Viljem Vek*, Ida Poljanšek, Urša Osolnik, Primož Oven[1]

Analysis of Extractives in Liquid and Headspace Samples of Silver Fir Using Gas Chromatography Coupled with a Mass Selective Detector

Analiza tekuće i parne faze ekstraktivnih tvari jelovine primjenom plinske kromatografije sa spektrometrom masa

ORIGINAL SCIENTIFIC PAPER
Izvorni znanstveni rad
Received – prispjelo: 15. 2. 2024.
Accepted – prihvaćeno: 7. 10. 2024.
UDK: 630*81; 674.032.475.2
https://doi.org/10.5552/drvind.2024.0193

ABSTRACT • *The main objective of the study is to present a chromatographic method that allows rapid and accurate quality control of extractives in liquid and headspace samples of wood and bark of European silver fir (Abies alba Mill.). The chemical identity of silver fir extractives in liquid and headspace samples was investigated. Mature silver firs were harvested and samples of sapwood, heartwood, knotwood and bark were prepared. Liquid samples were obtained by solvent extraction at higher temperature and pressure, while the volatile extractives of silver fir tissues were collected by headspace sampling. The extractives in the liquid silver fir samples were silylated prior to chromatographic analysis. The samples were then measured using gas chromatography (GC) coupled with a mass selective detector (MSD). The samples were introduced into the GC-MSD system using the Automatic Liquid Sampler (ALS) and the Headspace Sampler (HS). A total of 55 compounds were detected in the silver fir wood and bark samples. The liquid samples consisted of a variety of carboxylic/dicarboxylic acids, sugars with sugar alcohols (inositols) and sugar acids, with citric acid, quininic acid and sucrose being the most frequently represented. The liquid bark samples contained mainly sugar-like compounds, while the knotwood extracts contained large amounts of phenolic compounds and lignans. D-pitinol was confirmed as the most characteristic GC-MSD peak of the silver fir extracts. Analysis of the headspace of silver fir revealed α-pinene, camphene, D-limonene, β-myrcene, ocimene, 2-bornanone or D-camphor and α-terpineol as the characteristic monoterpenes. It was shown that the presented GC-MSD method is a suitable analytical tool for the chemical screening of low molecular weight compounds in both liquid and headspace samples of wood and bark. However, the analysis of higher molecular weight extractives requires a different analytical approach supported by other analytical methods such as LC/MS.*

KEYWORDS: *Abies alba Mill.; wood; bark; extractives; headspace; polyphenols; gas chromatography-mass selective detection (GC-MSD)*

* Corresponding author
[1] Authors are researchers at University of Ljubljana, Biotechnical Faculty, Department of Wood Science and Technology, Ljubljana, Slovenia. https://orcid.org/0000-0002-5091-0091

SAŽETAK • *Glavni cilj rada jest predstavljanje kromatografske metode koja omogućuje brzu i preciznu kontrolu kvalitete tekuće i parne faze ekstraktivnih tvari drva i kore jelovine (Abies alba Mill.). Ispitivan je kemijski sastav tekuće i parne faze ekstraktivnih tvari jelovine. Posječena su stabla zrele jele i pripremljeni su uzorci bjeljike, srži, kvrge i kore. Tekući uzorci ekstraktivnih tvari dobiveni su ekstrakcijom otapalom pri povišenoj temperaturi i tlaku, dok su hlapljive ekstraktivne tvari tkiva jelovine prikupljene injektiranjem parne faze. Ekstraktivne su tvari u tekućim uzorcima jelovine sililirane prije kromatografske analize. Uzorci su zatim izmjereni primjenom plinske kromatografije sa spektrometrom masa (GC-MSD) te su uneseni u GC-MSD sustav uz pomoć automatskog uzorkivača tekućina (ALS) i uzorkivača za hlapljive spojeve (HS). U uzorcima drva i kore jelovine detektirano je ukupno 55 spojeva. Tekući uzorci sastojali su se od različitih karboksilnih/dikarboksilnih kiselina, šećera sa šećernim alkoholima (inozitolima) i šećernih kiselina, a najčešće su bile zastupljene limunska kiselina, kininska kiselina i saharoza. Tekući uzorci kore sadržavali su uglavnom spojeve slične šećeru, dok su ekstrakti kvrga sadržavali velike količine fenolnih spojeva i lignana. D-pitinol je potvrđen kao najkarakterističniji GC-MSD vrh ekstrakata jelovine. Analiza parne faze ekstraktivnih tvari jelovine otkrila je α-pinen, kamfen, D-limonen, β-mircen, ocimen, 2-bornanon ili D-kamfor i α-terpineol kao karakteristične monoterpene. Pokazalo se da je predstavljena GC-MSD metoda prikladan analitički alat za kemijski pregled spojeva male molekularne mase u tekućoj i parnoj fazi ekstraktivnih tvari u uzorcima drva i kore. Međutim, analiza ekstraktivnih tvari veće molekularne težine zahtijeva drukčiji analitički pristup podržan drugim analitičkim metodama kao što je LC/MS.*

KLJUČNE RIJEČI: *Abies alba Mill.; drvo; kora; ekstraktivne tvari; parna faza; fenoli; plinska kromatografija sa spektrometrom masa (GC-MSD)*

1 INTRODUCTION
1. UVOD

Silver fir (*Abies alba* Mill.) is a tree species that plays an important role in the main processing streams of the timber industry in Europe. In addition, silver fir could even gain industrial importance with regard to the model prediction of future wood stock in European forests as a result of climate change (Dyderski *et al.*, 2018). The side streams of forestry and wood processing industry hold great underutilized potential (Verkasalo *et al.*, 2019). The idea of biorefining biomass products from wood industry side streams has been around for some time, and there are already value chains involving the extraction of valuable phytochemicals from low-value biomass of trees, such as knotwood or bark (Holmbom, 2011; Domazet *et al.*, 2023). The bioactive extractives are usually obtained from wood and bark tissues using various extraction methods and different more or less polar organic solvents (Holmbom, 1999). The choice of extraction method and analytical instruments depends on the objectives of the investigation and the type of extracts to be analyzed.

The literature review shows different analytical approaches for the analysis of extractives in the tissues of silver fir. The silver fir bark was extracted in different studies using various solvents and different extraction methods. These methods are based on the use of cold and/or hot water (Tavčar Benković *et al.*, 2014; Bianchi *et al.*, 2015), mixtures of water and ethanol (50:50, v/v) (Brennan *et al.*, 2020) or water and methanol (65:35, v/v) (Hamad *et al.*, 2019). The bark was also extracted sequentially, e.g. with hexane, then with acetone and finally with a mixture of toluene and ethanol (50:50, v/v), as shown by Brennan *et al.* (2020). Silver fir barks were extracted conventionally (Soxhlet, maceration, shaker) and with more advanced techniques such as sonication and accelerated solvent extraction (ASE). The bark extracts are analyzed with gas (GC-MS) and liquid chromatography (UPLC-PDA, HPLC-MS), with mass spectrometry (MALDI-TOF MS) and with NMR (^{13}C) spectroscopy (Bianchi *et al.*, 2015; Hamad *et al.*, 2019; Brennan *et al.*, 2020; Schoss *et al.*, 2022).

