of a number of lignocelullosic materials of residual origin from forest or agricultural activities. Protocatechuic acid (3,4 dihydroxybenzoic), with absorption maxima at 220, 260 and 294 nm (Natella, Nardini, Di Felice, & Scaccini, 1999), is naturally found in fruits and seeds (Sakakibara et al., 2003) and was detected in hydrothermal liquors from wood (Jönsson, Palmqvist, Nilvebrant, & Hahn-Hägerdal, 1998) and sugarcane bagasse (Martín et al., 2002). It is a more potent DPPH radical-scavenger than BHT (Keawpradub, Salaeh, & Muangwong, 2001), BHA and α-tocopherol (Fukumoto & Mazza, 2000), being effective to preserve (from oxidation) refined olive oil (Papadopoulus & Boskou, 1991), sunflower oil (Yanishlieva & Marinova, 1995), LDL (Cartron, Carbonneau, Fouret, Descomps, & Leger, 2001; Satué-Gracia, Andrés-Lacueva, Lamuela-Raventós, & Frankel, 1999; Zang et al., 2000) and β-carotene in a linoleic acid emulsion (Subba & Muralikrishna, 2002). At low dose, protocatechuic acid protects against cancer, although it presents liver and kidney toxicity at high dose (Nakamura, Torikai, & Ohigashi, 2001), and shows anti-inflammatory properties (Cartron et al., 2001) similar to those of cinnamic acids such as *p*-coumaric, caffeic, ferulic, gentisic, syringic and isovanillic acids (Fernández, Saenz, & García, 1998). 2,4 Dihydroxybenzoic acid, naturally found in vegetal products, shows ability as a DPPH radical-scavenger (McDonald, Prenzler, Antolovich, & Robards, 2001) and inhibits reactive oxygen species (ROS) formation in rat liver liposomes (Stupans, Kirlich, Tuck, & Hayball, 2002). Syringic acid, present in the crude extract and in F3, is a more potent DPPH radical-scavenger than BHA and BHT and comparable to ferulic acid, vanillic acid and *p*-coumaric acid (von Gadow et al., 1997), with anti-oxidant ability against oxidation in liver rat microsomes (Osawa, Ide, Su, & Namiki, 1987). This acid shows two absorption maxima (at 218 and 276–328 nm, see Robbins, 2003) and presents anti-inflammatory activity (Fernández et al., 1998). Other properties reported for this compound include higher radical-scavenging capacity than BHT (Kikuzaki, Hisamoto, Hirose, Akiyama, & Taniguchi, 2002), higher ability for protecting against linoleic acid oxidation than ferulic and *p*-coumaric acids (Bratt et al., 2003), higher capacity for preventing human LDL oxidation than *p*-coumaric acid (Andreasen, Landbo, Christensen, Hansen, & Meyer, 2001) and α-tocopherol (Pekkarinen, Stôckmann, Schwarz, Heinonen, & Hopia, 1999), and higher ability to protect against methyl linoleate oxidation than egg yolk phosphatidylcholine (Kikuzaki et al., 2002). Syringaldehyde and syringic acid are formed during degradation of syringyl propane units; syringaldehyde has been identified in hydrolysates from steam explosion of olive stones (Fernández-Bolaños, Felizón, Brenes, Guillén, & Heredia, 1998), wheat straw (Klinke et al., 2002), sugarcane bagasse (Martín et al., 2002) and wood (Ando et al., 1986; De Bari et al., 2002; Jönsson et al., 1998; Tran & Chambers, 1985; Tran & Chambers, 1986). Vanillin and vanillic acid, compounds naturally found in plant materials that can also be formed by degradation of the guaiacylpropane units, have been found in extracts from hydrolysates of olive wastes (Fernández-Bolaños et al., 1998), wheat straw (Klinke et al., 2002), sugarcane bagasse (Martín et al., 2002) and woods, such as pine (Clark & Mackie, 1984), poplar (Ando et al., 1986), red oak (Tran & Chambers, 1985; Tran &

