Zbornik gozdarstva in lesarstva 71, s. 159 - 172

GDK 892.62

Prispelo / *Received*: 20.05.2003
Sprejeto / *Accepted*: 21.10.2003

Izvirni znanstveni članek
Original scientific paper

ISOLATION AND CHARACTERIZATION OF ESSENTIAL OILS FROM THE CONES OF NORWAY SPRUCE (*PICEA ABIES* KARST.), EUROPEAN LARCH (*LARIX DECIDUA* MILL.) AND SCOTS PINE (*PINUS SYLVESTRIS* L.)

Janja ZULE[*], Vesna TIŠLER[**], Andrej ŽUREJ[**], Nikolaj TORELLI[***]

Abstract

Extraction and chemical characterization of essential oils from the cones of Norway spruce, European larch and Scots pine are presented in the article. Various monoterpenes have been identified and their relative concentrations calculated by means of gas chromatography. The contents of higher terpenes in individual oils have been also evaluated and the differences in their chemical compositions discussed.

Key words: Norway spruce, Scots pine, European larch, essential oils, terpenes, chemical characterization, gas chromatography

IZOLIRANJE IN KARAKTERIZIRANJE ETERIČNIH OLJ IZ STORŽEV NAVADNE SMREKE (PICEA ABIES KARST*), EVROPSKEGA MACESNA (LARIX DECIDUA* MILL.*) IN RDEČEGA BORA (PINUS SYLVESTRIS* L.*)*

Izvleček

Preučevana je bila ekstrakcija eteričnih olj iz svežih storžev navadne smreke, rdečega bora in evropskega macesna ter njihova kemijska karakterizacija z metodo plinske kromatografije. Identificirani so različni monoterpeni in določene njihove relativne koncentracije. Ovrednotene so tudi vsebnosti višjih terpenov in podane razlike v kemijski sestavi posameznih eteričnih olj.

Ključne besede: navadna smreka, rdeči bor, evropski macesen, eterična olja, terpeni, kemijska karakterizacija, plinska kromatografija

[*] Inštitut za celulozo in papir, Bogišičeva 8, 1000 Ljubljana, SVN
[**] Biotehniška fakulteta, Oddelek za lesarstvo, Rožna dolina, C. VIII/34, 1000 Ljubljana, SVN
[***] Gozdarski inštitut Slovenije, Večna pot 2, 1000 Ljubljana, SVN

VSEBINA
CONTENTS

1 **INTRODUCTION** 161
UVOD
2 **MATERIALS AND METHODS** 163
MATERIALI IN METODE
3 **RESULTS AND DISCUSSION** 165
REZULTATI IN RAZPRAVA
4 **CONCLUSIONS** 170
ZAKLJUČKI
5 **POVZETEK** 170
6 **REFERENCES** 172
VIRI

1 INTRODUCTION
UVOD

A cone is a reproductive structure in gymnosperms (and some pteridophytes), known technically as a strobilus. It consists of closely packed sporophylls bearing sporangia, arranged around the central axis. Conifers produce different male (staminate) and female (ovulate) strobili. The female strobilus (commonly recognized as a »cone «) comprises spirally arranged ovules and lignified ovuliferous scales. As opposed to broad-leaved (applied to angiosperm trees) conifers are gymnosperms with linear, commonly pungent leaves.

Cones usually need about a year to ripen, though in case of some species this period may be twice or three times longer.

Cones also contain, like wood, different extractive components. These are both hydrophilic and hydrophobic low molecular weight compounds, which compose wood material together with cellulose, different polysaccharides and lignin. By means of extraction they can be isolated from wood. Most commonly used extraction solvents are diethyl ether, methyl tert-butyl ether, dichloromethane, acetone, ethanol, methanol, hexane, toluol, tetrahydrofuran and water. Although the number of various extractive compounds may be typically very large, their total content in wood is relatively small and amounts up to few per cent at the best. Characteristic hydrophobic extractives of conifers are often designated by the common name wood resin (FENGEL / WEGENER 1989).