The silver fir wood, especially the knotwood, was most frequently extracted sequentially with less polar solvents, followed by extraction with polar solvents. This approach allows efficient removal of both lipophilic and hydrophilic extractives from silver fir wood and knots. For the extraction of knotwood, hexane and mixtures of water and acetone were used in the Soxhlet apparatus (Kebbi-Benkeder *et al.*, 2017) or in the ASE system (Willför *et al.*, 2004). Stemwood with sapwood and heartwood of silver fir was effectively extracted with a mixture of toluene and ethanol (2:1, v/v) and ethanol as reported by Hamada *et al.* (2018). An example of using only hot water as a solvent was shown by Schoss *et al.* (2022), who extracted sawdust consisting of both bark and wood tissue from branches using ultrasound. Silver fir branchwood samples were also extracted with aqueous methanol under reflux or/and successively with hexane and methanol under sonication as suggested by Patyra *et al.* (2022). It can be summarized from the literature that GC-MS and LC-MS are frequently used methods for the chemical screening of extractives in silver fir wood and bark. Thus, GC-MSD is a well-known analytical tool for the identification of low molecular weight compounds in wood samples.

In addition to the lipophilic and hydrophilic extractives of the liquid samples, the volatile terpenes of silver fir were analyzed both in the liquid and volatile

emission samples. As suggested by Salem *et al.* (2015), silver fir terpenes can be obtained by extracting wood and bark by soaking the ground samples in hexane for 14 days. Terpenes can also be extracted from wood by shaking the ground samples in hexane, as presented by Kačík *et al.* (2012). The volatile terpenes of silver fir are then separated and analyzed using various GC methods. An interesting sampling technique was demonstrated by Moukhtar *et al.* (2006), who measured volatile organic compound (VOC) emissions from silver fir branches using a large cuvette in which a selected branch was enclosed. The captured monoterpenes were then thermodesorbed and analyzed by GC-FID analysis (Moukhtar *et al.*, 2006).

However, due to the recent policy of the EU Commission Green Deal with green transition strategies, more attention is being paid to the use of so-called greener solvents with less hazardous effects on the environment and human health. Ethanol or water are suggested as such (Tekin *et al.*, 2018; Brennan *et al.*, 2021). However, extraction with such polar solvents usually results in complex extracts consisting of a variety of different compounds, e.g. from simple acids to sugar-like compounds to low and high molecular weight polyphenols. In this context, the choice of a suitable analytical approach for rapid and accurate chemical screening of the compounds in the wood and bark extracts is a difficult decision. The aim of the present study was to improve to some extent our published data on silver fir extractives by analyzing the wood and bark samples with GC-MSD for characteristic extractives that may have been overlooked or not detected in our previous TLC and HPLC analyses. The extractives in the liquid sample, i.e. the extracts of silver fir wood and bark, and the presence of volatile extractives in the headspace over the solid silver fir samples were analyzed by a gas chromatograph (GC) with a mass selective detector (MSD). Our aim is also to present a method for the chemical screening of extractives in liquid and headspace samples of wood and bark using a GC-MSD instrument in combination with simple and fast sample preparation.

2 MATERIALS AND METHODS
2. MATERIJALI I METODE

2.1 Chemicals
2.1. Kemikalije

The chemicals used for the silylation of silver fir extracts, i.e. pyridine, chlorotrimethylsilane (TMCS), N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), heneicosanoic acid (C21:0) and betulinol, were purchased from Merck (Sigma-Aldrich Chemie, Taufkirchen, Germany). Water, methanol and absolute ethanol were purchased from J.T. Baker (Phillipsburg, NJ, USA). All chemicals used are commercially available, all were of pure/analytical grade and used without further purification.

2.2 Material
2.2. Materijal

The material for this analysis was taken from mature silver firs (*Abies alba* Mill.) with a height of 30 to 35 meters and an age of up to 189 years. The silver firs were felled in the forests of Kočevska Reka, Slovenia (45°34'31.5" N, 14°46'27.8" E) (45°34'31.5" N, 14°46'27.8" E) (Vek *et al.* 2021, 2023). Silver fir wood and bark were randomly sampled from the 15 cm thick stem discs and homogenized into sapwood (SW), heartwood (HW), knotwood (KW) and bark (B) (Figure 1). Sapwood and heartwood on the stem discs were differentiated using an iodine-starch test by applying iodine solution to the surface of the discs with a brush. In the case of knotwood, only a dark-colored middle section was sampled, leaving out the adjacent light-colored tissue of knots. The bark samples were prepared as a mixture of inner (living) tissues, including phloem and periderm, and dead/outer bark with all tissues outside the youngest/deepest phellogen (rhytidome) (Figure 1). The samples were then dried overnight in an oven at 50 °C and ground using a Retsch SM 2000 cutting mill. For the GC-MSD analysis, the four samples were prepared by mixing the mass aliquots of each sample for the analysis of wood and bark tissue. The material for headspace analysis was taken directly from the stem discs using a drill, and the fresh samples were then stored in airtight vials until the start of chromatographic analysis. The samples of sapwood, heartwood, sapwood and bark prepared for solvent extraction were stored in PP bottles and kept in a cool and dark place until extraction. PP bottles were used for storage and to facilitate handling of the material prior to solvent extraction, and only glassware was used for later processing of the extracts.

2.3 Extraction
2.3. Ekstrakcija

The method for extracting wood and bark samples was similar to that already described (Vek *et al.* 2021, 2023). Prior to extraction, all wood and bark ground samples were freeze-dried overnight in a Telstar LyoQuest CC1930 lyophilizator at 0.045 mbar and - 85 °C. The silver fir samples were extracted in an automated extraction system at a temperature above the boiling point of the solvent and at higher pressure. The obtained extracts were filtered and the sample to solvent ratio of the final extracts was 1:100 (w/v). After extraction, all extracts were filtered into dark (amber) 20 mL bottles, which were hermetically sealed with screw caps with silicone/PTFE septum. Prior to the

Figure 1 A scheme of analysis: Trees of silver fir (*Abies alba* Mill.) were felled in the forests of Kočevska Reka, Ravne district. Sapwood (SW), heartwood (HW), knotwood (KW) and bark (B) of silver fir were prepared for extraction, the extracts were analyzed with a GC-MSD system equipped with automatic samplers for liquid samples and headspace samples.
Slika 1. Shema analize: stabla jelovine (*Abies alba* Mill.) posječena su u šumama Kočevske Reke, kotar Ravne. Bjelika (SW), srž (HW), kvrga (KW) i kora (B) jelovine pripremljeni su za ekstrakciju, a ekstrakti su analizirani sustavom GC-MSD opremljenim automatskim uzorkivačima za tekuću i hlapljivu fazu ekstraktivnih tvari.

analysis, all bottles containing the extracts were stored in a refrigerator at 4 °C.

