

OPTIMIZATION OF ACCELERATED SOLVENT EXTRACTION (ASE) OF SILVER FIR WOOD (ABIES ALBA MILL.)

OPTIMIZACIJA POSPEŠENE EKSTRAKCIJE S TOPILI (ASE) NA PRIMERU LESA BELE JELKE (ABIES ALBA MILL.)

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Abstract / *Izvleček*

Abstract: Qualitative and quantitative analysis of wood plant extractives depend on the efficiency of the extraction method used. The aim of this study was the application of the accelerated solvent extraction system ASE 350 to obtain hydrophilic extracts from silver fir wood (*Abies alba* Mill.) and optimization of the extraction protocol. Silver fir trees originated from Kočevska Reka. Dissected and milled samples of sapwood, heartwood and knots were extracted with the ASE 350 with water, ethanol (95 % aq), acetone (95 % aq) and ethyl acetate. The number of extraction cycles was determined. Analysis of extracts was done by UV/Vis spectrophotometry and HPLC. The most effective solvent for the extraction of phenolic components from heartwood was ethanol (95 % aq), in the case of sapwood water and acetone (95 % aq) in the case of knotwood. Only two 5-minute extraction cycles were required for sufficient extraction yield when using ASE 350 at 100 °C and 103.42 bar.

Keywords: accelerated solvent extraction (ASE), silver fir (*Abies alba*), hydrophilic extractives, UV/Vis, chromatography

Izvleček: Kvalitativna in kvantitativna analiza ekstraktivov iz lesa je v veliki meri odvisna od učinkovitosti uporabljene ekstrakcijske metode. Cilj te študije je bil uporabiti sistem za pospešeno ekstrakcijo ASE 350 za pridobivanje hidrofилnih ekstraktivov iz lesa bele jelke (*Abies alba* Mill.) ter optimizirati protokol ekstrakcije. Drevesa bele jelke so bila posekana v gozdovih Kočevske Reke. Izolirane in zmlete vzorce beljave, jedrovine in grč smo ekstrahirali z ASE 350 z vodo, etanolom (95 % aq), acetonom (95 % aq) in etil acetatom. Določili smo število ekstrakcijskih ciklov. Semi-kvantitativno analizo ekstraktov smo izvedli z UV/Vis spektrofotometrijo, kvalitativno analizo pa s HPLC. Najučinkovitejše topilo za ekstrakcijo fenolnih komponent iz jedrovine je bil etanol (95 % aq), v primeru beljave je bila to voda, pri grčah pa aceton (95 % aq). Za učinkovito ekstrakcijo z uporabo ASE 350 pri 100 °C in 103,42 bar sta bila potrebna le dva 5-minutna ekstrakcijska cikla.

Ključne besede: pospešena ekstrakcija s topili (ASE), bela jelka (*Abies alba*), hidrofилni ekstraktivi, UV/Vis, kromatografija

1 INTRODUCTION

1 UVOD

Wood is defined as the secondary xylem of woody plants, and in engineering terms as a polymer composite composed of structural components (95 % to 98 %) (Holmbom, 2011; Vek et al., 2019a). These include cellulose, lignin and hemicelluloses (Sjöström, 1993; Fengel and Wegener, 1989). In addition, wood tissues also contain smaller amounts of non-structural compounds called extractives (Sjöström, 1993; Fengel and Wegener, 1989). These are various low- or medium-molecular-weight substances found in cell lumina and intercellular

spaces (Oven et al., 2011). Some extractives are important from a physiological point of view as they play an important role in plant metabolism, like nonstructural carbohydrates, sterols and fatty acids (Taiz & Zeiger, 2002). The extractives that are considered as secondary metabolites contribute to the protection of plants against herbivorous animals and microorganisms (fungi), attract pollinators, and enable plant-to-plant competition and symbiosis between organisms (Taiz & Zeiger, 2002).

Extractives can be extracted from wood with water or organic solvents (Oven et al., 2011). Given the solvent in which they are soluble, extractives can be divided into lipophilic and hydrophilic (Vek et al., 2016). Hydrophilic extracts are compounds soluble in polar solvents. They include sugars, phenolic acids, flavonoids, stilbene, quinones, lignans and

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tannins (Willför et al., 2003a; Willför et al., 2004). However, lipophilic extracts are compounds soluble in non-polar organic solvents. The following groups of lipophilic extracts are present in trees: fatty acids, triacylglycerols, waxes, sterols and terpenoids (Vek et al., 2019a).

