EXTRACTION OF PHENOLIC AND LIPOPHILIC COMPOUNDS FROM *PINUS PINASTER* KNOTS AND STEMWOOD BY SUPERCRITICAL CO_2

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Abstract. Near and supercritical CO_2 was used for the extraction of phenolic and lipophilic compounds from *Pinus pinaster* knots and stemwood grown on the Northwest of Spain. The extraction of sapwood located at the tree heigh containing the knots of the first dead branch was carried out at 10-25 MPa, 30-50 °C and 1-20% ethanol to assess the influence of the operational conditions on the yields of total solubles, on the radical scavenging capacity and on the composition of the extracts. Under selected conditions (25 MPa, 50 °C, 10% ethanol), extraction yields of 4.1% of the initial dry wood were attained, the extracts contained up to 7.5% phenolic compounds (stilbenes, lignans and flavonoids), and showed one third of the radical scavenging capacity of Trolox. Native resin acids accounted for one fourth of the dry weight whereas flavonoids and lignans and, in lower amounts, stilbenes and juvabiones, accounted for almost 17% of the identified components. Stem sapwood enabled lower extract yield (2.1%) and radical scavenging capacity (one fourth of the radical scavenging capactity of Trolox) than that attained in sapwood from knots at a dead branch.

Keywords: Supercritical CO_2 extraction, *Pinus pinaster* knots and stemwood, phenolic compounds, lipophilic compounds, radical scavenging.

1. Introduction

The use of environmentally-friendly, biorenewable, non-toxic solvents for the extraction of bioactive products is desirable. Supercritical carbon dioxide (SC-CO_2) is an inexpensive solvent, suited for the extraction of oils and lipophilic compounds, but many products with biological activity (including natural phenolic antioxidants) are poorly soluble in this solvent. A suitable polar modifier may improve the performance and the economic feasibility of a given process, allowing the extraction of polar phenolic compounds. The use of increasing amounts of cosolvent facilitates the sucessive extraction of the low polar and the more polar compounds. The use of a progressively higher pressure and modifier proportion allows the extraction of phenols with increasing molecular weight, as reported for grape seed concentrates [1]. Ethanol significantly enhanced the extraction of flavonoids and terpenoids from *G. biloba* [2], lignans from magnolia cortex [3], and the extraction from pigeonpea leaves of antioxidant constituents, cajaninstilbene acid and pinostrobin [4]. The production of antioxidant extracts from vegetal materials is a research field of increasing

importance. By selecting the operational conditions during extraction and separation, active antioxidants with other biological activities can be produced. The extraction conditions reported for different raw materials ranged between 15 and 40 MPa and 310 and 353 K with 2-10% ethanol as cosolvent [5].

The knots are a part of the tree stem, which is discarded during mechanical and chemical pulping. Softwood knots are formed of shorter fibers and contain large amount of extractives than normal wood. Considerable amounts of phenolic substances, such as lignans, flavonoids, and stilbenes are present in softwood knots [6, 7]. The knot extracts from several tree species were stronger antioxidants than the bark extracts and corresponding pure compounds [7]. In particular, *Pinus* wood has been reported to contain more potent antibacterial and antifungal components than other woods. The antimicrobial activity of Scots pine wood extracts correlated to the stilbene content [8], which was up to seven times higher in the knots than in the stemwood [9]. The pine wood extractives are majorly composed of non-polar compounds [10].

The high pressure extraction (supercritical fluid extraction and pressurised fluid extraction) of antioxidants from spruce bark was reported [11], but scarce data on the SC-CO_2 extraction of *Pinus* wood was published. The flavonoid content and radical scavenging activity of supercritical CO_2 extracts from *Pinus* bark was reported both at laboratory and at pilot scale [13], and the production of supercritical CO_2 extracts from *Pinus pinaster* bark with ability to limit oxidation was studied [14].

The aim of this work is to analyze the detailed composition of phenolic and lipophilic compounds in the extracts from *Pinus pinaster* knots and stemwood grown on the Northwest of Spain. The pine extracts were produced using compressed CO_2 modified with ethanol operating under selected conditions leading to higher extraction yield and radical scavenging capacity.