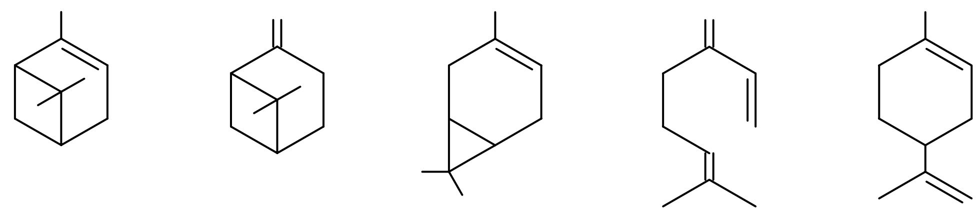
As such they play an important role in basic metabolytic processes in a living tree and serve as protective agents against environmental distress. Extractives influence chemical, biological and mechanical properties of wood. Their impact on light resistance, inflammability, combustibility, hygroscopicity as well as on gas and liquid penetration of water into wood structure is of great importance.

The most important groups of extractives are terpenes, glycerides, steryl esters, sterols, waxes, fatty acids, higher alcohols, resin acids and aromatic compounds like lignans, stilbenes and flavonoids. So far, more than 4000 different terpenes and their derivates only have been isolated from plant tissue and chemically characterized. They differ according to the number of basic structural $C_{10}H_{16}$ units that constitute them. Typical terpene groups are presented in Table 1.

Table 1: Classification of terpenes
Preglednica 1: Klasifikacija terpenov

Group *Skupina*	Number of $C_{10}H_{16}$ units *Število enot $C_{10}H_{16}$*	Molecular formula *Molekulska formula*
Monoterpenes *Monoterpeni*	1	$C_{10}H_{16}$
Sesquiterpenes *Seskviterpeni*	1,5	$C_{15}H_{24}$
Diterpenes *Diterpeni*	2	$C_{20}H_{32}$
Triterpenes *Triterpeni*	3	$C_{30}H_{48}$
Politerpenes *Politerpeni*	> 4	> $C_{40}H_{64}$

Structural units may form various spatially oriented rings, which couple with each other in different ways to give acyclic, monocyclic, bicyclic and tricyclic monoterpenes. Higher terpenes can possess an even higher number of rings. Technologically most important among these groups are monoterpenes and diterpenes. The former are very volatile and give off characteristic smells (TIŠLER / KVAS 1988, TIŠLER / DEIGELE 1991). The most representative members are α- and β-pinene, Δ-3-carene, p-cimene, camphene, myrcene, limonene, and some others. Structural formulas are presented in Figure 1.



α- pinene	β-pinene	Δ-3-carene	myrcene	limonene
α- pinen	β-pinen	Δ-3-karen	mircen	limonen

Figure 1: Monoterpenes from softwoods
Slika 1: Monoterpeni iglavcev

Terpenes are the main constituents of essential oils, which are synthesized in various parts of plant tissue. The most abundant quantities may be found in blossoms, leaves and cones. Other less important ingredients of essential oils are organic substances like aldehydes, esters and alcohols. Oils may be isolated from fresh plant materials with the aid of different extraction methods. They are often used as raw materials in chemical, pharmaceutical, cosmetic and food producing industries (STENIUS 1999, ZULE 2001, SJÖSTRÖM 1981).

The main purpose of our investigation was to isolate essential oils from the cones of Norway spruce (*Picea abies* Karst.), European larch (*Larix decidua* Mill) and Scots pine (*Pinus sylvestris* L.) and to establish kinds and quantities of individual monoterpenes present in each species. The results are applicable for determination of botanical origin of wood species and are as such very important for further chemotaxonomical research, since any deviation from the obtained values may indicate hybrid of the chosen sort of wood.

There were no published data available on terpene composition of cones.