Various procedures are used for extraction from wood. The most frequently used methods are conventional Soxhlet extraction (Vek et al., 2018), ultrasound-assisted extraction (Santos et al., 2019), microwave-assisted extraction (Quiles-Carrillo et al., 2019), accelerated solvent extraction and supercritical fluid extraction (Feng et al., 2016). Accelerated solvent extraction (ASE), a technique patented by Dionex Corporation, has received increased attention due to its suitability for extracting various plant material. In this technique, the liquid phase is used at high pressure and temperature but below a critical point. High

pressure is required to keep the solvent in a liquid state, and the pressure rarely affects the extraction process. This approach is considered an effective way to increase automation, but it can also shorten the process time and reduce the amount of solvent required for extraction (Priego-Capote & Delgado de la Torre, 2013). ASE has a comparable extraction yield to that at the Soxhlet extraction, with less time and solvents being consumed (Vek et al., 2018). Another advantage of the ASE system is its connectivity to other steps of analytical processes, such as filtration, preconcentration, derivatization, chromatographic separation or detection. ASE can also be used to extract compounds that have low solubility in the selected solvent (e.g., water). Prior to extraction with ASE, solid samples such as wood must be ground and sieved to obtain a homogeneous fraction. The sample thus prepared is loaded into an extraction cell which is inserted

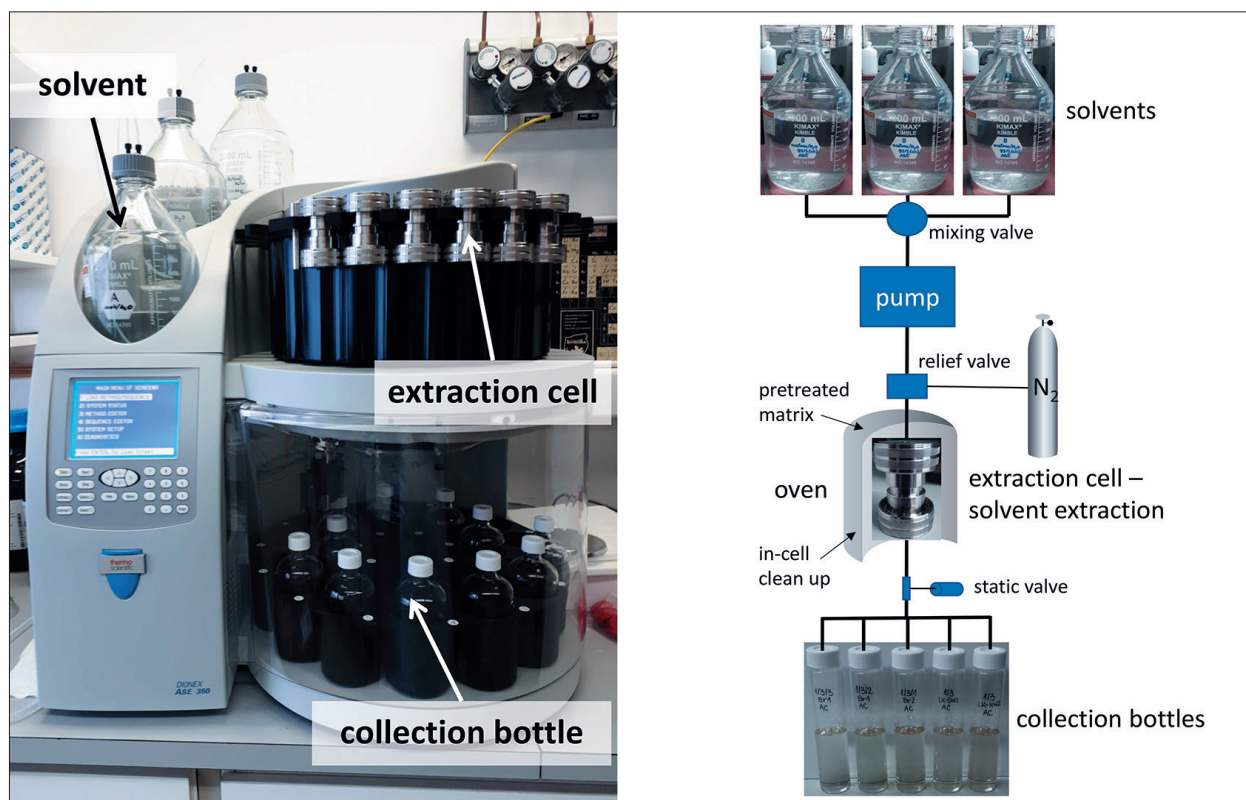


Figure 1. The device for accelerated solvent extraction, an ASE 350 (Thermo Scientific Dionex), in the laboratory of the Chair of the Chemistry of Wood and other Lignocellulosic Materials at the Department of Wood Science and Technology, Biotechnical Faculty, and a schematic diagram of the system (Thermo Scientific, 2013)