2. Materials and methods

2.1 Materials

Healthy 30 years old *Pinus pinaster* tree was felled in January 2012 near Ourense (NW, Spain), and disks (height, 5 cm) were cut and the following samples were prepared: stem sapwood, at 1.5 m from the ground (denoted SW) and sapwood of the disk containing the knots of the first dead branch (denoted DK_SW), at 6.5 m from the ground. The various samples were splintered, air-dried, milled to pass a 10-mesh screen, and stored at room temperature until use.

2.2 Supercritical CO_2 extraction

Extractions were performed using a supercritical fluid extractor (Thar Process, Inc.) with a 1000-mL extraction cell. For each experiment, the extraction cell was filled with 20 g pine wood (water content 6-13%). Extraction pressures in the range 10-25 MPa and temperatures in the range 30-50 ºC were assayed, using ethanol as modifier at concentrations in the range 1-20% (v/v). Solvent mass flow was fixed at 25 g/min and the extraction pressure was controlled by micrometering valves and the carbon dioxide used was Premier-X50S from. Dynamic extractions were performed when the experimental conditions in the extractor were achieved.

2.3 Trolox equivalent antioxidant capacity (TEAC)

This assay was perfomed according to the procedure by Re et al. [15]. The ABTS radical cation ($ABTS^{\bullet+}$) [2,2´-azinobis (3-ethyl-benzothiazoline-6-sulfonate)] was produced by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulfate allowing the mixture to stand in the dark at room temperature for 12-16 h before use. $ABTS^{\bullet+}$ solution was diluted with phosphate buffer saline (PBS) (pH 7.4) to an absorbance of 0.70 at 734 nm and equilibrated at 30 ºC. After addition of 1.0 mL of diluted $ABTS^{+}$solution to 10 μL of extract or Trolox standards in ethanol or PBS, the absorbance readings were taken for 6 min. Solvent blanks were run in each assay, and the percentage of absorbance inhibition was calculated as a function of the concentration of extracts and Trolox. The total amounts of extractives were determined gravimetrically.

2.4 Chromatographic methods

Stilbenes, flavonoids, lignans, juvabiones, resin acids, free fatty acids and free diterpenyl aldehydes were analyzed silylation following methods previously reported [9]. A 25 m x 0.20 mm i.d. column coated with

crosslinked methyl polysiloxane and a FID detector were used, with heneicosanoic as internal standard [16]. Steryl esters and triglycerides were analyzed on a 6 m x 0.53 mm i.d. column using cholesteryl heptadecanoate and 1,3-dipalmitoyl-2-oleyl glycerol as internal standards [17]. Identification of components was performed by GC-MS analysis of the silylated components with an HP 6890-5973 GC-MSD instrument, using a 25 m HP-1 column as above, and results were expressed as g/100g dried wood.

3. Results and discussion

3.1 Effect of cosolvent

The effect of cosolvent on the yields of total solubles (see Figure 1) as well as on the radical scavenging capacity and on the composition of extracts (see Table 1), were evaluated using the wood sample DK_SW. This sample was selected according to previous results on the investigation of the water-soluble components of *Pinus pinaster* wood [18], which presented the highest phenolic content radical scavenging activity. Operating at 30 ºC and 25 MPa, the extraction yields increased steadily with the ethanol concentration. Using 20% ethanol the extraction yield attained was almost three times the value with pure CO_2. In experiments performed at 50 ºC and 10 MPa, the yield was maximum operating with 10% ethanol.

The use of 1-20% ethanol as cosolvent resulted in extracts with ABTS radical scavenging capacities in the range 0.07-0.21 g Trolox/g extract. The highest concentration of phenolic compounds in the extracts (including stilbenes, flavonoids and lignans) was determined operating at 30 ºC and 25 MPa with 5% ethanol. The optimal modifier concentration for maximizing the lipophilic compounds content was 5% at 30 ºC, 25 MPa and 1% at 50 ºC, 10 MPa. The identified compounds accounted for up to 80% and 49% of the dry weight of the extracts, respectively. Pinosylvin monomethyl ether was the most abundant stilbene, followed by pinosylvin; hydroxy-dimethyl pinosylvin and hydroxy-monomethyl pinosylvin were found in trace amounts at concentrations of ethanol less than 5%.