2 MATERIALS AND METHODS
MATERIALI IN METODE

2.1 SAMPLES

The chosen cone samples were fresh, randomly selected biyearly female cones from 30 to 40 years old spruce, pine and larch trees. Individual species differed in colour, shape, size and weight. Sampling was performed in July in a region situated approximately 400 m above see level. Samples were stored immediately after collecting in plastic bags and kept until their laboratory analysis in a refrigerator at 4 ºC.

2.2 DETERMINATION OF DRY MATTER CONTENT

Cones were dried for 24 hours in a laboratory oven at 105 ºC. After cooling for 2 hours in the desiccator, they were weighed and the content of dry matter calculated.

2.3 ESSENTIAL OIL EXTRACTION

In order to isolate essential oils from samples, steam distillation was applied. Appropriate laboratory distillation equipment was used for this purpose.

200 g of previously ground cones and 750 mL of deionized water were poured into a 1000 mL vessel. The distillation procedure lasted for 2 hours, whereupon the volume of the extracted oil was measured by means of a graduated tube. The oil, still partly mixed with water, was quantitatively removed from the distillation vessel and transferred to the separating funnel. The whole procedure was repeated several times for each type of cone in order to obtain enough sample for further analyses.

2.4 DETERMINATION OF MONOTERPENES BY GAS CHROMATOGRAPHY

5 mL of essential oil water suspension was transferred to a 10 mL testing tube with a stopper.

1 mL of methyl tert-butyl ether was added, after which the tube was being intensively shaken for 1 minute. The obtained mixture was left standing until the upper organic phase was quantitatively separated from the lower water phase. The organic layer was transferred into another testing tube. The extraction procedure was repeated once more, and the second extract was added to the first. Thus the essential oils were separated from water suspensions.

Gas chromatograms of the extracts were recorded on the Hewlett Packard HP 5890 gas chromatograph at the following experimental conditions (Table 2).

Table 2: Experimental gas chromatography conditions
Preglednica 2: Eksperimentalne razmere kromatografiranja

Column *Kolona*	Ultra 1 (Crosslinked Methyl Silicone Gum; 25 m, 0,32 mm, 0,52 µm)
Mobile phase *Mobilna faza*	Nitrogen (flow 1,5 mL/min)
Injector *Injektor*	Split/splitless (temperature 250 ºC)
Detector *Detektor*	FID-flame ionisation (temperature 280 ºC)
Temperature programme *Temperaturni program*	80 ºC(2 min); 5 ºC/min; 200 ºC (2 min); 3 ºC/min; 280 ºC (10 min)
Sample volume *Volumen vzorca*	2 µL
Terpene standards *Terpenski standardi*	α-pinene, β-pinene, myrcene, Δ-3-carene, limonene

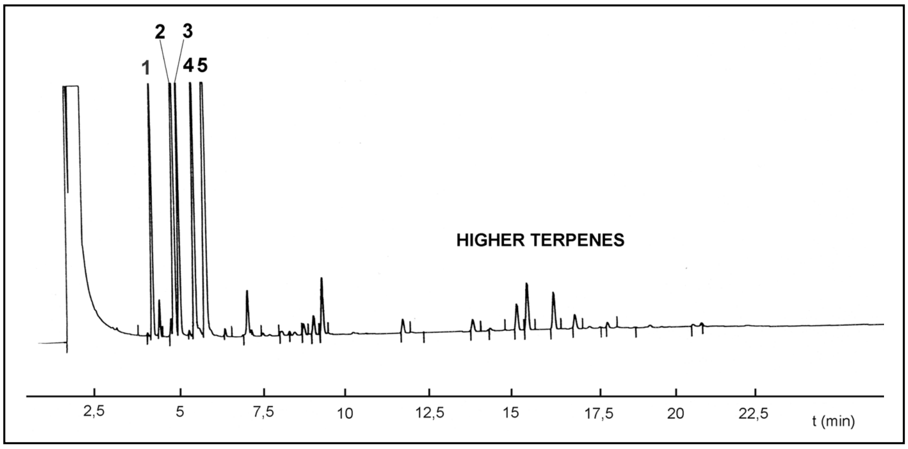
Identification of individual peaks on chromatograms was performed by comparison of retention times with appropriate values of standard monoterpene solutions. For this purpose, the chromatograms of several available monoterpenes in methyl tert-butyl ether solution were recorded under the same experimental conditions. Relative monoterpene and higher terpene contents were calculated from chromatograms by integrating their whole area and the areas of individual peak groups. In the same manner, relative contents of identified monoterpenes in monoterpene fractions were obtained.