Slika 1. Naprava za pospešeno ekstrakcijo ASE 350 (Thermo Scientific Dionex) v laboratoriju Katedre za kemijo lesa in drugih lignoceluloznih materialov Oddelka za lesarstvo na Biotehniški fakulteti in shematski prikaz sistema (Thermo Scientific, 2013)

into the ASE system. The extraction cell is restored to the selected temperature and then filled with the solvent, thereby increasing the pressure in the extraction cell. When the temperature and pressure are balanced then a static extraction cycle takes place and lasts for pre-set time. After static extraction is completed, the valve opens and the extract is poured into a collecting bottle. The extraction cell is washed with solvent or blown with inert gas (e.g. nitrogen) before and after extraction (Priego-Capote & Delgado de la Torre, 2013).

The aim of the study was optimization of extraction using an ASE 350 (Thermo Scientific Dionex) (Figure 1), which included selection of the best extraction solvent and examination of the number of extraction cycles needed to maximize extraction yield of different wood tissues of silver fir (*Abies alba* Mill.).

2 MATERIALS AND METHODS

2 MATERIALI IN METODE

2.1 CHEMICALS AND STANDARDS

2.1 KEMIKALIJE IN STANDARDI

All the solvents and reagents were of analytical grade. The extraction solvents (water, acetone, ethanol, and ethyl acetate) and solvents for chromatographic analysis were purchased from Sigma Aldrich (Steinheim, Germany). The Folin-Ciocalteu phenol reagent (2 N), sodium carbonate (anhydrous) and gallic acid monohydrate (HPLC assay, $\geq 99\%$) were provided by Merck (Sigma Aldrich Chemie).

2.2 PLANT MATERIAL AND PREPARATION OF SAMPLES

2.2 RASTLINSKI MATERIAL IN PRIPRAVA VZORCEV

Three silver fir trees (*Abies alba* Mill.) originating from the forest of Kočevska Reka were included in the study. Tree felling was carried out in mid-December 2018. The sample discs were taken from the upper trunks.

The discs were planed, thereafter we marked the boundary between the sapwood and heartwood, counted the growth rings, measured their diameters, and marked the sampling points. Wood tissues from the trunk, knots and branches were sampled. Individual samples were dried at

40 °C for 24 hours and then disintegrated on a Retsch SM 2000 cutting mill using a sieve with 1 mm openings. The ground samples were stored in a dark and cool place until the beginning of the chemical analyzes. Before extraction, all samples were freeze-dried to an absolutely dry state for 24 h at - 85° C and 0.045 mbar.

2.3 EXTRACTION

2.3 EKSTRAKCIJA

Homogenized heartwood (HW), sapwood (SW), and knotwood (KW) samples from all sample discs were extracted at elevated temperature and pressure (100 °C and 103.42 bar) on an accelerated solvent extraction system, an ASE 350. We used different solvents: water, EtOH / H₂O (95/5, v/v), EtOAc, and Me₂CO / H₂O (95/5, v/v). After selecting the appropriate solvent, the samples were extracted with five extraction cycles (5 min per cycle). We captured the extracts from each cycle separately. The extracts were evaluated colorimetrically by using UV/Vis spectrophotometry.

2.4 SPECTROPHOTOMETRIC ANALYSIS

2.4 SPEKTROFOTOMETRIČNA ANALIZA

Total phenols were measured according to the protocol already described in Vek et al. (2013, 2014), Scalbert et al. (1989) and Singleton and Rossi (1965). Diluted Folin-Ciocalteu phenol reagent (*aq*) and an aqueous solution of sodium carbonate (75 g/l) were added to each wood extract. After incubation of the reaction mixtures, the absorbance was measured at 765 nm with a UV/Vis spectrophotometer Lambda (Perkin-Elmer). The results were determined by the standard curve of gallic acid and expressed in milligrams of gallic acid equivalents per gram of dried wood sample (mg GAE/g).