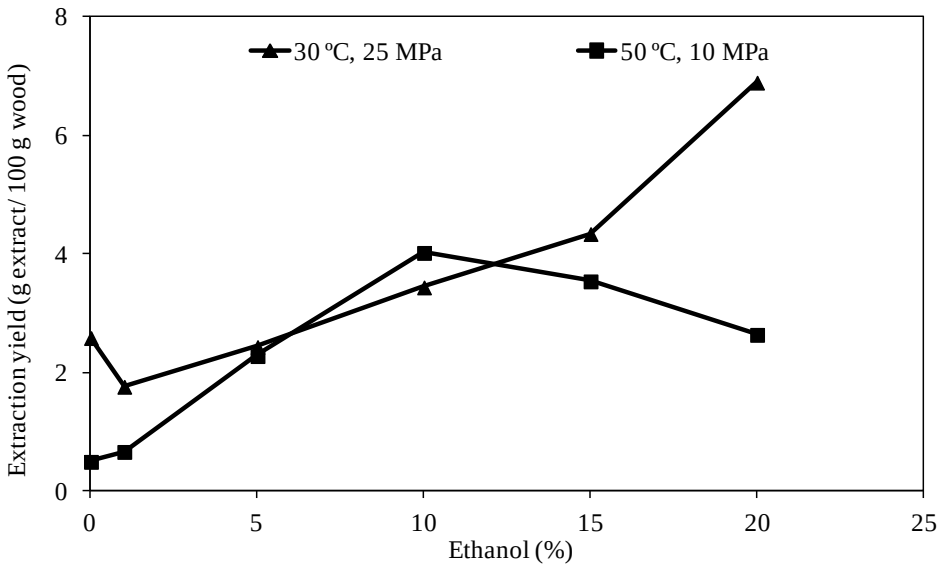

Figure 1. Effect of cosolvent on the yields of total solubles from *Pinus pinaster* sapwood dead knots (DK_SW) obtained by SC-CO_2 extraction at 30 ºC and 25 MPa and at 50 ºC and 10 MPa for 1 h.

Pinocembrin was the most abundant flavonoid, followed by pinobanksin-3-acetate and pinobanksin. Pinoresinol and nortrachelogenin were the main lignans identified. Epijubavione was the jubavione identified and pimaral was the diterpenyl aldehyde identified. Dehydroabietic acid was the most abundant native resin acid, followed by abietic acid, pimaric acid, palustric acid, isopimaric acid, neoabietic acid, levopimaric acid, sandaracopimaric acid and abietatetranoic acid. The most important substituted resin acids were x-hydroxydehydroabietic acid, 7-oxodehydroabietic acid and, x-hydroxyabietic acid. Oleic acid was the most abundant fatty acid, followed by linoleic acid; palmitic, stearic, margaric and linolenic acids.

Table 1. Effect of cosolvent on the TEAC and concentration of phenolic and lipophilic extractives in extracts from *Pinus pinaster* DK_SW sample obtained by SC-CO_2 extraction at 30 ºC and 25 MPa and at 50 ºC and 10 MPa for 1 h.