2.4 Chromatographic analysis of silver fir wood and bark extractives
2.4. Kromatografska analiza ekstraktivnih tvari drva i kore jelovine

2.4.1 Preparation of silver fir samples for GC-MSD analysis
2.4.1. Priprema uzoraka jelovine za GC-MSD analizu

Prior to analysis by gas chromatography coupled with mass selective detector (GC-MSD), liquid samples of silver fir wood and bark were chemically modified by silylation as described by Willför *et al.* (2004). The extracts of SH, HW, KW and B were pipetted into a boro- glass tube. Heneicosanoic acid (C21) and betulinol were used as internal standards (ISTD). The extracts were dried to constant weight in a vacuum chamber at 0.050 bar and RT. The silver fir extracts were then silylated by adding 20 µL pyridine, 20 µL TMCS and 80 µL BSTFA. The reaction mixture was kept at 75 °C for 45 minutes. Silylation was used to chemically modify the extractives of the liquid silver fir samples into silyl derivatives, making the modified compounds more volatile and thermally stable and thus easier to analyze qualitatively and quantitatively (Sigma-Al-

drich©, 1997). The silylated samples were then transferred to 1.5 mL glass vials with 100 µL inserts using glass Pasteur pipettes.

For headspace analysis with GC-MSD, 1 gram of ground fresh sample was placed in a 20 mL glass vial, which was sealed airtight with an aluminum cap and a 20 mm PTFE/silicone septum. Headspace is the gas space in a valet above the fresh sample. Headspace analysis is therefore the analysis of the components present in this gas (Anthias Consulting, 2018).

2.4.2 Gas chromatography of headspace and liquid samples of silver fir
2.4.2. Plinska kromatografija parne i tekuće faze ekstraktivnih tvari jelovine

Injection of 1 µL of derivatized silver fir extracts containing internal standards (ISTD, C21:0 and betulinol) was performed in a split mode of 1:20. The sample was introduced into the column via an ultra-inert inlet liner for split injection with glass wool measuring 4 mm (ID) × 78.5 mm (length) and a volume of 870 µL. The inlet heater temperature was set to 250 °C.

The headspace of 1.00 g of a silver fir sample in 20 mL vials was sampled using the Agilent 7697A Headspace Sampler (HS). The HS oven temperature was set to 80 °C, the HS loop temperature to 90 °C, and the HS transfer line temperature to 100 °C. The samples were extracted from the vials for 1.00 minute. The headspace extractives were then transferred to the GC system at a temperature of 115 °C. Due to this increase in temperature from the HS oven to the transfer line unwanted condensation was avoided. The silver fir headspace samples were introduced into the column through an ultra-inert inlet liner for splitless injection without glass wool; this splitless liner had the same dimensions as the split liner mentioned above. The inlet temperature was 250 °C.

The silver fir extractives of the liquid and headspace samples were analyzed with an Agilent 8890 gas chromatograph (GC) coupled with an Agilent 5997B mass selective detector (MSD). The GC-MSD system is supported by the 7693A Automatic Liquid Sampler (ALS), which is a 16-vial injector tower complemented by the Agilent 7693A Autosampler with a 150-vial tray, and the Agilent 7697A Headspace Sampler with a 12-vial tray. The chromatography of the silver fir extractives, both the derivatized extractives and the headspace extractives, was carried out on an Agilent 19091S-433 Ultra Inert (5 % phenyl)-Methylpolysiloxane Fused Silica Capillary Column (HP-5ms) with a film thickness of 0.25 µm, an inner diameter (ID) of 0.25 mm and a length of 30 m. Helium was used as carrier gas at flow of 1.2 mL/min. The separation of the extractives from the liquid samples was achieved by the following temperature program: 100 °C (1 min)→ 4 °C min^{-1} to 220 °C →20 °C min^{-1} to 320 °C (8 min). The headspace samples of silver fir were separated in a similar temperature range as mentioned above using the temperature program: 40 °C (2 min) → 15 °C min^{-1} to 200 °C →10 °C min^{-1} to 280 °C (5 min) →10 °C min^{-1} to 300 °C (5 min). The separated extractives were detected with the following MSD parameters: MS source and transfer line temperatures were 230 and 250 °C, respectively, MS quadrupole temperature was 150 °C, ionization was performed in electron impact (EI) mode at an ionization energy of 70 eV, and the scan time segments were set for the mass range of 20 m/z to 800 m/z. The solvent delay was set to 3 min. GC/MS chromatograms were analyzed using Agilent MassHunter Workstation 10.0 software, and peak assignment and peak identification were performed by comparison with spectra from the NIST 2017 Mass Spectral Library. The results of the qualitative analysis of the extractives present in the liquid and headspace samples of silver fir were confirmed with a high probability value (Prob. %). The chromatograms obtained were also compared with the GC/MS analysis data for silver fir samples from the literature.

3 RESULTS AND DISCUSSION
3. REZULTATI I RASPRAVA

3.1 Chemical identities of extractives in liquid silver fir samples
3.1. Kemijski sastav ekstraktivnih tvari u tekućim uzorcima jelovine

GC-MSD analysis revealed the presence of 33 compounds (excluding the two ISTDs) in the liquid samples. The extractives identified in the liquid silver fir samples are listed in Table 1. GC-MSD of the silylated compounds revealed the presence of various simple and carboxylic acids, monosaccharides, disaccharides and polyphenols, including simple phenols, flavonoids and lignans (Table 1). Figure 2 and Figure 3 show clear differences in the GC-MSD chromatograms of stemwood, knotwood and bark extracts of silver fir. With GC-MSD, significantly more compounds were separated and detected from the samples of silver fir KW and B (Figure 2), whereby the chromatograms of KW and B were richer than those of SW and HW (Figure 3). The extended peaks 26 and 35 with retention times of 18.480 minutes and 26.240 minutes are assigned to the internal standards (ISTD), i.e. heneicosanoic acid (linear/unbranched C21:0 fatty acid) and betulin or betilinol (triterpene/sterol) (Figure 2 and Figure 3). The ISTDs are used to quantitatively evaluate the separated compounds of the chemically analyzed samples, but quantitative analysis was not the aim of the present preliminary investigation. Detailed information on the amounts of the identified compounds in the silver fir wood and bark extracts will be

presented in our future reports. In the present study, the characteristic and abundant peaks of ISTD are mostly used for easier orientation and reading of the chromatograms. As can be seen from Figure 2, the obvious difference between the extracts of KW (DK in the upper chromatogram) and B is in the MSD responses for the extractives/peaks eluted before the retention time of 18.480 minutes, i.e. before the ISTD C21:0 fatty acid, and the detector responses for the extractives/peaks eluted between two ISTDs (C21:0 and betulinol), between 18.480 and 26.240 minutes (Figure 2).

The B extracts of silver fir were found to be characterized by a large number of carboxylic/dicarboxylic acids, sugar acids, sugar alcohols and monosaccharides, with inositols/sugar alcohols (peaks #20 and #23), citric acid (peak #19), quininic acid (peak #21) and sucrose (peak #17) being among the most abundant peaks (Figure 2) (Table 1). The GC-MSD analysis also showed the presence of flavonoids (peak #29 and #31) and lignans (peak #33), which were found in rather low amounts (Figure 2) (Table 1). However, it has already been proven that silver fir bark contains polyphenols with a simple chemical structure, such as gallic acid, homovanillic acid, protocatechuic acid, vanillic acid, ferulic acid, p-coumaric acid, catechin, epicatechin, taxifolin, isorhamnetin (or 3-methylquercetin), gallocatechin, epigallocatechin, taxiresinol, lariciresinol, secolariciresinol, but also more complex

Table 1 Chemical composition of hydrophilic extractives of silver fir (*Abies alba* Mill.)
Tablica 1. Kemijski sastav hidrofilnih ekstrakata jelovine (*Abies alba* Mill.)