2.5 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

2.5 TEKOČINSKA KROMATOGRAFIJA VISOKE LOČLJIVOSTI (HPLC)

Chromatographic analysis was performed on a Thermo Scientific system for high performance liquid chromatography (Accela HPLC) equipped with a photodiode array detector (PDA). Separation of samples was done on a Thermo Accucore ODS column (4.6 id × 150 mm, 2.6 μm). Water (A) and methanol (B), both containing 0.1 % of formic

acid, served as a mobile phase. The flow rate of the mobile phase was set at 1000 $\mu\text{l}/\text{min}$. The gradient used was 5–95 % of the solvent (B). Both the autosampler containing sample trays and the column oven were thermostated at 5 $^{\circ}\text{C}$ and 30 $^{\circ}\text{C}$, respectively. Absorbance was measured at 275 nm and UV spectra were recorded from 200 nm to 400 nm. Peak identities were investigated by comparison of retention times and UV spectra of separated compounds with those of analytical standards. The chromatographic method was linear in the selected concentration range ($R^2 \geq 0.99$). The samples were measured in triplicate. The identification of phenolic compounds was done with the aid of external standards.

3 RESULTS AND DISCUSSION

3 REZULTATI IN DISKUSIJA

Chemical analysis included determination of total phenol content and identification of individual phenolic compounds (lignans, flavonoids and phenolic acids).

The aim of this study was to maximize the yields of the target compounds. Figure 2 shows the contents of total phenols in the aqueous (H_2O), ethanol ($\text{EtOH}/\text{H}_2\text{O}$), ethyl acetate (EtOAc) and acetone ($\text{Me}_2\text{CO}/\text{H}_2\text{O}$) extracts of heartwood (HW), sapwood (SW), and knotwood (KW) of silver fir (*A. alba*).

The extraction yields were different with the various solvents used for the different categories of wood. The most effective solvent for the extraction of phenolic components from heartwood was ethanol/water (95/5, v/v), while in the case of sapwood it was water and in that of knotwood it was acetone/water (95/5, v/v) (Figure 2). Ethyl acetate gave the lowest extraction yield in the case of all tissues. Acetone/water (95/5) was chosen as the most promising solvent for further sequential extractions, because the extraction yield of knotwood is the focus of the industrial partner who supported this investigation.

Further experimental work focused on examination of the number of extraction cycles needed to maximize the extraction yield. Figure 3 shows the contents of total phenols in the extracts obtained at five successive extraction cycles.

