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LIPOPHILIC EXTRACTIVES AND MAIN COMPONENTS OF BLACK PINE CONES

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During the last decades, considerable attention has been paid first to pine cone extracts, especially for production of commercial, medicinal, and preservative compounds. The carbohydrate composition of some pine cones and the resin acid composition as well as fungi-toxic activities of cone extractives were investigated by Michales et al. [1]. The characterization of pine cone polysaccharides and lignin was made by Eberhardt and Young [2]. The antioxidant activity and total phenol content of different components from pine species have been estimated [3]. The chemical constituents of pine cones from species *Pinus annandii* and *Pinus sylvestris* were identified by Yang et al. [4] and Ganenko et al. [5] respectively.

Black pine (*Pinus nigra* subsp. *pallasiana*) is widely distributed in Turkey. It is one of the most popular trees for timber use. Because of the biomass potential value, the aim of the present study was to elucidate the composition of both main and extractive components of Turkish black pine cones. In order to determine the extent of the change in the chemical composition, fresh and several years seasoned old cones were studied separately.

The amount of ash, extractives, and main components of fresh and old cones are given in Table 1. The ash and extractive content of old sample was higher than that of fresh one. It was assumed that the inorganic residues from the forest ground could not be completely removed by washing the old cones and at least a small part of the ash might have its origin in the forest soil.

Considering the solubilities of hot water, 1% NaOH, alcohol-cyclohexane, and alcohol, new cones delivered slightly higher values. The Klason lignin content of cones was estimated in two ways; first, extractives were removed from cone meal with a two-step organic solvent extraction and thereafter the extracted material was hydrolyzed with 72% sulfuric acid, and secondly a third-step extraction with 1% NaOH was conducted before sulfuric acid treatment. From Table 1 it is apparent that the acid-insoluble parts of the fresh and old cones differ from each other remarkably when the material was prepared with or without 1% NaOH treatment.

After the third-step alkali extraction, the results from both specimens were very close and they probably indicate real lignin values. The lower lignin amount after alkali extraction reveals that phenolic compounds in the cones led to overestimations in the Klason lignin content. Both the Klason- and acetyl bromide lignin content were determined in some pine cones [2]. They found that the Klason lignin content of cones had a wider range of values (26.9-42.4%). However, when they used the acetyl bromide method to determine lignin content, their results were lower than those of the Klason content. These acetyl bromide-lignin estimations are in good agreement with our results obtained after 1% NaOH treatment. Because conifer cones contain proanthocyanidin polymers and they precipitate after condensation in the acidic reactions, Eberhardt and Young explained the possibility that a portion of the total content of proanthocyanidins may not be removed during the extraction process.

Remaining insoluble after extraction, these materials would result in an overestimation of the actual lignin contents of cones [6].

The acid soluble lignin amount in old samples was a bit higher than that of new ones. Perhaps due to some enzymatic degradation by microorganisms, the acid soluble lignin content increases depending on the seasoning of cones on the forest ground.

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TABLE 1. Solubilities and Lignin Content of Cones, %

Component	Fresh	Old
Ash (% of original material)	0.7	3.9
Solubilities:		
Hot water	5.6	5.9
1% Sodium hydroxide	18.7	19.9
Cyclohexane extracts (CE)	2.47	4.75
- Neutrals (as % of CE)	49.1	14.3
- Saponifiables (as % of CE)	50.83	26.96
Alcohol-cyclohexane	2.3	2.9
Alcohol	0.6	0.8
Lignin (Klason)*	29.7	38.6
Lignin (Acid-Soluble)*	0.4	0.7
Lignin (Klason)**	22.9	23.1

*Based on material extracted with EtOH-cyclohexane and EtOH; **Based on material extracted with EtOH-cyclohexane, EtOH, and 1% NaOH.

TABLE 2. Carbohydrate Composition of Cones, %

Component	Fresh cones		Old cones	
	Monosac.	Polysac.*	Monosac.	Polysac.*
Glucose	39.7	35.7	34.0	30.6
Xylose	5.6	4.9	5.8	5.1
Galactose	3.2	2.9	2.6	2.3
Arabinose	2.5	2.2	2.3	2.0
Mannose	16.8	15.1	13.3	12.0
Σ	67.8	60.9	58.1	52.1
Klason+Acid-soluble Lignin		30.2		39.3
Sum		91.1		91.4

*Based on extract-free material.

The sugar composition of fresh and old cones is shown in Table 2. As in the wood, glucose, the hydrolysis product of chiefly cellulose, was the main sugar in the hydrolyzates. The old cone showed higher levels of xylose but lower quantities of glucose, mannose, and galactose than the new cones. Decrease in the amount of these monosaccharides can be attributed to the favored degradation of the original polysaccharide, galactoglucomannan, during the seasoning of cones. Similar to our results, the resistance of cellulose and xylans to the decay of woods by brown-rot fungi was observed [7].

