

Effect of Extraction Technique on Composition of Volatile Constituents of Oleoresin from *Pinus Brutia* Ten.

Utjecaj tehnike ekstrakcije na sastav hlapljivih sastojaka oleoresina iz drva *Pinus brutia* Ten.

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ABSTRACT • In this study, volatile constituents of oleoresin from *Pinus brutia* Ten. were extracted by solid phase microextraction (SPME) and hydrodistillation. Gas chromatography-mass spectroscopy (GC/MS) was performed to determine volatile constituents of turpentine oil (obtained by hydrodistillation) and extract from oleoresin (obtained by SPME). Eleven volatile compounds constituted the total of turpentine oil obtained by hydrodistillation, whereas SPME extract contained 32 volatile compounds. Three distinctive volatile constituents in the turpentine oil were α -pinene (49.12 %), β -pinene (19.12 %) and Δ^3 -carene (15.33 %). They were represented in the amount of 44.35 %, 18.59 % and 14.58 % in the SPME extract. The volatile constituents of turpentine oil were monoterpenes and sesquiterpenes. Monoterpenes, sesquiterpenes, terpene oxides, alcohols, aldehydes, esters and ethers were identified as main groups in the SPME extract by GC/MS. The results of chromatographic analysis showed that solid phase microextraction was a more effective extraction technique than hydrodistillation for isolating volatile constituents from oleoresin.

Keywords: volatile constituents, oleoresin, hydrodistillation, solid phase microextraction (SPME), GC/MS

SAŽETAK • Za ovu su studiju hlapljivi sastojci oleoresina iz drva *Pinus brutia* Ten. izdvojeni mikroekstrakcijom čvrste faze (SPME) i hidrodestilacijom. Radi određivanja hlapljivih sastojaka terpentinskog ulja (dobivenoga hidrodestilacijom) i ekstrakta oleoresina (dobivenoga SPME-om), provedena je plinska kromatografija i masena spektrometrija (GC/MS). Jedanaest hlapljivih spojeva sadržavalo je terpentinsko ulje dobiveno hidrodestilacijom, dok je ekstrakt dobiven SPME-om sadržavao 32 hlapljiva spoja. Tri karakteristična hlapljiva sastojka u terpentinskom ulju bila su α -pinene (49,12 %), β -pinene (19,12 %) i Δ^3 -karen (15,33 %). Ti su spojevi u ekstraktu SPME-a bili zastupljeni u udjelu od 44,35, 18,59 i 14,58 %. Hlapljivi sastojci terpentinskog ulja bili su monoterpeni i seskviterpeni, a kao glavne skupine u ekstraktu SPME-a uz pomoć GC/MS metode identificirani su monoterpeni, seskviterpeni, terpeni oksidi, alkoholi, aldehidi, esteri i eteri. Rezultati kromatografske analize pokazali su da je za izolaciju hlapljivih sastojaka oleoresina mikroekstrakcija čvrste faze učinkovitija tehnika ekstrakcije od hidrodestilacije.

Ključne riječi: hlapljivi sastojci, oleoresin, hidrodestilacija, mikroekstrakcija čvrste faze (SPME), GC/MS

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1 INTRODUCTION

1. UVOD

Oleoresin is obtained as exudates from pine trees by tapping the bark (bark chipping). Turpentine, essential oil, is produced by steam/hydro distillation from oleoresin. Turpentine is also known commercially as the "spirits of turpentine", "pine tree terpenic", "pine oleoresin", "gum turpentine", "terpenes oil", "turpentine from Bordeaux" or "oil of turpentine". α -pinene and β -pinene are the major constituents of turpentine. Pinenes are mostly used as starting materials to produce more valuable components (Jantam and Ahmad, 1999; McMorn *et al.*, 2000; Rezzi *et al.*, 2005; Wang *et al.*, 2006; Limberger *et al.*, 2007; Mercier *et al.*, 2009). Besides pinenes, turpentine contains other terpenes such as camphene, Δ^3 -carene, tricyclene, myrcene, *p*-cymene, limonene, β -caryophyllene and aromadendrene. Moreover, turpentine compounds are useful raw material for the production of pharmaceuticals, plasticizers, repellents, insecticides, solvents, perfumery, food additives, antiviral, antimicrobial and antioxidant compounds (Roberge *et al.*, 2001; Macchioni *et al.*, 2003; Sun, 2007; Burdock and Carabin, 2008; Mayekiso *et al.*, 2008; Behr and Johnen, 2009; Zulak and Bohlmann, 2010; Adams *et al.*, 2011; Teshome, 2011; Back *et al.*, 2012; Rudback *et al.*, 2012; Gillette *et al.*, 2012; Limberger *et al.*, 2012).