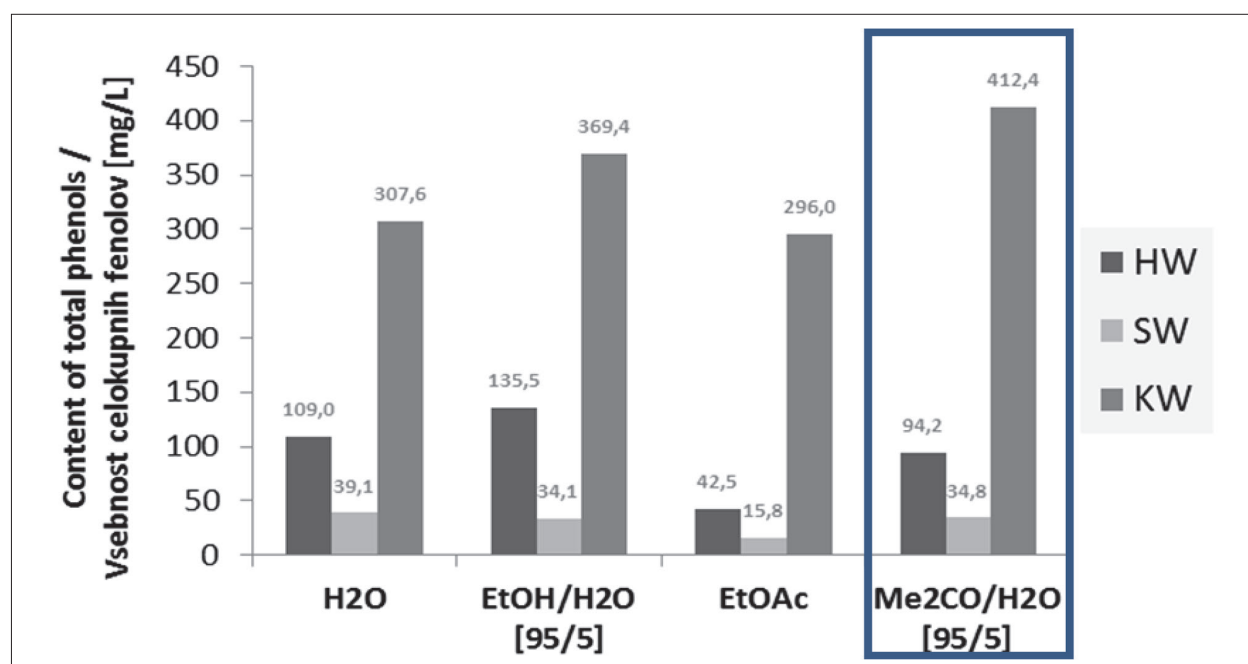


Figure 2. Content of total phenols in the aqueous (H_2O), ethanol ($\text{EtOH}/\text{H}_2\text{O}$), ethyl acetate (EtOAc), and acetone ($\text{Me}_2\text{CO}/\text{H}_2\text{O}$) extracts of heartwood (HW), sapwood (SW), and knotwood (KW) of silver fir (*A. alba*) (the frame indicates the most suitable solvent)

Slika 2. Vsebnost celokupnih fenolov v vodnih (H_2O), etanolnih ($\text{EtOH}/\text{H}_2\text{O}$), etil acetatnih (EtOAc) in acetonskih ($\text{Me}_2\text{CO}/\text{H}_2\text{O}$) ekstraktih jedrovine (HW), beljave (SW) in lesa grč (KW) bele jelke (*A. alba*) (z okvirjem je označeno najbolj primerno topilo)