3 RESULTS AND DISCUSSION
REZULTATI IN RAZPRAVA

The largest portion of dry matter was found in larch cones (43.6 %), a somewhat lower value was determined in pine (38.6 %), and the lowest in spruce cones (35.2 %). Volatile extractive compounds are not included in the measured dry matter content, since they evaporate in oven during the drying process. Pronounced differences in values can be ascribed to the fact that the cones of conifers differ according to their chemical composition.

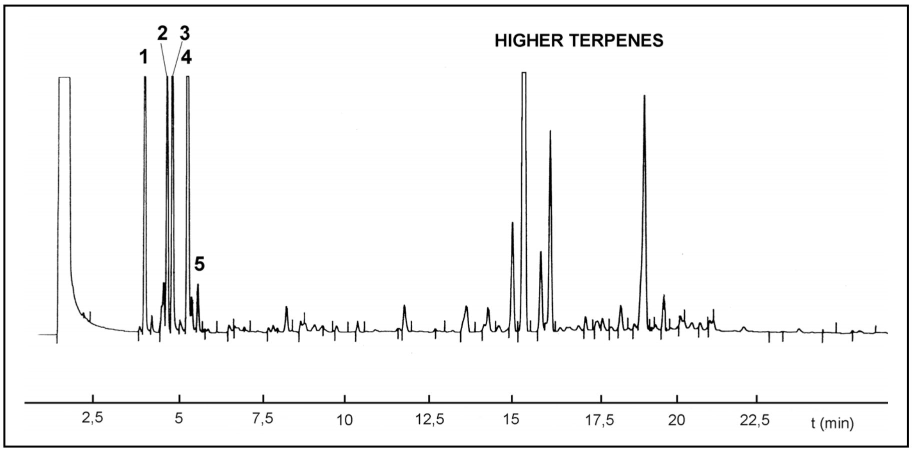
Volume measurements of obtained essential oils showed that in case of spruce and pine cones, less oil could be released, that is 0.10 mL per 200 g of fresh sample, while this value amounts to 0.12 mL for larch samples.

Gas chromatograms of spruce, pine and larch essential oils are presented in Figures 2, 3 and 4.



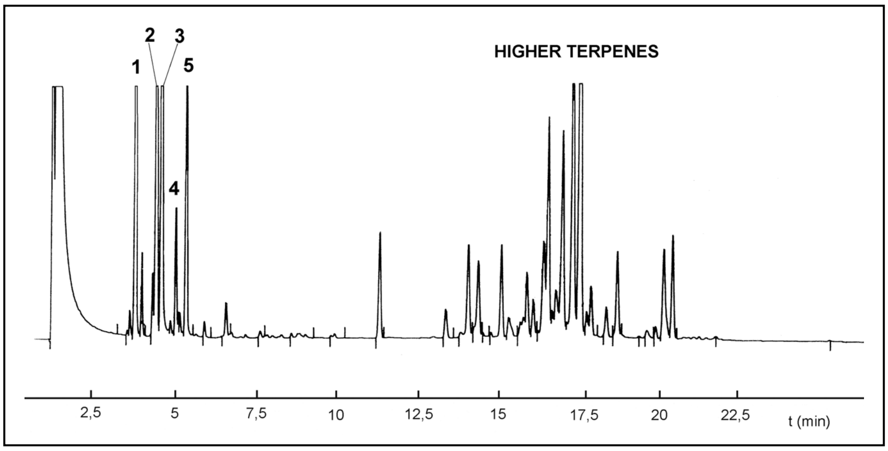
(1 - α-pinene, 2 - β-pinene, 3 - myrcene, 4 - Δ-3-carene, 5 – limonene)