Brutian pine (*Pinus brutia* Ten.) is especially used for the production of oleoresin in Turkey (Oz *et al.*, 2012; Deniz, 2013; Oz *et al.*, 2015). It grows widely in the Mediterranean region, Aegean region, Marmara region and also in some localities of the western Black Sea region in a total area of about 5.8 million ha in Turkey (OGM, 2015).

Solid phase microextraction (SPME) is a sampling technique, whereby a constituent is adsorbed onto the surface of the coated silica fiber. Afterwards, constituents are desorbed into a suitable chromatography instrument coupled with an appropriate detector for identification and quantification. SPME is usually performed by gas chromatography (GC) in the applications. In SPME-GC analysis, the fiber is introduced into the injection port of GC device and constituents are thermally desorbed from the coating for chromatographically determination (Malik *et al.*, 2006). SPME was especially applied in environmental chemistry (Fattore *et al.*, 1996; Abalos *et al.*, 2002; Mousavi *et al.*, 2007), for example for determining organic pollutants in environmental samples (Penalver *et al.*, 1999) and phthalate esters in environmental waters (Polo *et al.*, 2005). It was regularly used to isolate volatile and semivolatile components (Zhang and Pawliszyn, 1993; James and Stack, 1996), for example for determining volatile components of some medicinal and aromatic plants (Bicchi *et al.*, 2007; Yasar *et al.*, 2016) and semivolatile organics in environmental solids (Hageman *et al.*, 1999).

The determination of volatile constituents of oleoresin, after isolation of turpentine oil by hydrodistillation, is a commonly used procedure (Rezzi *et al.*, 2005; Wang *et al.*, 2006; Tümen and Reunanen, 2010;

Oz *et al.*, 2015). Besides hydrodistillation technique, the volatile constituents of oleoresin were isolated from brutian pine using SPME in this study. This was followed by determination of volatile constituents of turpentine oil (obtained by hydrodistillation) and extract (obtained by SPME) by gas chromatography-mass spectroscopy (GC/MS). Finally, both applications were compared to reveal which extraction technique is more effective for isolating the volatile constituents of oleoresin.

2 MATERIALS AND METHODS

2. MATERIJALI I METODE

2.1 Materials

2.1. Materijali

Oleoresin of brutian pine (*Pinus brutia* Ten.) was obtained from Karaisali-Adana (37°17'40.0"N 35°09'15.0"E) in Turkey. Tapping was applied using an acid paste method after bark chipping. The bark of each tree was chipped 10 cm in width and 5 cm in height using a wounding apparatus at 1m height above ground. Afterwards, a collector was placed under the wound for gathering flowed oleoresin and the chemical stimulant. The acid paste was applied as chemical stimulant using plastic injectors. Acid paste contained 65 % solution of sulphuric acid, coal, dust, barley-rice bran and dyotamide soil. Sample trees were aged 35, 35 and 37 years, and they were 16, 17 and 18 meters in height and 30, 35 and 38 cm in diameter.