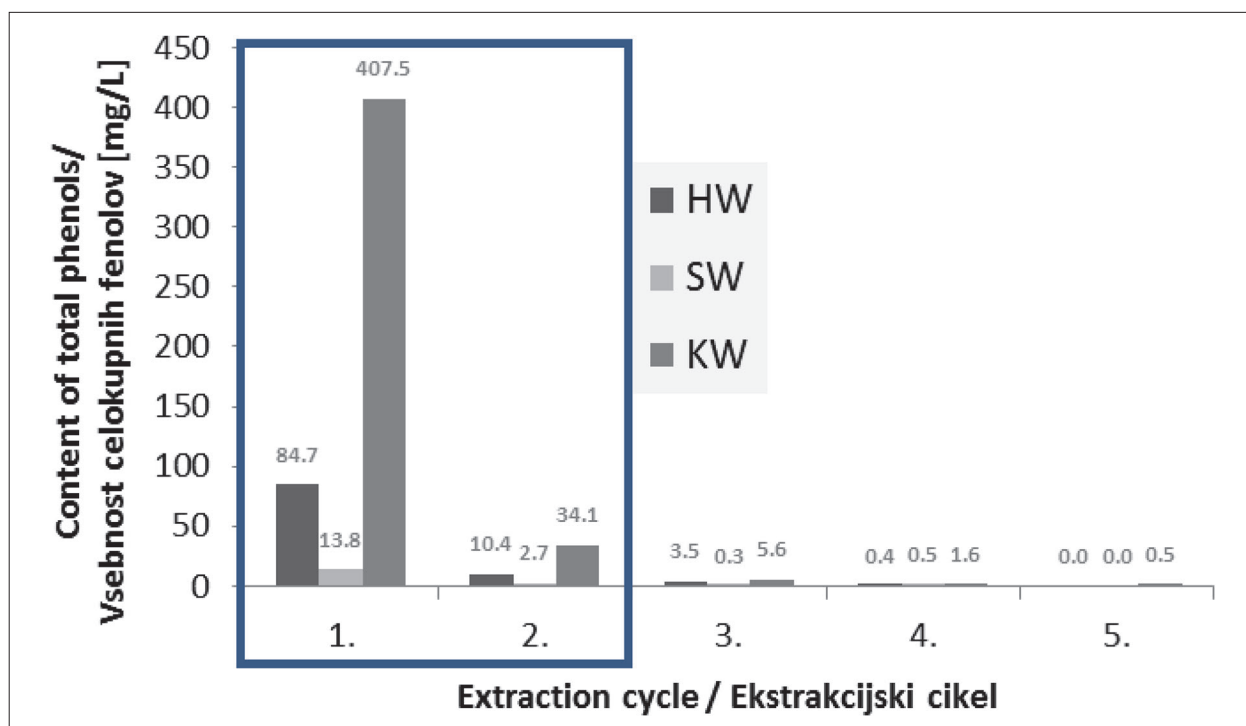


Figure 3. Content of total phenols in the acetone extracts of heartwood (HW), sapwood (SW) and knotwood (KW) of silver fir (*A. alba*) obtained in different successive extraction cycles (the frame indicates the appropriate number of cycles).

Slika 3. Vsebnost celokupnih fenolov v acetonskih ekstraktih jedrovine (HW), beljave (SW) in lesa grč (KW) bele jelke (*A. alba*), pridobljenih v različnih zaporednih ekstrakcijskih ciklih (z okvirjem je označeno primerno število ciklov).

Figure 3 reveals that the extraction yield decreased with each consecutive cycle. Most extracts were obtained from wood with the first extraction cycle (Vek et al., 2019b). The amount of total phenols in the extracts of knotwood was 407.5 mg/L after the first extraction cycle, 34.1 mg/L after the second, and less than 10 mg/L were obtained from the following cycles. In the extracts of heartwood and sapwood, the content of total phenols was already 10 mg/L or lower in the second extraction cycle. The results of this analysis show that only two extraction cycles are required for sufficient extraction yield, which corresponds to the protocol described by Willför et al. (2003b).

The chromatograms in Figure 4 show the compounds contained in the acetone extracts of living knots of silver fir (*A. alba*) after the first, second, third and fourth static extraction cycles in the ASE system.

The highest peaks represent the lignans, which are the dominant group of compounds in

the extracts of silver fir. They are also present in the extract of the second extraction cycle. In addition to lignans, live knots also contain phenolic acids and flavonoids, which are represented by lower peaks in the chromatogram, and are no longer extracted with the second cycle, with the exception of taxifolin. As the most polar compound, the flavonoid epicatechin ($t_r = 8.0$ min) was the first to elute, followed by phenolic acids homovanilic acid ($t_r = 8.1$ min) and coumaric acid ($t_r = 9.5$ min), flavonoid taxifolin ($t_r = 9.7$ min), ferulic acid ($t_r = 10.0$), lignans isolariciresinol (Iso-Lari, $t_r = 10.2$ min), lariciresinol ($t_r = 11.5$ min), secoisolariciresinol ($t_r = 11.7$ min), pinoresinol ($t_r = 12.6$ min), and matairesinol ($t_r = 12.9$ min), and finally the less polar flavonoid quercetin was eluted from the column ($t_r = 13.6$ min). Our results on composition of phenolic extractives of silver fir wood are in accordance with the data from the literature (Willför et al., 2004; Benkovic et al., 2017).