The chemical composition of neutrals and acidic fractions from cones are shown in Table 3. The yield of lipophilic extractives in fresh and old cone was 2.47% and 4.75% respectively. While the amount of saponifiables and neutrals in fresh cone extract was estimated to be 50.8% and 49.1%, these values were determined as 27.0% and 14.3% in the extract of old cones. Thus, about 60% of cyclohexane extract obtained from old cones could not be recovered during the saponification procedure.

The neutrals were composed mainly of diterpene alcohols, diterpenes, and sterols. Monoterpenes and sesquiterpenes were minor constituents of the neutrals. The extracts exhibited some quantities of borneol, junipene, and caryophyllene oxide. The most abundant diterpenes in cones were neoabietol, neoabietal, dehydroabietol, pimarol, and some diterpene alcohols (MW=286, 288). The fresh cone contained much higher levels of these compounds than the old one. Neoabietol and neoabietal (4.87% and 3.93% in fresh cone) were even below detectable limits in the old cone. As in wood, β-sitosterol was the major component in both extracts, its amount being relatively increased in old one.

TABLE 3. Composition of Acidic and Neutral Fractions from Lipophilic Extractives of Cones (TMS: Trimethyl Silyl Ether, ME: Methyl Ester), %

Neutrals	Fresh	Old	Saponifiables	Fresh	Old
Verbenone	0.10	0.43	α-Campholenic acid ME	0.05	0.15
Unident. (MW 224) & Borneol TMS	1.31	2.09	Dodecanoic acid ME	0.24	0.15
Monoterpene alcohol TMS (MW 224)	2.16	2.80	Tetradecanoic acid ME	0.27	0.28
Junipene	0.43	2.14	Hydroxydodecanoic acid ME	0.29	0.17
β-Caryophyllene	0.68	0.05	Me-tetradecanoic acid ME	0.05	0.11
α-Humulene	0.21	0.07	Pentadecanoic acid ME	0.13	0.26
Sesquiterpene oxide	0.15	0.67	9-Hexadecenoic acid ME	0.21	0.25
Caryophyllene oxide	2.06	2.00	Hexadecanoic acid ME	2.25	3.80
Humuladienone	0.28	0.23	Hydroxy acid (C14) ME & unid. (MW 274)	1.05	1.11
Sesquiterpene alcohol TMS	0.61	1.51	Methylhexadecanoic acid ME	0.11	0.19
Dodecenyl acetate	Tr.	0.16	Heptadecanoic acid ME	0.18	0.25
Sesquiterpene alcohol (MW 292)	0.97	3.05	Tetradecadioic acid di-ME	0.07	0.08
Hexadecanol TMS	0.11	0.23	Hydroxyhexadecanoic acid ME	0.16	0.16
Pimaradiene	0.19	0.19	Octadecatrienoic acid ME	2.34	0.62
Norabietatriene (MW 256)	0.29	0.31	Octadecadienoic acid ME (isomer)	0.49	0.65
Norditerpene (MW 258)	0.13	Tr.	9,12-Octadecadienoic acid ME	6.77	2.51
Manoyl oxide & unid.	0.27	0.30	9-Octadecenoic acid ME	4.26	5.53
Nordehydrabietane (MW 256)	1.17	0.86	Octadecanoic acid ME	0.87	1.21
Rimuene	0.56	Tr.	Hexadecadioic di-ME & unident.	0.34	0.82
Manoyl oxide isomer	0.56	1.59	Me-pimarate	9.0	3.80
Dehydroabietane	0.95	0.47	Me-sandaracopimarate	3.03	2.11
Unident. & Diterpene (MW 272)	0.62	0.41	Ambreinolide	1.26	0.82
Diterpene alcohol TMS (MW 360)	0.23	0.55	Me-isopimarate & Me-palustrate	7.01	8.8
Neoabiatadiene (MW 272) & unid.	1.29	1.14	Me-levopimarate	1.05	Nill
Pimaral isomer	0.56	0.25	Me-dehydroabietate	7.83	8.13
Diterpene alcohol TMS (MW 360)	0.30	0.45	Me-abietate	4.35	0.79
Dehydroditerpenyl TMS (MW 358)	Tr.	2.16	Hydroxyditerpene acid ME	3.33	5.14
Diterpene alcohol TMS (MW 362)	7.08	0.11	Neoabietic acid ME	4.49	Tr.
Pimarol TMS	2.47	1.68	Dimethyldihydroagathate	2.59	4.76
Dehydroabietal	2.18	1.01	Hydroxyabietatrienoic acid ME	1.93	3.12
Diterpene alcohol TMS (MW 360)	6.16	3.53	Hydroxyabietic acid ME	3.48	1.32
Dehydroabietol TMS	9.95	8.0	Docosanoic acid ME	2.16	3.98
Manoyl oxide derivative (MW 430)	0.87	0.88	Diterpenoids (MW 330)	4.74	3.37
Diterpene alcohol TMS (MW 360)	3.37	0.73	Oxodehydroabietic acid ME (MW 328)	3.29	7.08
Neoabietal	3.93	Nill	Me-dehydroabietate derivative	0.64	0.72
Neoabietol TMS	4.87	Nill	Tricosanoic acid ME	0.42	0.39
Docosanol TMS & unident.	0.91	0.90	Tetracosanoic acid ME	0.50	0.80
Tetracosanol TMS	0.65	1.29	Hydroxytricosanoic acid ME	0.17	0.25
Campesterol TMS	0.50	0.85	Pentacosanoic acid ME	0.05	0.11
Unident. sterol TMS (MW 484)	0.26	0.49	Hydroxytetracosanoic acid ME	0.21	0.33
Sitosterol TMS	5.71	9.80	Hexacosanoic acid ME	0.05	0.09
Sitostanol TMS	0.16	0.27	Unknown compounds	18.2	25.8
Unid. sterol TMS (MW 574)	0.17	0.43	Sum	99.9	100.0
Methyl cycloartenol TMS	0.28	0.29			
Unknown compounds	34.3	45.6			
Sum	100.0	100.0			