	30 ºC, 25 MPa						50 ºC, 10 MPa					
	0%	**1%**	**5%**	**10%**	**15%**	**20%**	**0%**	**1%**	**5%**	**10%**	**15%**	**20%**
TEAC (g Trolox/g extract)	0.07	0.11	0.18	0.18	0.16	0.19	0.01	0.06	0.06	0.08	0.12	0.21
CONCENTRATION (g/100 g extract)												
Phenolic compounds	1.8	5.0	7.9	6.8	3.7	4.5	1.7	2.3	2.4	2.2	3.6	5.1
Stilbenes	0.3	1.1	1.0	0.8	0.5	0.7	0.6	0.2	0.3	0.3	0.5	1.1
Flavonoids	1.2	2.1	4.4	3.3	1.4	1.8	1.0	1.4	1.5	1.3	2.0	2.0
Lignans	0.3	1.8	2.5	2.7	1.8	2.0	0.1	0.7	0.6	0.6	1.1	2.0
Lipophylic compounds	27.3	61.4	71.7	40.8	31.5	27.6	25.7	46.6	33.6	28.3	36.2	43.9
Juvabiones	0.3	0.7	0.9	0.6	0.4	0.4	0.3	0.5	0.4	0.4	0.5	0.7
Natural resinic acids	15.5	37.1	43.1	23.8	18.3	17.0	10.2	24.1	17.7	15.9	20.9	26.2
Modified resinic acids	1.4	4.1	5.1	4.0	2.8	2.9	0.5	2.6	1.6	1.7	2.4	3.7
Diterpenyl aldehydes	0.1	0.1	0.2	0.1	0.1	0.1	0.2	0.2	0.2	0.1	0.1	0.1
Fatty acids	6.6	14.8	17.2	9.0	7.8	5.8	11.9	15.6	11.5	9.1	10.4	10.8
Steryl esters	1.3	1.6	1.8	1.0	0.7	0.5	0.5	0.8	0.6	0.3	0.6	0.8
Triglycerides	2.1	3.0	3.4	2.3	1.4	0.9	2.1	2.8	1.3	0.8	1.3	1.6
Total amount	29.1	66.4	79.6	47.6	35.2	32.1	27.4	48.9	35.7	30.6	39.8	49.1

3.2 Effect of pressure and temperature

Ethanol (10%) was used as a co-solvent in further experiments to assess the influence of pressure (10-25 MPa) and temperature (30-50 ºC) on the extraction yield of solubles (Figure 2). The extraction yield increased when pressure increased from 10 to 15 MPa, and decreased at higher pressures. The highest extraction yield (6.1 %) was obtained at 30 ºC and 15 MPa, which was higher than those obtained by autohydrolysis (1.2-1.7%) [18] and similar to the extraction in ASE (data not published). The extract obtained at 50 ºC, 25 MPa with 10% ethanol presented the highest radical scavenging capacity (see Table 2). The influence of pressure was favourable for increasing the purity of the different components, being more marked at the highest temperature The extraction yied, TEAC and composition of phenolic and lipophilic extractives from DK_SW sample obtained under optimial conditions (50 ºC, 25 MPa with 10% ethanol) are shown in Table 2, comparative data from extraction of SW are presented. SW sample enabled lower yield (2.1%) and radical scavenging capacity (0.2 g Trolox/g extract) than that attained in DK_SW sample.

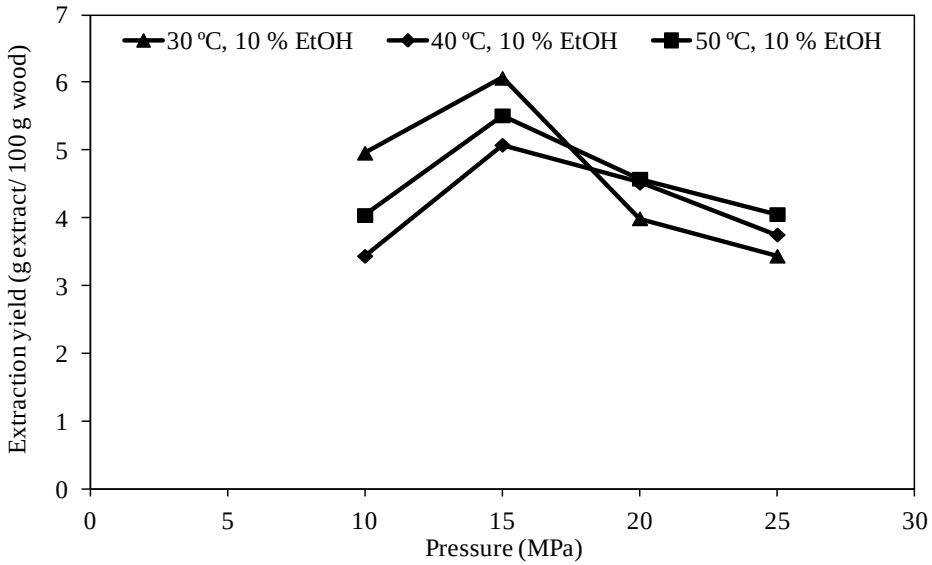

Figure 2. Effect of pressure and temperature on the yields of total solubles from *Pinus pinaster* sapwood dead knots (DK_SW) obtained by SC-CO_2 containing 10% ethanol as a modifier.