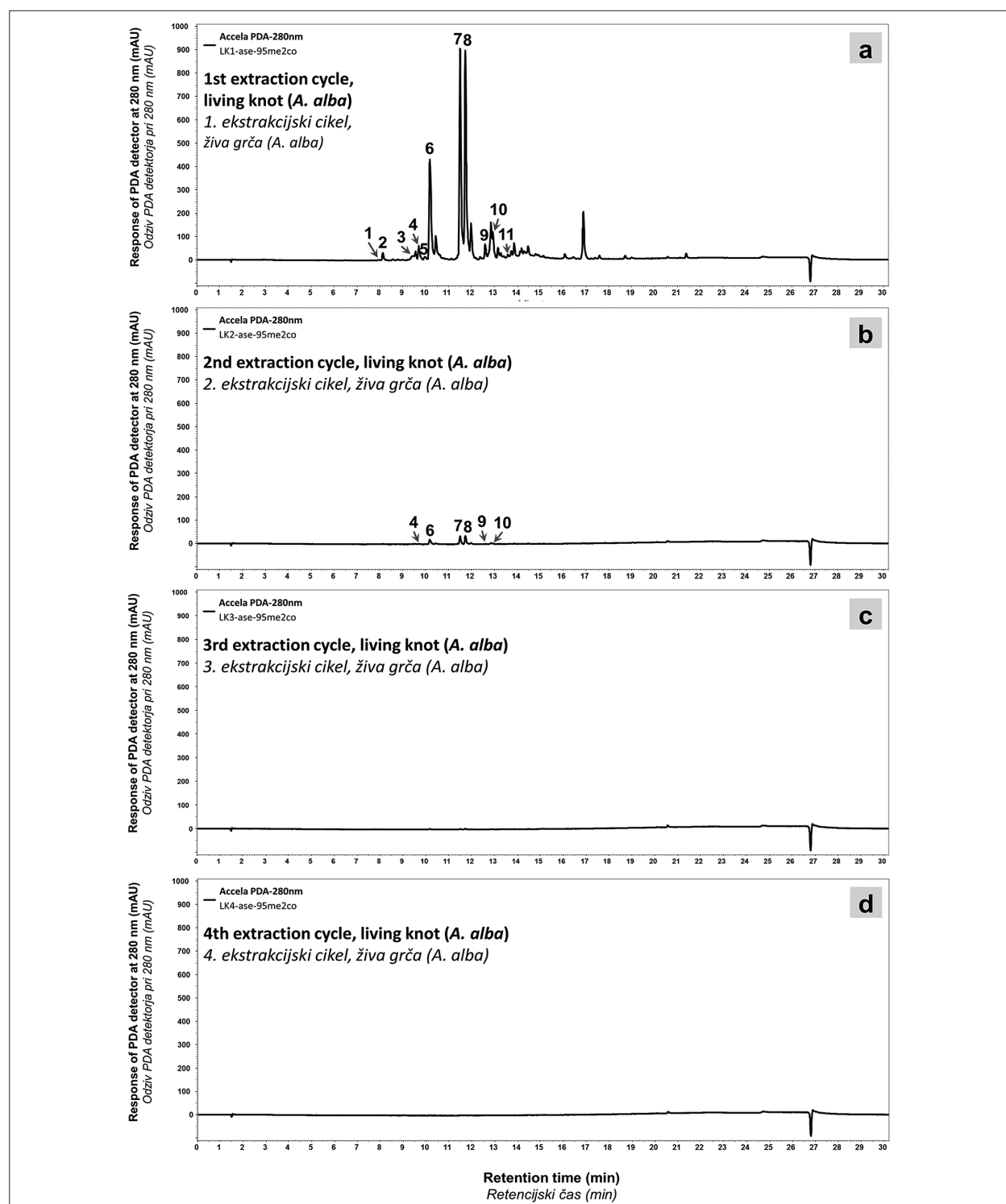


Figure 4. HPLC-PDA chromatograms of silver fir extracts (*A. alba*) taken at 280 nm. Acetone extracts of living knots after a) the first, b) second, c) third and d) fourth static extraction cycles in the ASE system (the numbers of marked compounds given in Table 1).

Slika 4. Kromatogrami HPLC-PDA ekstraktov bele jelke (*A. alba*), posneti pri 280 nm. Acetonski ekstrakt lesa žive grče po a) prvem, b) drugem c) tretjem in d) četrtem statičnem ekstrakcijskem ciklu v sistemu ASE (s številkami označene spojine so podane v preglednici 1).

Table 1. Identification of the marked peaks on the chromatogram
Preglednica 1. Identifikacija označenih vrhov na kromatogramu

Peak number / Št. vrha	Identified phenolic compound / Identificirana fenolna spojina	Compound type / Tip spojine	R _t of HPLC analysis / R _t HPLC-analize [min]
1.	Epicatechin / Epikatehin	Flavonoid / Flavonoid	7.96
2.	Homovanillic acid / Homovanilinska kislina	Phenolic acid / Fenolna kislina	8.14
3.	Coumaric acid / Kumarilna kislina	Phenolic acid / Fenolna kislina	9.46
4.	Taxifolin / Taksifolin	Flavonoid / Flavonoid	9.69
5.	Ferulic acid / Ferulna kislina	Phenolic acid / Fenolna kislina	9.99
6.	Isolariciresinol / Izolaricirezinol	Lignan / Lignan	10.20
7.	Lariciresinol / Laricirezinol	Lignan / Lignan	11.50
8.	Secoisolariciresinol / Sekoizolaricirezinol	Lignan / Lignan	11.74
9.	Pinoresinol / Pinorezinol	Lignan / Lignan	12.60
10.	Matairesinol / Matairezinol	Lignan / Lignan	12.91
11.	Quercetin / Kvercetin	Flavonoid / Flavonoid	13.60