The acidic fraction (saponifiables) comprises practically fatty and resin acids. Among fatty acids, palmitic, octadecatrienoic, linoleic, oleic, and docosanoic acids were most abundant. Somewhat higher quantities of palmitic, stearic, and docosanoic acids were notable in old cones, while unsaturated fatty acids amounted to higher values in fresh ones. This may be because unsaturated fatty acids were degraded or perhaps converted to other compounds during the seasoning of cones.

In old cones, the levopimaric and neoabietic acids were almost absent, and the concentrations of pimaric, sandaracopimaric, palustric, and abietic acids were lower. In contrast, the amount of hydroxyditerpene acids, dehydoabietic, hydroxyabietatrienoic, and oxodehydroabietic acids, exhibited an increase in the old cone. As a previous study showed, levopimaric, neoabietic, abietic, and palustric acids can be oxidized into dehydroabietic acid, either directly or with abietic acid as an intermediary step [8].

About 10 kg of fresh pine cones from *Pinus nigra* var. *pallasiana* were collected from Belgrad Forest near Istanbul. The old cones, which were weathered between 1-3 years on the forest ground, were collected and sent by forest directory in Eskisehir district. Residual soil and dust were removed from old cones by rinsing with cold distilled water and thereafter air-dried. The fresh cones were warmed in the oven (50-60°C) until concomitant seeds were released. Representative amounts of air dry cones were ground in a Wiley mill and sieved to obtain the fraction between 40 and 100 mesh [9].

To determine the total amount of extractives, this fraction was extracted in Soxhlet with ethanol-cyclohexane (1:2) and then with ethanol successively (Tappi T 204-om-88). Solubilities of hot water and 1% NaOH were determined according to Tappi test methods (Tappi T 207-om-88, Tappi T 212-om-88). For isolation of lipophilic extractives, about 20 g of the cone meal was extracted in a Soxhlet apparatus with cyclohexane for 8 h. The extract was first saponified with 0.5 N KOH solution at 70°C, then separated into two fractions, as neutrals and saponifiables, by means of solvent extraction with petroleum ether. The neutral fraction was silylated with a mixture of trimethylchlorosilane and *bis*-(trimethylsilyl)-trifluoracetamide (1:3) [10], while saponifiables were methylated by diazomethane. Acidic and neutral fractions were analyzed in GC-MS (Shimadzu, QP 5050 A) on a 30 m DB-1 capillary column. The temperature program for the separation of acids was 80°C for 3 min, then 8°C/min to 240°C, 4°C/min to 280°C, 10 min at 280°C. The temperature progression for the neutrals was 90°C for 0.75 min, 8°C/min to 250°C, 5 min at 250°C, 10°C/min to 280°C, 5 min at 280°C, 5°C/min to 300°C, 12 min at 300°C. The He-flow rate was maintained at 1.2 mL/min for acids and 1.5 mL/min for neutrals with a split ratio of 1/10. The commercial libraries Nist 21, Nist 107, and Wiley 229 were used for the identification of the compounds.

Lignin content as acid-insoluble residue (Klason) was determined according to Runkel and Wilke [11], and the amount of acid soluble lignin was also estimated (Tappi UM 250) [12]. In order to determine the polysaccharide composition, the extracted sample was hydrolyzed with 77% H_2SO_4 [13]. The hydrolyzate was than neutralized with $Ba(OH)_2$, and monosaccharides were analyzed in HPLC (600 Controller and 717 Autosampler, 410 RI Detector by Waters, column: Aminex HPX 87P, Biorad).

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