Peak *Vrh #*	**Retention time, min** *Retencijsko vrijeme,* min	**Group of extractives** *Skupine ekstraktivnih tvari*	**Identified compound** *Identificirani spoj*
1	3.190	Polysiloxane	Tetrasiloxane
2	3.381	Carboxylic acid	Lactic Acid
3	3.505	Carboxylic acid	Glycolic acid
4	4.055	Dicarboxylic acid	Oxalic acid
5	4.570	Polysiloxane	Pentasiloxane
6	5.762	Sugar alcohol	Glycerol
7	6.193	Carboxylic acid	Butanedioic acid
8	6.474	Dicarboxylic acid	Glyceric acid
9	8.415	Dicarboxylic acid	Malic acid
10	8.713	Carboxylic acid	2,3,4-Trihydroxybutyric acid
11	8.795	Monosaccharide	Ribofuranose-type 1
12	9.346	Carboxylic acid	2,3,4-Trihydroxybutyric acid
13	9.441	Monosaccharide	3-O-Methyl-β-D-Glucopyranose
14	10.013	Monosaccharide	Ribofuranose-type 2
15	10.765	Monosaccharide	Xylose
16	11.496	Dihydroxybenzoic acid	Vanillic Acid
17	11.894	Sugar acid	Ribonic acid
18	12.066	Cyclohexanecarboxylic acid	Shikimic acid
19	12.227	Carboxylic acid	Citric acid
20	12.456	Sugar alcohol	D-Pinitol
21	12.733	Cyclohexanecarboxylic acid	Quininic acid
22	13.317	Sugar alcohol	Acrylic acid
23	13.780	Sugar alcohol	Inositol 1
24	14.185	Sugar acid	D-Gluconic acid
25	14.988	Sugar alcohol	Inositol 2
26	18.480	Internal standard ISTD	C21:0 Heneicosanoic acid
27	19.670	Disaccharide	Sucrose
28	19.916	Fatty acid	Tricosanoic acid
29	21.184	Flavonoid	Catechin
30	21.263	Disaccharide	Melibiose
31	21.476	Flavonoid	Gallocatechin-type 1
32	21.588	Flavonoid	Gallocatechin-type 2
33	21.748	Lignan	Isolariciresinol
34	25.193	Disaccharide	Disaccharide-type
35	26.240	Internal standard ISTD	Betulinol

ISTD, internal standard. The identified compounds with their peaks are shown in the GC-MSD chromatograms at Figure 2 and Figure 3, with the peak numbers given in the table.
ISTD – interni standard. Identificirani spojevi sa svojim vrhovima prikazani su u GC-MSD kromatogramima na slici 2. i 3., s brojevima vrhova navedenima u tablici.

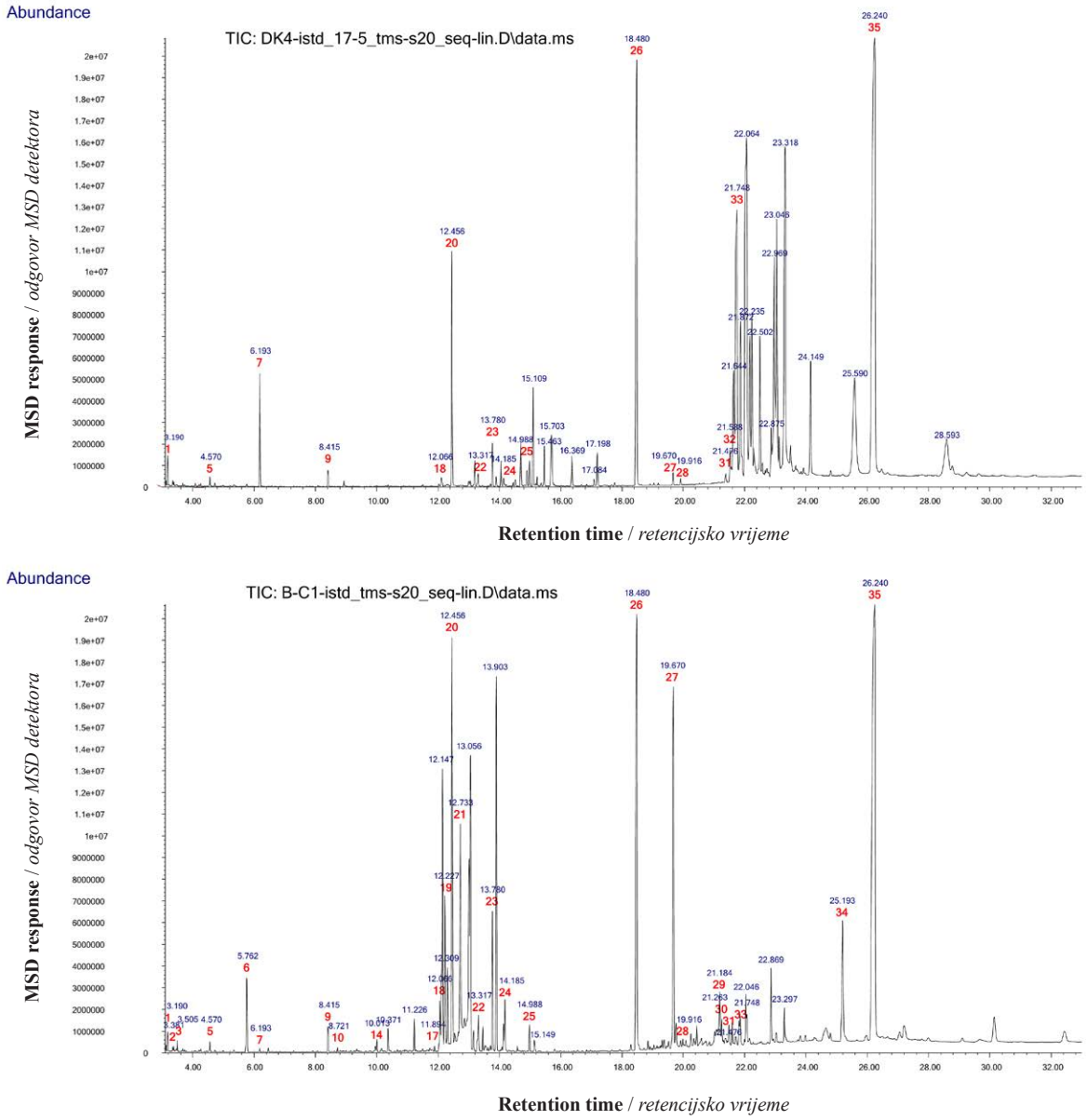

Figure 2 GC-MSD chromatograms (TIC) of silylated extracts of silver fir (*Abies alba* Mill.) (DK – knotwood, B – bark, TIC – total ion chromatogram, #1 – #35, for peak assignment see Table 1)
Slika 2. GC-MSD kromatogrami (TIC) siliranih ekstraktivnih tvari jelovine (*Abies alba* Mill.) (DK – kvrga, B – kora, TIC – ukupni ionski kromatogram za pikove #1 – #35 prikazane u tablici 1.)

compounds such as gallocatechin gallate, various flavonoid glycosides (e.g. quercetin glycoside, isorhamnetin glucoside, astringin), gallocatechin dimers and various prodelphinidins. It has also been confirmed that dimeric and trimeric procyanidins are part of the hydrophilic extractable fraction of silver fir samples (Patyra *et al.*, 2022). The compounds mentioned are characterized by a higher molecular mass, therefore LC-MS methods are the usual choice of analytical approach for the analysis of polyphenols from silver fir bark (Tavčar Benković *et al.*, 2014; Bianchi *et al.*, 2015; Brennan *et al.*, 2020; Patyra *et al.*, 2022; Vek *et al.*, 2023).