2.2 SPME and GC/MS analysis

2.2. SPME i GC/MS analiza

The solid phase microextraction (SPME) apparatus, equipped with a fiber coated and a 75 μ m-thick layer of Carboxen/Polydimethylsiloxane (CAR/PDMS), was used for the isolation of volatile constituents from oleoresin. 2 g of oleoresin were used in the experiment. Firstly, oleoresin was placed in a 10 mL vial, which was sealed with a silicone septum and a crimp cap and heated at 60 °C for 15 min. Secondly, SPME fiber was pushed through the headspace of a sample vial. The volatile constituents were adsorbed at 60 °C for 30 min. Then fiber was inserted directly into the injection port of the Shimadzu 2010 Plus GC/MS. GC/MS device was equipped with a Restek Rx-5Sil MS capillary column (30 m x 0.25 mm i.d., 0.25 μ m film thickness) coupled to a mass spectrometer with an ion trap detector in full scan under electron impact ionization (70 eV). Helium was used as carrier gas with a flow rate of 1.61 mL/min. The injection and detection were performed at the temperature of 250 °C. Quadrupole temperature was also 250 °C. Mass range was between 35 and 450 m/z. The temperature of column was kept at 40 °C for 2 min, subsequently raised to 250 °C at a rate of 4 °C/min and then held at 250 °C for 5 min. The software used was LabSolutions GCMSsolution Version 2.7.

2.3. Isolation of turpentine oil and GC/MS analysis

2.3. Izolacija terpentinskog ulja i GC/MS analiza

For the isolation of turpentine oil, 100 g of oleoresin were submitted to hydrodistillation for 5 h using

Table 1 Extraction parameters for hydrodistillation and SPME
Tablica 1. Parametri ekstrakcije za hidrodestilaciju i SPME analizu

Extraction type <i>Vrsta ekstrakcije</i>	Time <i>Vrijeme</i> min	Sample weight <i>Masa uzorka</i> g	Solvent <i>Otapalo</i>
Hydrodistillation / <i>hidrodestilacija</i>	300	100	Distilled water / <i>Destilirana voda</i>
SPME Sampling / <i>SPME uzorkovanje</i>	45	2	-

a Clevenger-type apparatus. Hydrodistillation yielded 30.4 % (w/w) turpentine oil from oleoresin. 30 μ L turpentine oil were added to 970 μ L hexane (GC grade) and 1 μ L of this solution was submitted to injection port of GC/MS. GC/MS was performed under the same conditions as described above.

2.4 Identification of constituents

2.4. Identifikacija sastojaka

Retention indices of volatile constituents were determined using a series of saturated *n*-alkanes. *n*-alkanes were injected after each sample at the same chromatographic conditions as described above for GC/MS. The volatile constituents were identified by comparison of their mass spectra with the Wiley, NIST, Tutor and FFNSC libraries or with data already available in the literature (Adams, 2007). Percentage amounts were calculated based on peak areas from the GC/MS chromatogram by the computer software described above.

2.5 Statistical analysis

2.5. Statistička analiza

MiniTab 16 software was used for statistical analyses. Independent samples *t* test analysis was applied to determine the statistical significance. For each variable, independent samples *t* test was separately done.

3 RESULTS AND DISCUSSION

3. REZULTATI I RASPRAVA

SPME was definitely a more efficient sample preparation technique as compared to classical hydrodistillation, and it could be performed with a much smaller amount of oleoresin than hydrodistillation (Table 1). For this reason, SPME technique offers more advantages than conventional hydrodistillation for extraction.

GC/MS chromatograms of volatile constituents of turpentine oil obtained by hydrodistillation and extract obtained by SPME from oleoresin of *Pinus brutia* Ten. are demonstrated in Figure 1 and 2.

Identified volatile constituents of turpentine oil and SPME extract of oleoresin from *Pinus brutia* Ten. are given in Table 2. Through the application of SPME for the extraction of volatile compounds from oleoresin, it was possible to determine a total of 32 compounds by direct injection on GC/MS. α -pinene, β -pinene and Δ^3 -carene were the major constituents (44.35 %, 18.59 % and 14.58 %, respectively) of oleoresin. According to GC/MS analysis of the hydrodistilled turpentine oil, a total of 11 volatile constituents were characterized. At the highest percentage of monoterpenes, they were represented by α -pinene (49.12 %), β -pinene (19.2 %) and Δ^3 -carene (15.33 %).