Figure 2: Gas chromatogram of spruce cone essential oil
Slika 2: Plinski kromatogram eteričnega olja smrekovih storžev



(1 - α-pinene, 2 - β-pinene, 3 - myrcene, 4 - Δ-3-carene, 5 – limonene)

Figure 3: Gas chromatogram of pine cone essential oil
Slika 3: Plinski kromatogram eteričnega olja borovih storžev



(1 - α-pinene, 2 - β-pinene, 3 - myrcene, 4 - Δ-3-carene, 5 – limonene)

Figure 4: Gas chromatogram of larch cone essential oil
Slika 4: Plinski kromatogram eteričnega olja macesnovih storžev

By accurate observation of the chromatograms of essential oil samples it could be concluded that all three oils showed some common similarities, but at the same time they also demonstrated some pronounced differences in their chemical composition. In all three samples, the characteristic monoterpenes α-pinene, β-pinene, myrcene, Δ-3-carene and limonene were present, though their relative concentrations in essential oils were different. Retention times (t_r) that unambiguously define individual monoterpenes are collected in Table 3, and their relative concentrations in the corresponding monoterpene fraction in the first part of the chromatogram up to the retention values of 7 minutes are listed in Table 4.

Table 3: Monoterpene retention times of spruce, pine and larch cone essential oils
Preglednica 3: Retencijski časi monoterpenov v eteričnih oljih storžev smreke, bora in macesna

Essential oil, cones *Eterično olje, storži*	1 α-pinene α-*pinen* t_r (min)	2 β-pinene β-*pinen* t_r (min)	3 myrcene *mircen* t_r (min)	4 Δ-3-carene Δ-3-*karen* t_r (min)	5 limonene *limonen* t_r (min)
Spruce/*Smreka*	3,59	4,28	4,45	4,93	5,28
Pine/*Bor*	3,60	4,28	4,45	4,94	5,27
Larch/*Macesen*	3,58	4,27	4,44	4,92	5,25

Table 4: Relative constitution of monoterpene fraction in spruce, pine and larch cone essential oils

Preglednica 4: Relativna sestava monoterpenske frakcije v eteričnih oljih storžev smreke, bora in macesna

Essential oil, cones *Eterično olje, storži*	α-pinene *α-pinen* %	β-pinene *β-pinen* %	myrcene *mircen* %	Δ-3-carene *Δ-3-karen* %	limonene *limonen* %
Spruce/*Smreka*	11	30	11	15	31
Pine/*Bor*	28	13	20	35	2
Larch/*Macesen*	30	27	30	2	7

The differences in the composition of the lower or monoterpene fractions of the samples were very interesting indeed. Though all five compounds were present in samples and constituted the largest part of this fraction, their relative concentrations differed. Thus β-pinene and limonene predominated in spruce, α-pinene and Δ-3-carene in pine and α-pinene, β-pinene and myrcene in larch cones. There were also traces of other lower terpenes in samples, but in no case their total concentrations exceeded 4 %.

In all samples higher terpenes were also present. Their peaks appeared at retention times higher than 10 minutes in chromatograms. In the case of spruce essential oil, they were hardly visible, but their types and concentrations seemed to be quite abundant in pine and larch oils. There was more similarity between higher fractions of spruce and pine samples, while larch showed a quite different and chemically very rich structure. It is worth mentioning that only one component, with retention time of 15.40 – 15.46 minutes, was common to all three samples (Table 5) and that it predominated in spruce and pine, while the most important in larch was the one with retention time of 17.94 minutes.

Individual sesquiterpenes and diterpenes were not identified, as there were no appropriate standard compounds available.

Retention times of higher terpenes are listed in Table 5, where the most abundant substances in each sample are designated by bold numbers. Relative contents of lower and higher terpene fractions in essential oils are presented in Table 6.