On the other hand, the KW extracts were richer in phenolic compounds that eluted at retention times intermediate to those of the two ISTDs (Figure 2). Of the compounds that eluted at retention times between 18.80 and 26.40 minutes (Figure 2), the presence of gallocatechins and isolariciresinol could be confirmed using the NIST spectral database. Silver fir knots have previously been described as a rich source of lignans, of which secoisolariciresinol, lariciresinol, nortrachelogenin, liovil, hydroxymatairesinol, matairesinol, cyclolariciresinol, and pinoresinol were qualitatively assessed by GC-MS analysis (Willför *et al.*, 2004). Analysis on a short GC column also revealed the presence of sesquilignans, dilignans and higher oligolignans in silver fir knotwood (Willför *et al.*, 2004). Similar results were reported by Brennan *et al.* (2021), who used GC-MS analysis and their spectral database to

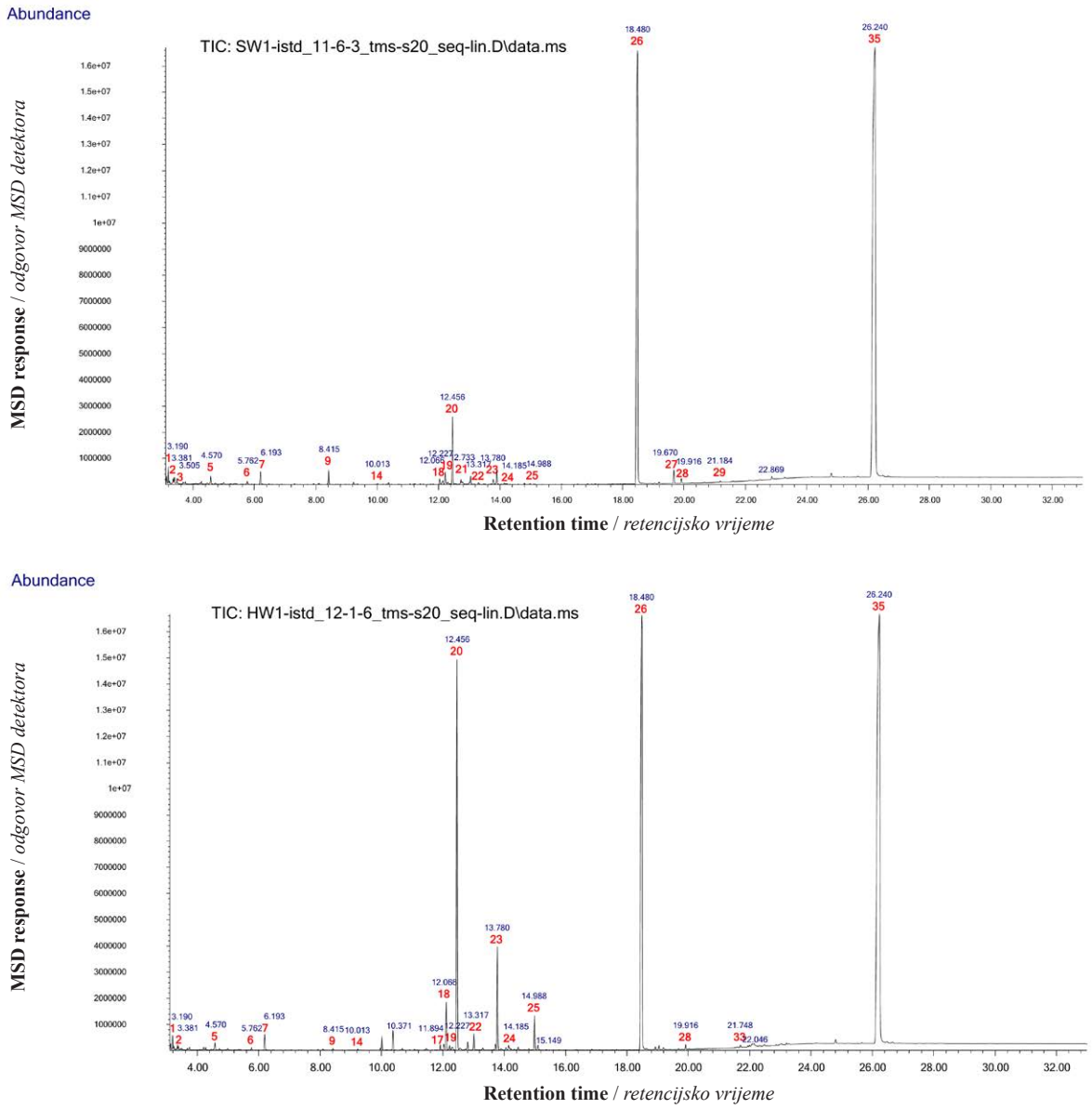

Figure 3 GC-MSD chromatograms (TIC) of silylated extracts of silver fir (*Abies alba* Mill.) (SW – sapwood, HW – heartwood, TIC – total ion chromatogram, #1 – #35, for peak assignment see Table 1)
Slika 3. GC-MSD kromatogrami (TIC) siliranih ekstraktivnih tvari jelovine (*Abies alba* Mill.) (SW – bjeljika, HW – srž, TIC – ukupni ionski kromatogram za pikove #1 – #35 prikazane u tablici 1.)

detect silylated secoisolariciresinol, lariciresinol, hydroxymatairesinol and isolariciresinol in silver fir knotwood extracts. In addition, the extraction of silver fir KW also produces sugars with mono- and disaccharides as well as sugar alcohols (Table 1, Figure 2). D-pitinol (peak #20) was confirmed as a characteristic GC-MSD peak for both the KW and B samples, which agrees well with the literature data (Brennan *et al.*, 2021) (Figure 2).

Our recent investigations using other chromatographic methods (TLC, HPLC) on the silver fir samples from Kočevska also showed the presence of simple phenolic acids (homovanillic acid, coumaric acid, ferulic acid), flavonoids (epicatechin, taxifolin, quercetin) and, of course, lignans, as already mentioned in the literature review. With regard to the GC-MSD results (Table 1, Figure 2), our HPLC-PDA analysis of lignans in KW proved to be a convenient and rapid method for the separation and detection of not only isolariciresinol, but also lariciresinol, secoisolariciresinol, pinoresinol and matairesinol, with secoisolariciresinol being confirmed as the most abundant (Vek *et al.*, 2021). Therefore, the peaks between the retention times of 21.00 and 23.00 minutes could represent the presence of these lignans, with secoisolariciresinol being the predominant peak of the KW extracts presumably at 22,064 minutes (Figure 2) (Willför *et al.*, 2004).