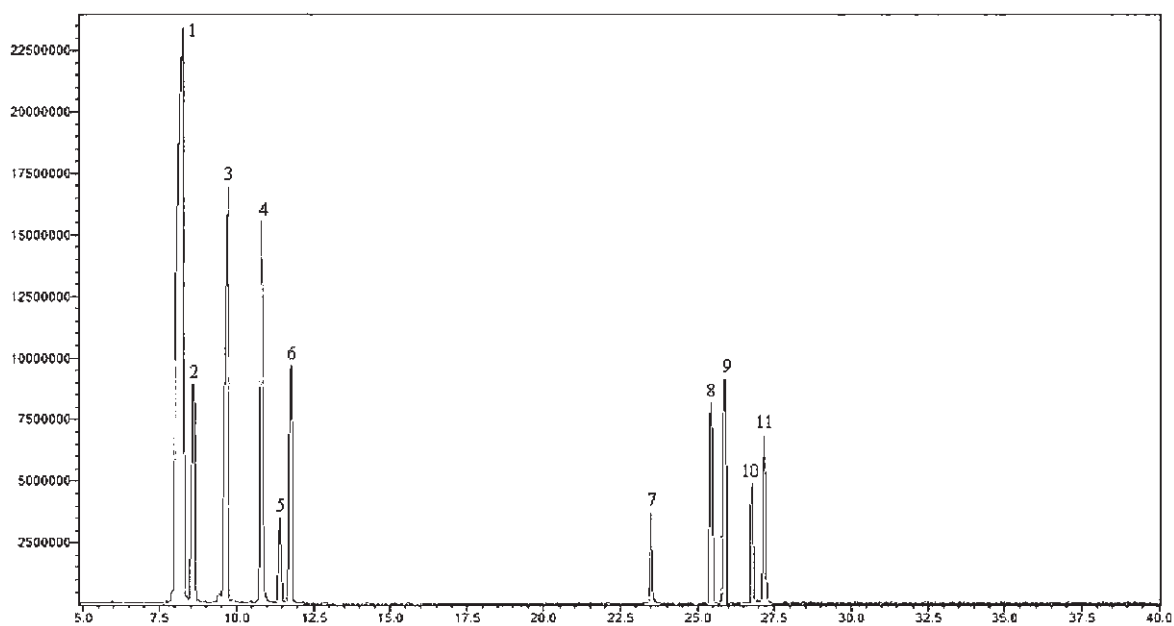


Figure 1 GC/MS chromatogram of turpentine oil obtained by hydrodistillation of oleoresin from *Pinus brutia* Ten. (1: α -pinene, 2: camphene, 3: β -pinene, 4: Δ^3 -carene, 5: *p*-cymene, 6: limonene, 7: α -cubebene, 8: α -gurjunene, 9: β -caryophyllene, 10: aromadendrene, 11: α -humulene)

Slika 1. GC/MS kromatogram terpentinskog ulja dobivenoga hidrodestilacijom oleoresina iz drva *Pinus brutia* Ten. (1: α -pinen, 2: camfen, 3: β -pinen, 4: Δ^3 -karen, 5: *p*-cimen, 6: limonen, 7: α -kubeben, 8: α -gurjunen, 9: β -kariofilen, 10: aromadendren, 11: α -humulen)