4 CONCLUSION

4 ZAKLJUČEK

The results of the analysis show that only two extraction cycles, each 5 min long, are required for sufficient extraction yield when using an accelerated solvent extraction system ASE 350 at T = 100 °C and P = 103.42 bar, to remove the majority of phenolic components present in a different wood tissues of silver fir tree (*A. alba*). Accelerated solvent extraction is considered an effective extraction method, one which essentially reduces the extraction time and consumption of solvents, while maximizing the extraction efficiency in comparison to traditional extraction methods. The extraction protocol established in this study will be further employed in extensive investigation of the hydrophilic extractives content in the wood and bark of silver fir from different growth sites, to meet the needs of our industrial partner.

5 SUMMARY

5 POVZETEK

Učinkovitost uporabljene ekstrakcijske metode v veliki meri vpliva na kvalitativno in kvantitativno analizo lesnih ekstraktivov. Pri ekstrakciji iz lesa se poslužujemo različnih ekstrakcijskih postopkov. Med najpogosteje uporabljene tehnike štejemo ekstrakcijo v Soxhletovem aparatu, ultrazvočno ekstrakcijo, ekstrakcijo z mikrovalovi, pospešeno

ekstrakcijo in superkritično ekstrakcijo. V pričujoči študiji smo uporabili eno od sodobnejših tehnik, s komercialnim poimenovanjem ASE (akronim za accelerated solvent extraction). Pospešena ekstrakcija ASE je (Singleton & Rossi 1965) metoda, pri kateri se uporablja tekoča faza pri visokem tlaku in temperaturi, vendar pod kritično točko. Visok tlak je potreben za ohranjanje topila v tekočem stanju, vendar ta le redko vpliva na postopek ekstrakcije. S tem pristopom lahko povečamo avtomatizacijo, skrajšamo čas postopka in zmanjšamo količino topila, potrebnega za ekstrakcijo.

Cilj študije je bil uporabiti sistem za pospešeno ekstrakcijo ASE 350 za pridobivanje hidrofilnih ekstraktivov iz različnih kategorij lesa bele jelke (*Abies alba* Mill.) ter optimizirati protokol ekstrakcije. V ta namen smo s sistemom za pospešeno ekstrakcijo ASE 350 izvedli ekstrakcije z različnimi topili in v več statičnih ekstrakcijskih ciklih ter izvedli analize posameznih ekstraktov.

V študijo so bila vključena drevesa bele jelke, ki so bila posekana v gozdovih Kočevske Reke. Vzorčne kolute smo odvzeli iz zgornjega dela dreves. V mizarški delavnici smo izolirali vzorce beljave, jedrovine in grč ter jih zmlleli na delce, velike 1 mm ali manj. Z namenom identifikacije najučinkovitejšega ekstrakcijskega topila smo zmlate vzorce ekstrahirali z ASE 350 pri 100 °C in 103,42 bar z vodo, etanolom / vodo (95/5), acetonom / vodo (95/5) in etil acetatom. Z zajemanjem ekstrakta vsakega

ekstrakcijskega cikla posebej in analizo le-teh smo nato določili število ekstrakcijskih ciklov. S tem smo optimizirali čas ekstrakcije in porabo topila. Semikvantitativno analizo ekstraktov smo izvedli z UV/Vis spektrofotometrijo, kvalitativno analizo pa s HPLC z uporabo zunanjih standardov.

Najučinkovitejše topilo za ekstrakcijo fenolnih komponent iz jedrovine je bil etanol / voda (95/5, v/v), v primeru beljave je bila to voda, pri grčah pa aceton / voda (95/5, v/v). Rezultati te analize so pokazali, da sta za učinkovito ekstrakcijo fenolnih spojin s pospešenim sistemom za ekstrakcijo ASE 350 pri $T = 100\text{ }^{\circ}\text{C}$ in $P = 103,42\text{ bar}$, potrebna le dva ekstrakcijska cikla, trajajoča po 5 minut. Spojine, ki smo jih identificirali v lesu bele jelke, so bile epikatehin, homovanilinska kislina, kumarilna kislina, taksifolin, ferulna kislina, izolaricirezinol, laricirezinol, sekoizolaricirezinol, pinorezinol, matairezinol in kvercetin. Prevladujoča skupina spojin so bili lignani izolaricirezinol, laricirezinol in sekoizolaricirezinol.

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