Furthermore, our results confirmed that the extraction of silver fir stemwood samples, i.e. SW and HW samples, yielded a significantly lower amount of

extractives than the extracts from B and KW (Figure 2), as shown by the low detector responses for the separate extractives of SW and HW extracts (Figure 3). Similar results have been reported by other research groups (Willför *et al.*, 2004; Ul'yanovskii *et al.*, 2022). The hydrophilic extracts of silver fir SW and HW consisted mainly of various carboxylic acids (lactic acid #2, glycolic acid #3, butanedioic acid #7, malic acid #9, shikimic acid #18, citric acid #19, quininic acid #21), various sugar compounds (sugar alcohols, sugar acids, monosaccharides and disaccharides), of which D-pinitol was the most abundant peak (Figure 3, Table 1). Phenolic compounds (#29 and #33) were only present in trace amounts in the liquid SW and HW samples (Figure 3). It is evident that the peaks for the internal standard (peak 26 and peak 35) were high compared to the peaks of the other compounds present in both the SW and HW extracts (Figure 3). However, due to the same preparation of the liquid samples for chromatographic analysis, the differences in the chemical composition and the amount of extractives between the SW, HW, KW and B samples are very clear (Figure 2 and Figure 3).

Finally, regarding our liquid silver fir samples, all hydrophilic extracts (SW, HE, KW and B) were also characterized by the presence of small amounts of tricosanoic acid (peak #28), a C23:0 fatty acid (Figure 2 and Figure 3). Other research groups have also reported the presence of compounds with lower polarity, i.e. epimanool, neoabietic acid and abietic acid, extracted from silver fir wood with more polar solvents such as ethanol or aqueous methanol (Brennan *et al.*, 2021; Patyra *et al.*, 2022).

3.1 Chemical identities of silver fir extractives in headspace samples
3.1. Kemijski sastav parne faze ekstraktivnih tvari uzoraka jelovine

The GC-MSD analysis of volatile extractives in silver fir samples confirmed the presence of 22 compounds. The extractives identified in the silver fir headspace (HS) samples are listed in Table 2. Compared to the analysis of the liquid silver fir sample (Figure 2 and Figure 3), the headspace analysis could be performed in a much shorter time – the chromatographic run took 12.5 minutes as can be seen in the figure. As mentioned above, the samples analyzed in this preliminary headspace analysis were selected for their good sensory properties and pleasant characteristic odor. The headspace GC-MSD analysis revealed the presence of volatile monoterpenes, sesquiterpenes as well as volatile esters of low molecular weight carboxylic acids (Table 2, Figure 4).

Table 2 Chemical composition of volatile extractives in headspace samples of silver fir (*Abies alba* Mill.)
Tablica 2. Kemijski sastav parne faze ekstraktivnih tvari uzoraka jelovine (*Abies alba* Mill.)

Peak *Vrh #*	**Retention time, min** *Retencijsko vrijeme,* min	**Group of extractives** *Skupine ekstraktivnih tvari*	**Identified compound** *Identificirani spoj*
1	2.757	Polysiloxane	Disiloxane, hexamethyl-
2	4.671	Monoterpene, bicyclic	α-Pinene
3	4.806	Monoterpene, bicyclic	α-Pinene
4	4.881	Monoterpene, bicyclic	Camphene
5	4.901	Monoterpene, bicyclic	Dehydrosabinene
6	5.136	Monoterpene, acyclic	β-Myrcene
7	5.442	Monoterpene, cyclic	D-Limonene
8	5.479	Monoterpene, cyclic	D-Limonene
9	5.702	Monoterpene, bicyclic	γ-Terpinene
10	5.968	Monoterpene, bicyclic	L-Fenchone
11	6.163	Monoterpene, bicyclic	Fenchol, exo-
12	6.217	Monoterpene	Ocimene
13	6.249	Monoterpene	α-Campholenal
14	6.427	Monoterpene	2-Bornanone/D-Camphor
15	6.578	Monoterpene, bicyclic	endo-Borneol
16	6.647	Monoterpene	Terpinen-4-ol
17	6.738	Monoterpene	α-Terpineol
18	6.790	Monoterpene	Myrtenol
19	8.070	Sesquiterpene	Ylangene
20	8.099	Sesquiterpene	Copaene
21	8.421	Sesquiterpene	Caryophyllene
22	9.421	LMW carboxylic acid	Valeric acid, volatile esters

LMW, low-molecular weight compound. The identified compounds with their peaks are shown in the GC-MSD chromatogram in Figure 4, with the peak numbers given in the table.
LMW – spoj niske molekularne mase. Identificirani spojevi sa svojim vrhovima prikazani su u GC-MSD kromatogramu na slici 4., s brojevima vrhova navedenima u tablici.

As shown in Figure 4, the characteristic volatile compounds of the silver fir headspace samples were monoterpenes, which eluted from the column between 4.20 and 7.00 minutes. The peaks in the GC-MSD chromatogram (Figure 4) were assigned to the monoterpenes α-pinene (peak #2 and #3), camphene (peak #4), D-limonene (#7 and #8), β-myrcene (#6), ocimene (#12), (+)-2-bornanone or D-(+)-camphor (#14), and α-terpineol (#17) (Table 2). It is obvious that the concentration of α-pinene and D-limonene in the extracted headspace sample was very high, so the interactions of the compounds with the stationary phase led to the formation of the split peaks, which means that the molecules of both α-pinene and D-limonene came out of the column and eluted at two retention times (Figure 4). Therefore, improving the existing headspace sampling method and determining the exact timing for sampling the headspace of wood and bark from the HS vials will be one of the important goals of our future research activities.

Monoterpenes from various tree tissues are extracted by simple steam distillation, supercritical CO_2 distillation, solid phase extraction (SPE), purge and trap (P&T), thermal desorption (TD) and solid phase microextraction (SPME) as well as by solvent extraction (Fengel and Wegener, 1989; Moukhtar *et al.*, 2006; Holmbom, 2011; Kačík *et al.*, 2012; Salem *et al.*, 2015; Turner, 2018). As described by Kačík *et al.* (2012) α-pinene, camphene, β-pinene, α-phellandrene, cymene, limonene, fenchol, borneol, thymol, myrtenal and verbenone are extracted from silver fir wood using hexane as a solvent. Using the present GC-MSD method, we were also able to detect sesquiterpene structures, i.e. terpenes consisting of three isoprene units, in the silver fir headspace samples; these volatile extractives were eluted after 8 minutes, as shown in Figure 4. Ylangene (peak #19), copaene (#20) and caryophyllene (#21) were described as sesquiterpenes with the spectral database used (Table 2). Brennan *et al.* (2021) report the detection of juvabiones, i.e. dehydrojuvabione and juvabione, among the eluted sesquiterpenes in the liquid sample of silylated KW silver fir extracts by GC-MSD analysis. However, the first detailed report on the presence of juvabiones (4'-dehydrojuvabione, todomatuic acid and 4'-dehydrotodomatuic acid) in extracts from silver fir stemwood and knotwood was presented two decades ago (Willför *et al.*, 2004). Interestingly, dehydrojuvabione was also detected in methanolic extracts of silver fir wood using the LC-DAD-ESI-MS/MS method, demonstrating that liquid chromatography/mass spectrometry is also an effective analytical approach for the analysis of terpenes in liquid samples (Patyra *et al.*, 2022).