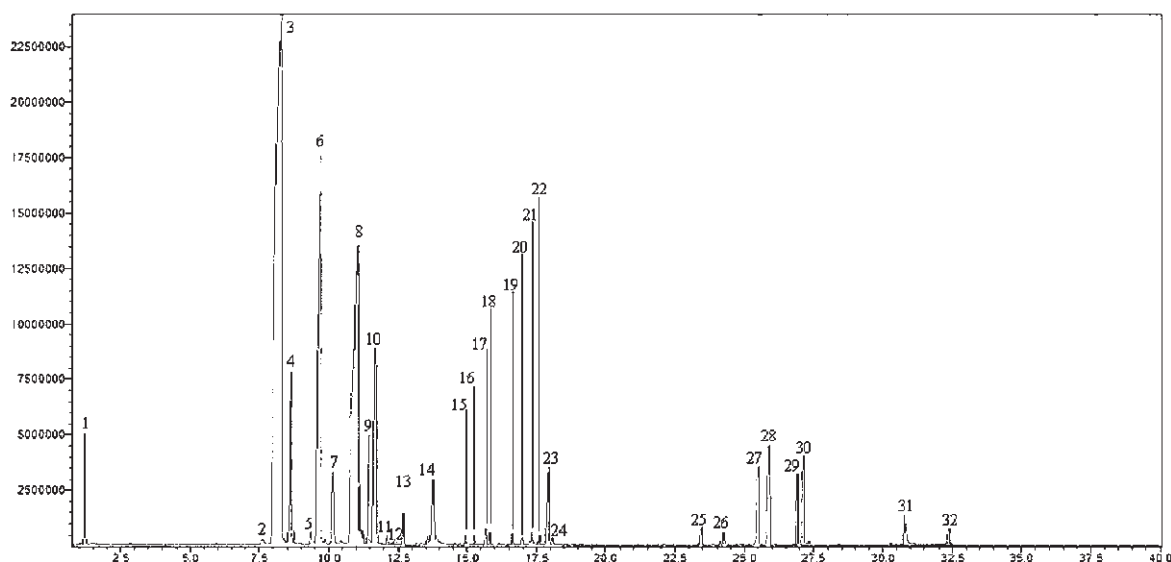


Figure 2 GC/MS chromatogram of SPME extract of oleoresin from *Pinus brutia* Ten. (1: isopropyl acetate, 2: tricyclene, 3: α -pinene, 4: camphene, 5: benzaldehyde, 6: β -pinene, 7: myrcene, 8: Δ^3 -carene, 9: *p*-cymene, 10: limonene, 11: butyl 2-methylbutyrate, 12: (E)- β -ocimene, 13: γ -terpinene, 14: *p*- α -dimethylstyrene, 15: 2,4-dimethylanisole, 16: α -campholene aldehyde, 17: terpin-3-en-1-ol, 18: trans- β -terpineol, 19: trans-limonene oxide, 20: isoborneol, 21: terpinen-4-ol, 22: *p*-cymen-8-ol, 23: α -terpineol, 24: verbenone, 25: α -cubebene, 26: cyclosativene, 27: α -gurjunene, 28: β -caryophyllene, 29: aromadendrene, 30: α -humulene, 31: caryophyllene oxide, 32: humulene oxide)

Slika 2. GC/MS kromatogram SPME ekstrakta oleoresina iz drva *Pinus brutia* Ten. (1: izopropil acetat, 2: triciklen, 3: α -pinen, 4: kamfen, 5: benzaldehid, 6: β -pinen, 7: mircen, 8: Δ^3 -karen, 9: *p*-cimen, 10: limonen, 11: butil 2-metilbutirat, 12: (E)- β -ocimen, 13: γ -terpinen, 14: *p*- α -dimetilstiren, 15: 2,4-dimetilanisol, 16: α -kamfolen aldehid, 17: terpin-3-en-1-ol, 18: trans- β -terpineol, 19: trans-limonen oksid, 20: izoborneol, 21: terpinen-4-ol, 22: *p*-cimen-8-ol, 23: α -terpineol, 24: verbenon, 25: α -kuben, 26: ciklosativen, 27: α -gurjunen, 28: β -kariofilen, 29: aromadendren, 30: α -humulen, 31: kariofilen oksid, 32: humulen oksid)

α -pinene, camphene, β -pinene, Δ^3 -carene, *p*-cymene, limonene, α -cubebene, α -gurjunene, β -caryophyllene, aromadendrene and α -humulene constituted the total of turpentine oil by hydrodistillation, whereas these compounds appeared as 90.25 % in the extract by SPME. The present findings show that 9.75 % of volatile constituents cannot be extracted by hydrodistillation of oleoresin from *Pinus brutia* Ten.

According to independent samples *t* test, a significant difference was found between hydrodistillation and SPME sampling for α -pinene, camphene, *p*-cymene, α -cubebene, α -gurjunene, β -caryophyllene and aromadendrene ($p < 0.05$). However, there was no significant variance between hydrodistillation and SPME sampling for β -pinene, Δ^3 -carene, limonene and α -humulene ($p > 0.05$) (Table 2).