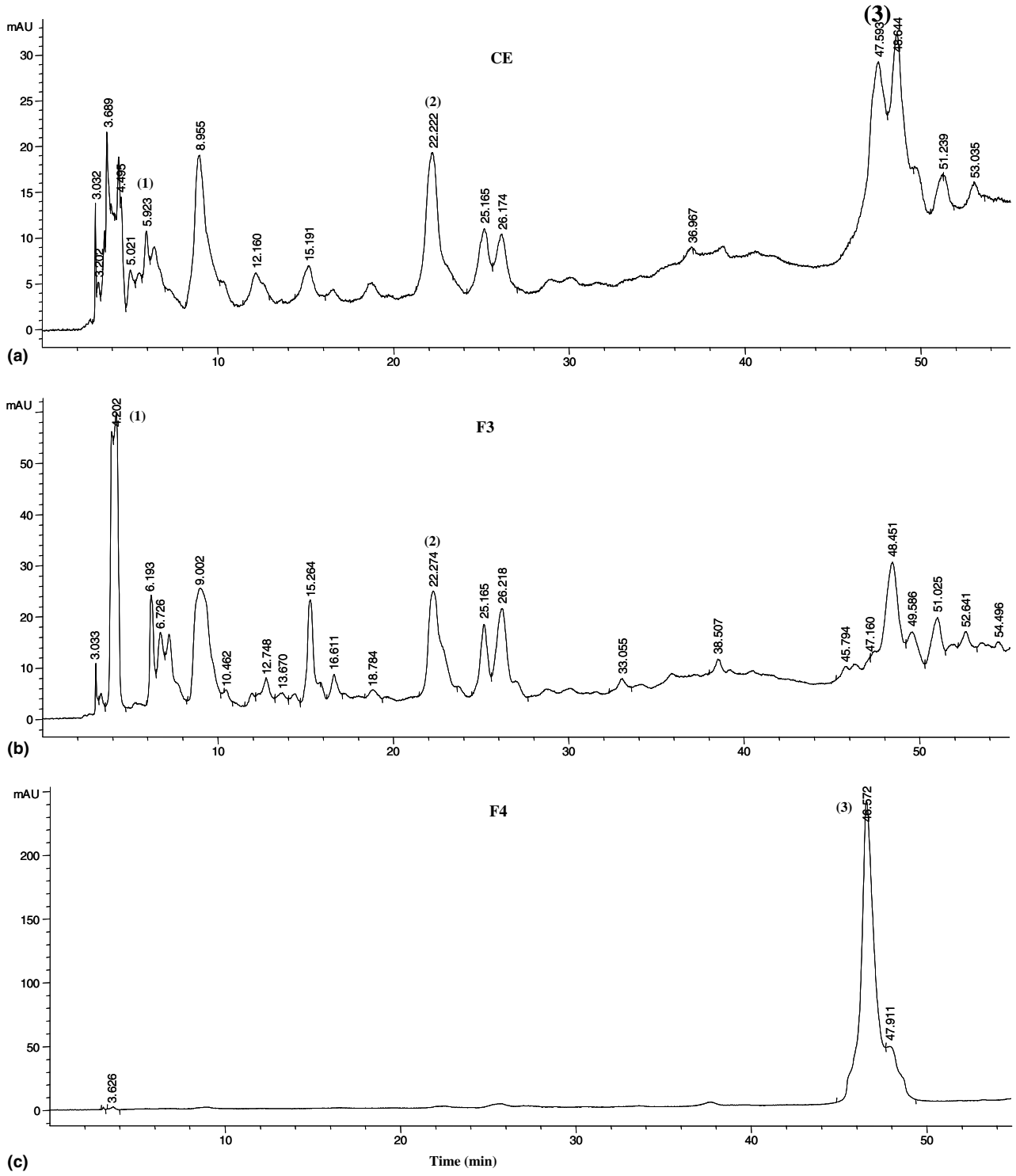

Fig. 3. Chromatogram of: (a) crude ethyl extracts, (b) fraction F3 and (c) fraction F4. (1) Gallic acid, (2) syringic acid, (3) ellagic acid.

Chambers, 1986), willow (Jönsson et al., 1998) and aspen (De Bari et al., 2002). Gallic acid is a potent alkyl radical-cavenger (Milic, Djilas, & Canadanovic-Brunet, 1998), showing stronger DPPH radical-scavenging activity than BHA, ascorbic acid (Brand-Williams, Cuvelier, & Berset, 1995; Chung et al., 1999), BHT or α-tocopherol (Fukumoto & Mazza, 2000). Its ability to inhibit oxidation in emulsion is lower than that of typical anti-oxidants (Subba & Muralikrishna, 2002), but higher than that of chlorogenic, ferulic and caffeic acids (Fukumoto & Mazza, 2000). Gallic acid protects low density lipoproteins and egg yolk phosphatidylcholine

against oxidation (Andreasen et al., 2001; Kikuzaki et al., 2002), inhibits the formation of ROS in human and rat liver microsomes (Stupans et al., 2002) and protects esters from oxidation (Brand-Williams et al., 1995; Cuvelier, Richard, & Berset, 1992). Fraction 4 contains ellagic acid, which is very effective in protecting lipids (Priyadarsini, Khopde, Kumar, & Mohan, 2002) and LDL (Meyer, Heinonen, & Frankel, 1998) from oxidation. Ellagic acid is an effective in vivo metal chelating agent suitable for suppressing nickel-induced renal and hepatic biochemical alterations (Ahmed, Rahman, Saleem, Athar, & Sultana, 1999), and can induce gastrointestinal enzyme activities contributing to a better detoxification of potentially carcinogenic compounds (van der Logt, Roelofs, Nagengast, & Peters, 2003). The DPPH radical-scavenging capacity of this compound was the highest among different phenolic compounds tested by Fukumoto and Mazza (2000). The ellagic acid content of woods increases with thermal treatment (Sarni, Montounet, Puech, & Rabier, 1990), probably from ellagitannin degradation, behaviour similar to that observed for other compounds, such as gallic acid and vanillic acid (Canas, Leandro, Spranger, & Belchior, 1999). The anti-oxidant activity of F4 is ascribed to both the comparatively high concentration of phenolics and the anti-oxidant potency of ellagic acid.

In conclusion, the fractionation of the crude extracts from ethyl acetate solubles of *Eucalyptus* wood hydrolysates allowed separation of four fractions (F1–F4) with different properties and composition. The anti-oxidant activity was concentrated in fractions F3 and F4. Fraction 3 presented anti-oxidant capacity similar to that of the crude extract, whereas a very remarkable anti-oxidant activity was determined for fraction F4, which had ellagic acid as its main phenolic component.

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