However, the collection of volatile extractives from wood and bark using the headspace sampling method described is a rapid and accurate technique that requires no additional pretreatment of the sample prior to analysis. When taking headspace samples, it is important that the sample is fresh and has not been subjected to any thermal treatment that could negatively influence the results of the subsequent chromatographic analysis. Our results are in good agreement with existing literature data, and small differences in the composition of the volatile extracts of silver fir samples

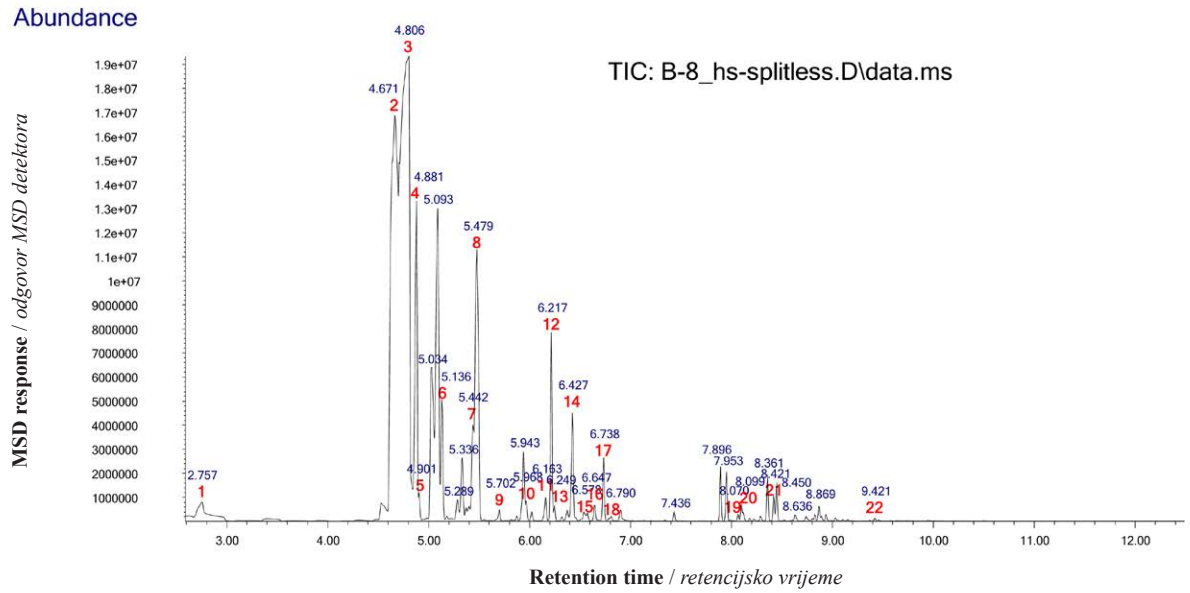

Figure 4 GC-MSD chromatograms (TIC) of headspace extractives of silver fir (*Abies alba* Mill.) (B – bark, TIC – total ion chromatogram, #1 – #22, for peak assignment see Table 2)
Slika 4. GC-MSD kromatogrami (TIC) parne faze ekstraktivnih tvari jelovine (*Abies alba* Mill.) (B – kora, TIC – ukupni ionski kromatogram za pikove #1 – #22 prikazane u tablici 2.)

could be due to the fact that the terpenoid profiles of conifers are geographically, taxa, genotype, temperature and light dependent (Manninen *et al.*, 2002; Moukhtar *et al.*, 2006; Kačík *et al.*, 2012).

4 CONCLUSIONS
4. ZAKLJUČAK

This research report presents a rapid and accurate method for analyzing the solvent-soluble and volatile extractives of silver fir tissues. The demonstrated gas chromatograph-mass selective detector (GC-MSD) method allows both the analysis of liquid wood and bark samples, where the extracts have been previously silylated, and the analysis of the headspace (HS) of freshly prepared silver samples. A total of 55 extractives in silver fir wood and bark samples were qualitatively evaluated using the HS-GC-MSD method. Gas chromatography of silylated compounds confirmed that silver fir bark extracts are characterized by a large number of carboxylic/dicarboxylic acids, sugar acids, sugar alcohols and monosaccharides, with inositols/sugar alcohols, citric acid, quininic acid and sucrose reaching the most abundant peaks. Larger amounts of phenolic extractives in the bark were not found, which does not mean that the bark does not contain phenolic extractives, but that the GC results should also be supported by other analytical methods. The liquid samples of knotwood extracts were rich in phenolic compounds and lignans. These compounds include gallocatechins and isolariciresinol. D-pitinol was confirmed as the most characteristic chromatographic peak in the GC traces of both the silver fir wood and bark samples. On the other hand, headspace analysis revealed the monoterpenes α-pinene, camphene, D-limonene, β-myrcene, ocimene, 2-bornanone or D-camphor and α-terpineol as the characteristic volatile extractives of silver fir. Headspace sampling has proven to be a fast and convenient method for analyzing volatile extractives, but still needs some fine-tuning. Finally, gas chromatography in combination with a mass selective detector and automatic samplers for liquid and headspace samples is an excellent analytical tool for the chemical screening of low molecular weight compounds in wood and tree bark. However, the analysis of higher molecular weight extractives requires a different analytical approach.

Acknowledgements – Zahvala

The authors would like to thank the Slovenian Research and Innovation Agency (ARIS) for supporting the project L4-2623 (ArsAlbi), the project V4-2017 and the programme group P4-0015. Special thanks go to Ars Pharmae d.o.o. for supporting the research activities and co-financing the HS-GC-MSD system. Many thanks also to the company Kočevski les d.o.o. for providing the silver fir material and to Samo Grbec, Anže Lopatič and Peter Hrovatič for their help and support in the field and in the laboratory.