The most abundant simple phenolics were isoferulic acid and 3,4-dihydroxycinnamic acids; 3-hydroxybenzoic acid, vanillic alcohol, 4-hydroxybenzyl alcohol, 4-hydroxycinnamic acid, dihydroconiferyl alcohol, vanillic acid and pinitol were found at lower concentrations. Similar results were found in

hydrothermal extracts, where isoferulic and 3,4-dihydroxycinnamic acids reached concentrations up to 0.1-0.2% of the extract [18].

Table 2. Extraction yield, TEAC and composition of phenolic and lipophilic components in extracts from *Pinus pinaster* wood samples (DK_SW and SW) produced by SC-CO_2 operating at 25 MPa, 50 ºC, with 10% ethanol as a modifier 1 h. Trace amounts: tr < 0.04 g/100 g extract.

		DK_SW	SW
EXTRACTION YIELD (g extract/100 g wood)		4.06	2.12
TEAC (g Trolox/ g extract)		0.34	0.22
CONCENTRATION (g/100 g extract)			
Stilbenes		1.02	0.61
	Pinosylvin monomethyl ether	0.52	0.20
	Pinosylvin	0.26	0.17
	Hydroxy-dimethylpinosylvin	0.11	0.15
	Hydroxy-monomethylpinosylvin	0.12	0.09
Flavonoids		3.64	2.42
	Pinocembrin	1.32	0.83
	Pinobanksin	0.36	0.32
	Pinobanksin-3-acetate	0.57	0.43
	Dihydrokaempferol	-	-
	Taxifolin	-	-
Juvabiones		0.64	0.17
	Epijuvabione acid	0.64	0.17
Lignans		2.88	1.46
	Todolactol	-	tr
	Isolariciresinol	-	tr
	Secoisolariciresinol	tr	tr
	Nortrachelogenin	1.28	0.68
	Pinoresinol	1.55	0.67
Native resin acids		25.18	18.97
	Pimaric acid	3.14	2.48
	Sandaracopimaric acid	0.50	0.41
	Isopimaric acid	1.86	1.23
	Palustric acid	2.70	2.01
	Levopimaric acid	0.78	0.27
	Dehydroabietic acid	9.59	8.87
	Abietic acid	4.75	2.89
	Abietatetraenoic acid	0.46	0.24
	Neoabietic acid	1.40	0.57
Modified resin acids		4.06	2.99
	x-Hydroxyabietic acid	0.72	0.69
	7-Oxodehydroabietic acid	0.81	0.54
	x-Hydroxydehydroabietic acid	1.71	1.16
	Dihydroxy-dehydroabietic acid	0.26	0.22
	x-Hydroxy-7-oxodehydroabietic acid	0.56	0.37
Diterpenyl aldehydes		0.06	0.04
	Pimaral	tr	tr
Fatty acids		8.56	20.94
	Caprylic acid (8:0)	tr	tr
	Palmitic acid (16:0)	1.04	2.07
	Margaric acid (17:0)	0.14	0.37
	Linolenic acid (18:3)	tr	0.13
	Linoleic acid (18:2)	2.01	5.21
	Oleic acid (18:1)	4.96	12.56
	Stearic acid (18:0)	0.28	0.52
Steryl esters		0.84	2.42
Triglycerides		2.12	0.66
Total amount		48.99	50.68

4. Conclusions

Extracts from knots of *Pinus pinaster* trees grown on the Northwest of Spain were produced by supercritical carbon dioxide extraction. The addition of ethanol as cosolvent increased the extraction yield up to 6.9% of the dry wood weight. The extracts produced under optimized conditions (50ºC, 25 MPa and 10% ethanol) showed a radical scavenging capacity of one third of that of Trolox.

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