The present chemical composition of volatile compounds in the turpentine oil sample from *Pinus brutia* Ten. [α -pinene (49.12 %), β -pinene (19.12%), Δ^3 -carene (15.33 %), other monoterpenes (6.79 %) and sesquiterpenes (9.56 %)] is higher than findings by Papajannopoulos *et al.* (2001), who determined α -pinene (13.7 %), β -pinene (5.1 %), and other terpenes (4.5 %) using GC/MS and α -pinene (8.47 %), β -pinene (2.99 %), Δ^3 -carene (3.37 %) and other terpenes (1.49 %) using GC.

The findings by Ulukanli *et al.* (2014) were comparable with this study. They found α -pinene (25.4 %), β -pinene (9.69 %) and Δ^3 -carene (0.16 %) in the turpentine oil sample from *Pinus brutia* Ten.

Oz *et al.* (2015) determined, as major compounds, α -pinene, β -pinene and γ -terpinene with quantities of 19.7 %, 13.3 % and 10.2 % in the turpentine oil of oleoresin from *Pinus brutia* Ten. These results support the present findings except for γ -terpinene and Δ^3 -carene. γ -terpinene was not obtained in the turpentine oil of this study. Δ^3 -carene was presented as 3.7 % in the work by Oz *et al.* (2015), whereas Δ^3 -carene was determined as a major compound in the turpentine oil of this study.

The main groups of determined volatile constituents of turpentine oil and SPME extract from oleoresin of *Pinus brutia* Ten. are listed in Table 3. Monoterpenes and sesquiterpenes were identified in the turpentine oil, whereas monoterpenes, sesquiterpenes, terpene oxides, alcohols, aldehydes, esters and ethers were characterized in the extract by SPME. Based on independent samples *t* test, a significant difference was found between hydrodistillation and SPME sampling for monoterpenes and sesquiterpenes ($p < 0.05$).

4 CONCLUSIONS

4. ZAKLJUČAK

Using SPME for the extraction from oleoresin, 32 volatile constituents were determined by direct injection in GC/MS, whereas 11 volatile constituents were identified in the turpentine oil by GC/MS after hydrodistillation of oleoresin from *Pinus brutia* Ten. Three major volatile constituents in the turpentine oil were α -pinene

Table 2 Volatile constituents of turpentine oil and SPME extract from oleoresin of *Pinus brutia* Ten.

Tablica 2. Hlapljivi sastojci terpentinskog ulja i SPME ekstrakta iz oleoresina iz drva *Pinus brutia* Ten.