5 REFERENCES
5. LITERATURA

1. Bianchi, S.; Kroslakova, I.; Janzon, R.; Mayer, I.; Saake, B.; Pichelin, F., 2015: Characterization of condensed tannins and carbohydrates in hot water bark extracts of European softwood species. Phytochemistry, 120: 53-61. https://doi.org/10.1016/j.phytochem.2015.10.006
2. Brennan, M.; Fritsch, C.; Cosgun, S.; Dumarcay, S.; Colin, F.; Gerardin, P., 2020: Quantitative and qualitative composition of bark polyphenols changes longitudinally with bark maturity in *Abies alba* Mill. Annals of Forest Science, 77 (1): 14. https://doi.org/10.1007/s13595-019-0916-x
3. Brennan, M.; Hentges, D.; Cosgun, S.; Dumarcay, S.; Colin, F.; Gérardin, C.; Gérardin, P., 2021: Intraspecific variability of quantity and chemical composition of ethanolic knotwood extracts along the stems of three industrially important softwood species: *Abies alba*, *Picea abies* and *Pseudotsuga menziesii*. Holzforschung, 75 (2): 168-179. https://doi.org/10.1515/hf-2020-0108
4. Domazet, M.; Zaloker, U.; Vek, V., 2023: Ars Pharmae®: Innovative natural approach to prevent and reduce diseases of modern times; food supplements from wood extractives. BioRural Knowledge-exchange Workshops (online), 2023.
5. Dyderski, M. K.; Paź, S.; Frelich, L. E.; Jagodziński, A. M., 2018: How much does climate change threaten European forest tree species distributions? Global Change Biology, 24 (3): 1150-1163. https://doi.org/10.1111/gcb.13925
6. Fengel, D.; Wegener, G., 1989: Wood: chemistry, ultrastructure, reactions. Berlin – New York, Walter de Gruyter. https://doi.org/10.1515/9783110839654
7. Hamad, A. M. A.; Ates, S.; Olgun, C.; Gur, M., 2019: Chemical Composition and Antioxidant Properties of Some Industrial Tree Bark Extracts. BioResources, 14 (3): 5657-5671. https://doi.org/10.15376/biores.14.3.5657-5671
8. Hamada, J.; Petrissans, A.; Ruelle, J.; Mothe, F.; Colin, F.; Petrissans, M.; Gerardin, P., 2018: Thermal stability of *Abies alba* wood according to its radial position and forest management. European Journal of Wood and Wood Products, 76 (6): 1669-1676. https://doi.org/10.1007/s00107-018-1353-5
9. Holmbom, B., 1999: Extractives. In: Sjöström, E.; Alén, R. (eds.): Analytical methods in wood chemistry, pulping and papermaking. Berlin, Springer-Verlang, pp. 316. https://doi.org/10.1007/978-3-662-03898-7_5
10. Holmbom, B., 2011: Extraction and utilisation of non-structural wood and bark components. In: Alén, R. (ed.): Biorefining of forest resources. Helsinki, Paper Engineers' Association/Paperi ja Puu Oy, pp. 178-224.
11. Kačík, F.; Veľková, V.; Šmíra, P.; Nasswettrová, A.; Kačíková, D.; Reinprecht, L., 2012: Release of Terpenes from Fir Wood during Its Long-Term Use and in Thermal Treatment. Molecules, 17 (8): 9990-9999. https://doi.org/10.3390/molecules17089990
12. Kebbi-Benkeder, Z.; Manso, R.; Gérardin, P.; Dumarçay, S.; Chopard, B.; Colin, F., 2017: Knot extractives: a model for analysing the eco-physiological factors that control the within and between-tree variability. Trees, 31 (5): 1619-1633. https://doi.org/10.1007/s00468-017-1573-z

13. Manninen, A. M.; Tarhanen, S.; Vuorinen, M.; Kainulainen, P., 2002: Comparing the Variation of Needle and Wood Terpenoids in Scots Pine Provenances. Journal of Chemical Ecology, 28 (1): 211-228. https://doi.org/10.1023/A:1013579222600
14. Moukhtar, S.; Couret, C.; Rouil, L.; Simon, V., 2006: Biogenic Volatile Organic Compounds (BVOCs) emissions from *Abies alba* in a French forest. Science of The Total Environment, 354 (2): 232-245. https://doi.org/10.1016/j.scitotenv.2005.01.044
15. Patyra, A.; Dudek, M. K.; Kiss, A. K., 2022: LC-DAD – ESI-MS/MS and NMR Analysis of ConiferWood Specialized Metabolites. Cells, 11 (20): 3332. https://doi.org/10.3390/cells11203332
16. Salem, M.; Zeidler, A.; Böhm, M.; Mohamed, M.; Ali, H., 2015: GC/MS Analysis of Oil Extractives from Wood and Bark of *Pinus sylvestris*, *Abies alba*, *Picea abies* and *Larix decidua*. BioResources, 10: 7725-7737. https://doi.org/10.15376/biores.14.3.5657-5671
17. Schoss, K.; Benedetič, R.; Kreft, S., 2022: The Phenolic Content, Antioxidative Properties and Extractable Substances in Silver Fir (*Abies alba* Mill.) Branches Decrease with Distance from the Trunk. Plants, 11 (3): 333. https://doi.org/10.3390/plants11030333
18. Tavčar Benković, E.; Grohar, T.; Žigon, D.; Švajger, U.; Janeš, D.; Kreft, S.; Štrukelj, B., 2014: Chemical composition of the silver fir (*Abies alba*) bark extract Abigenol® and its antioxidant activity. Industrial Crops and Products, 52: 23-28. https://doi.org/10.1016/j.indcrop.2013.10.005
19. Tekin, K.; Hao, N.; Karagoz, S.; Ragauskas, A. J., 2018: Ethanol: A Promising Green Solvent for the Deconstruction of Lignocellulose. ChemSusChem, 11 (20): 3559-3575. https://doi.org/10.1002/cssc.201801291
20. Turner, D., 2018: Hands-on Sample Preparation. Headspace, Solid-Phase Micro-Extraction (SPME), Thermal Desorption (TD), Purge and Trap (P&T). Hands-on Sample Preparation Training Course, Ljubljana, 2018.
21. Ul'yanovskii, N. V.; Onuchina, A. A.; Faleva, A. V.; Gorbova, N. S.; Kosyakov, D. S., 2022: Comprehensive characterization of chemical composition and antioxidant activity of lignan-rich coniferous knotwood extractives. Antioxidants, 11 (12): 2338. https://doi.org/10.3390/antiox11122338
22. Vek, V.; Keržič, E.; Poljanšek, I.; Eklund, P.; Humar, M.; Oven, P., 2021: Wood Extractives of Silver Fir and Their Antioxidant and Antifungal Properties. Molecules, 26 (21): 6412. https://doi.org/10.3390/molecules26216412
23. Vek, V.; Šmidovnik, T.; Humar, M.; Poljanšek, I.; Oven, P., 2023: Comparison of the content of extractives in the bark of the trunk and the bark of the branches of silver fir (*Abies alba* Mill.). Molecules, 28 (1): 225. https://doi.org/10.3390/molecules28010225
24. Verkasalo, E.; Leppälä, J.; Muhonen, T.; Korpinen, R.; Möttönen, V.; Kurppa, S., 2019: Novel industrial ecosystems and value chains to utilize side-streams of wood products industries – Finnish approach. Pro Ligno Journal, 15 (4): 157-165.
25. Willför, S.; Nisula, L.; Hemming, J.; Reunanen, M.; Holmbom, B., 2004: Bioactive phenolic substances in industrially important tree species. Part 2: Knots and stemwood of fir species. Holzforschung, 58 (6): 650-659. https://doi.org/10.1515/HF.2004.119

Corresponding address:

VILJEM VEK
University of Ljubljana, Biotechnical Faculty, Department of Wood Science and Technology, Jamnikarjeva ulica 101, Ljubljana, SLOVENIA, e-mail: viljem.vek@bf.uni-lj.si