Table 5: Retention times of terpenes with higher molecular weight in spruce, pine and larch cone essential oils

Preglednica 5: Retencijski časi višjih terpenov v eteričnih oljih smreke, bora in macesna

Essential oil, cones *Eterično olje, storži*	A t_r (min)	B t_r (min)	C t_r (min)	D t_r (min)	E t_r (min)	F t_r (min)	G t_r (min)	H t_r (min)
Spruce/*Smreka*	/	/	/	/	15,08	**15,42**	/	16,26
Pine/*Bor*	/	13,69	14,35	/	15,10	**15,46**	15,98	16,28
Larch/*Macesen*	11,51	13,62	14,33	14,65	/	15,40	/	
Essential oil, cones *Eterično olje, storži*	I t_r (min)	J t_r (min)	K t_r (min)	L t_r (min)	M t_r (min)	N t_r (min)	O t_r (min)	P t_r (min)
Spruce/*Smreka*	/	/	/	/	/	/	/	/
Pine/*Bor*	/	/	/	/	/	19,27	/	/
Larch/*Macesen*	16,91	17,38	17,71	**17,94**	19,12	/	20,61	20,89

Table 6: Relative contents of terpenes with lower and higher molecular weights in spruce, pine and larch cone essential oils

Preglednica 6: Relativna vsebnost nižjih in višjih terpenov v eteričnih oljih storžev smreke, bora in macesna

Essential oil, cones *Eterično olje, storži*	Lower terpenes, % *Nižji terpeni,* %	Higher terpenes, % *Višji terpeni*, %
Spruce/*Smreka*	90	10
Pine/*Bor*	48	52
Larch/*Macesen*	62	38

From Table 6 it is evident that pine cones contained the highest relative content of higher terpenes, while lower terpenes significantly predominated in spruce samples. The most interesting fact about the analyses was that larch cones showed most diverse chemical structure and that highest amounts of oil could be extracted from them.

4 CONCLUSIONS ZAKLJUČKI

A comparison of qualitative and quantitative composition of essential oils extracted from spruce, pine and larch cones showed that all three oils contained the same five monoterpenes, namely α-pinene, β-pinene, myrcene, Δ-3-carene, and limonene. However, their concentrations differed in each individual oil sample. The most abundant portion of monoterpenes was found in essential oil of spruce cones. There were pronounced differences among the samples in content and chemical constitution of higher terpene fractions. The largest quantity of higher molecular weight compounds were found in pine samples, while by far the most diverse chemical structure was typical of larch samples.

The investigation clearly indicated that spruce, pine and larch cones could be applied as raw materials for extraction of essential oils. Despite the fact that larch cones contain the largest quantity of essential oil, pine cones seem to be more appropriate for technological use, since they are a few times heavier and more easily accessible in nature. Industrial use of cones as valuable sources of lower and higher terpenes is still to be considered, which means that further research will have to be conducted.

Comparison of obtained results with previous research could not have been done as there were no published literature data on terpene composition of cones.

5 POVZETEK

Storž ali češarek je večinoma olesenelo socvetje oz. soplodje iglavcev. Strokovno se imenuje strobilus. Sestavljajo ga tesno zbiti sporofili, razmeščeni okrog osi. Storžnjaki ali konifere (Pinopsida) so golosemenke (Gymnospermae) z igličastimi listi (»iglavci«). Storž golosemenk je lahko olesenel ali mesnat. Storži za dozoritev navadno potrebujejo približno leto dni, pri nekaterih rodovih pa lahko zorijo tudi dlje.

Storži lahko tako kot les vsebujejo različne ekstraktivne komponente. To so nizkomolekularne organske spojine, ki so dobro topne predvsem v organskih topilih, nekatere pa tudi v vodi. Ekstraktivi v lesu opravljajo osnovne funkcije metabolitskega razvoja kot tudi obrambne reakcije glede na svojo okolico, hkrati pa vplivajo na kemične, biološke in mehanske lastnosti lesa. Med ekstraktive prištevamo tudi skupino terpenov. Ti se med seboj razlikujejo glede na število osnovnih enot $C_{10}H_{16}$, ki jih sestavljajo, in glede na prostorsko orientiranost molekul. Najenostavnejši so monoterpeni z eno osnovno enoto, poznamo pa tudi višje terpene z več osnovnimi gradbenimi enotami. Terpeni so glavne sestavine eteričnih olj, ki jih najdemo v različnih rastlinskih tkivih in dajejo karakterističen vonj. Kot surovine se uporabljajo predvsem v kemični, živilski, kozmetični in farmacevtski industriji.