No	RI ^A	Compound Sastojak	Hydrodistillation Hidrodestilacija % ^B	SPME Sampling SPME uzorkovanje % ^B	t-test results Rezultati t-testa		
					F	t	p
1	635	isopropyl acetate	-	1.72 (0.06)*	-	-	-
2	918	tricyclene	-	0.26 (0.04)	-	-	-
3	937	α -pinene	49.12 ^a (0.65)	44.35 ^b (1.09)	0.466	5.323	0.0060
4	947	camphene	2.74 ^c (0.18)	2.17 ^d (0.12)	0.276	3.708	0.0207
5	956	benzaldehyde	-	0.17 (0.03)	-	-	-
6	975	β -pinene	19.12 ^c (0.52)	18.59 ^c (0.13)	2.118	1.392	0.2365
7	989	myrcene	-	1.45 (0.05)	-	-	-
8	1010	Δ^3 -carene	15.33 ^f (0.44)	14.58 ^f (0.35)	0.102	1.882	0.1330
9	1023	<i>p</i> -cymene	1.10 ^g (0.11)	1.47 ^h (0.12)	0.041	-3.229	0.0320
10	1029	limonene	3.03 ⁱ (0.20)	3.11 ⁱ (0.16)	0.066	-0.444	0.6803
11	1040	butyl 2-methylbutyrate	-	0.15 (0.03)	-	-	-
12	1045	(E)- β -ocimene	-	0.03 (0.01)	-	-	-
13	1056	γ -terpinene	-	0.53 (0.05)	-	-	-
14	1088	<i>p</i> - α -dimethylstyrene	-	1.11 (0.03)	-	-	-
15	1113	2,4-dimethylanisole	-	0.16 (0.03)	-	-	-
16	1121	α -campholene aldehyde	-	0.19 (0.03)	-	-	-
17	1133	terpin-3-en-1-ol	-	0.39 (0.07)	-	-	-
18	1136	trans- β -terpineol	-	0.25 (0.02)	-	-	-
19	1159	trans-limonene oxide	-	0.17 (0.02)	-	-	-
20	1168	isoborneol	-	0.14 (0.03)	-	-	-
21	1177	terpinen-4-ol	-	0.21 (0.03)	-	-	-
22	1184	<i>p</i> -cymen-8-ol	-	0.17 (0.05)	-	-	-
23	1193	α -terpineol	-	1.51 (0.07)	-	-	-
24	1202	verbenone	-	0.14 (0.01)	-	-	-
25	1347	α -cubebene	1.24 ^j (0.19)	0.32 ^k (0.14)	0.176	5.571	0.0051
26	1370	cyclosativene	-	0.26 (0.04)	-	-	-
27	1406	α -gurjunene	2.39 ^l (0.23)	1.15 ^m (0.16)	0.283	6.347	0.0032
28	1418	β -caryophyllene	2.80 ⁿ (0.09)	1.73 ^o (0.05)	0.637	14.791	0.0001
29	1444	aromadendrene	1.42 ^p (0.12)	1.11 ^r (0.05)	1.241	3.324	0.0293
30	1452	α -humulene	1.71 ^s (0.10)	1.67 ^s (0.12)	0.098	0.361	0.7366
31	1581	caryophyllene oxide	-	0.45 (0.07)	-	-	-
32	1604	humulene oxide	-	0.29 (0.02)	-	-	-

A – Retention indices calculated against *n*-alkanes / *indeksi zadržavanja izračunani prema n-alkanima*; B – Percentages calculated from GC/MS data. / *postotci izračunani iz GC/MS podataka*; a-s – Variances according to *t* test / *varijance prema t-testu*; * Standard deviations / *standardne devijacije*

(49.12%), β -pinene (19.12%) and Δ^3 -carene (15.33%). They were represented by the amounts of 44.35%, 18.59% and 14.58% in oleoresin volatiles isolated by

SPME. Monoterpenes and sesquiterpenes were determined in the turpentine oil, while monoterpenes, sesquiterpenes, terpene oxides, alcohols, aldehydes, esters and

Table 3 Main groups of determined constituents of turpentine oil and SPME extract from oleoresin of *Pinus brutia* Ten.

Tablica 3. Glavne skupine određenih sastojaka terpentinskog ulja i SPME ekstrakta iz oleoresina drva *Pinus brutia* Ten.

No	Main group Glavna skupina	Hydrodistillation Hidrodestilacija %	SPME Sampling SPME uzorkovanje %	t-test results Rezultati t-testa		
				F	t	p
1	Monoterpene <i>monoterpene</i>	90.44 ^a (1.08)*	87.79 ^b (0.22)	2.429	3.407	0.0271
2	Sesquiterpene <i>seskviterpen</i>	9.56 ^c (0.56)	6.24 ^d (0.35)	0.386	7.147	0.0020
3	Terpene oxide <i>terpen oksid</i>	-	0.91 (0.05)	-	-	-
4	Alcohol / <i>alkohol</i>	-	2.67 (0.09)	-	-	-
5	Aldehyde / <i>aldehid</i>	-	0.36 (0.03)	-	-	-
6	Ester / <i>ester</i>	-	1.87 (0.06)	-	-	-
7	Ether / <i>eter</i>	-	0.16 (0.04)	-	-	-

a-d – Variances according to *t* test / *varijance prema t-testu*; * Standard deviations / *standardne devijacije*

ethers were identified as main groups in the extract by SPME. Regarding the quantities of identified volatile compounds occurring in resin of *Pinus brutia* Ten., SPME has been shown as a more appropriate extraction technique than conventional hydrodistillation.

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