Namen naše raziskave je bil izolirati eterična olja iz storžev navadne smreke, evropskega macesna in rdečega bora ter ugotoviti vrste in količine monoterpenov v njih.

Rezultati so uporabni za ugotavljanje botanične pripadnosti drevesnih vrst in so pomembni za nadaljnje kemotaksonomske raziskave, saj večji odmiki od dobljenih vrednosti lahko nakazujejo hibridnost določene drevesne vrste.

Pri pregledu strokovne literature nismo zasledili nobenih podatkov o kvalitativni in kvantitativni sestavi eteričnih olj v storžih iglavcev.

Kot vzorce smo uporabili sveže smrekove, borove in macesnove storže 30 do 40 let starih dreves. Eterična olja smo izolirali po metodi destilacije z vodno paro. Kvalitativno in kvantitativno sestavo olj smo določili s pomočjo plinske kromatografije. Posamezne monoterpene smo identificirali s primerjavo z ustreznimi standardnimi substancami, relativne vsebnosti enostavnih in višjih terpenov pa z integracijo celotnih kromatogramov in posameznih skupin vrhov.

Meritve so pokazale, da vsebujejo največ olja macesnovi storži. V vseh vzorcih smo zasledili enostavne terpene α-pinen, β-pinen, mircen, Δ-3-karen in limonen, vendar so se njihove relativne koncentracije v nižji terpenski frakciji razlikovale. Smreka je vsebovala največ β-pinena (30 %), v boru je prevladoval Δ-3-karen (35 %), macesen pa je imel največ α-pinena (30 %) in mircena.(30 %). Količinsko največ višjih terpenov je vsebovalo eterično olje borovih storžev, največ različnih spojin pa je bilo v eteričnem olju macesna. Medtem ko je bila kvalitativna sestava monoterpenske frakcije v vseh primerih precej podobna, pa je bila le ena komponenta višje terpenske frakcije skupna vsem vzorcem. Zaradi pomanjkanja standardnih spojin posameznih komponent višje frakcije nismo mogli identificirati.

Raziskava je pokazala, da bi smrekovi, borovi in macesnovi storži lahko bili vir za pridobivanje eteričnih olj. Čeprav macesnovi storži vsebujejo največ eteričnih olj, pa so borovi storži nekajkrat težji in laže dostopni, zato bi bila njihova uporaba za tehnološke namene verjetno najbolj upravičena.

6 REFERENCES
VIRI

FENGEL, D. / WEGENER, G., 1989. Wood, Chemistry, Ultrastructure, Reactions. – Berlin – New York, Walter de Gruytter, 613 p.

SJÖSTRÖM, E., 1981. Wood Chemistry, Fundamentals and Applications. –New York, Academic Press, 223 p.

STENIUS, P. (ed.), 2000. Papermaking Science and Technology. Book 3: Forest Products Chemistry: Structure and Chemical Composition of Wood, Gummerus Printing, Jyväskylä, 43 p.

TIŠLER, V. / KVAS, P., 1988. Kemijska zgradba terpentina iglic *Picea abies* (L.) Karst iz različnih območij v Sloveniji. –Gozdarski vestnik 46, 2: 79 – 82.

TIŠLER, V. / DEIGELE, C., 1991. Kemijska analiza terpenov iglic in njihova zastopanost v vretenih *Picea abies* (L.) Karst. –Zbornik gozdarstva in lesarstva 37: 201 – 209.

ZULE, J., 2001.Problematika lesnih ekstraktivnih spojin v papirni industriji. –Les 53, 4: 113 – 118.