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Influence of Lyophilisation and Oven-Drying on Extraction Yield of Oregonin from European Black Alder (*Alnus glutinosa* (L.) Gaertn.) Bark

Utjecaj liofilizacije i sušenja u sušioniku na prinos ekstrahiranog oregonina iz kore crne johe (*Alnus glutinosa* (L.) Gaertn.)

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ABSTRACT • *Oregonin ((5S)-1,7-bis(3,4-dihydroxyphenyl)-5-(β-D-xylopyranosyloxy)-heptan-3-one) is the first discovered and reported naturally occurring diarylheptanoid glycoside. It exhibits high biological activity, but it is also the compound of interest because of its ability to form reddish-orange and reddish-brown colours. In this research, European black alder (Alnus glutinosa (L.) Gaertn.) bark was separately subjected to lyophilisation and oven-drying before oregonin extraction with two different solvents. According to the results, methanol has proven to be more suitable as solvent compared to deionised water for oregonin extraction by maceration from black alder bark matrix regardless of the dehydration method. Accordingly, in the case of methanol, much higher yields of oregonin were obtained after lyophilisation, than after oven-drying. Furthermore, extraction by deionised water produced slightly higher yield of oregonin after oven-drying than after lyophilisation, as opposed to methanol. However, in much lower oregonin concentrations. Among other things, oregonin propensity to degradation at elevated temperature could probably be applied for improving and facilitating alder wood hydrothermal processing by minimizing uneven discolouration.*

Keywords: *European black alder, bark, oregonin, hydrothermal treatment, lyophilisation (freeze-drying), oven-drying, extraction yield, methanol, deionised water*

SAŽETAK • *Oregonin ((5S)-1,7-bis(3,4-dihidroksifenil)-5-(β-D-ksilopiranosiloksi)-heptan-3-on) prvi je otkriveni i objavljeni prirodni diarilheptanoid glikozid. Pokazuje visoku biološku aktivnost, a važan je i zbog svoje sposobnosti da stvara crvenonarančaste i crvenosmeđe boje. U ovom je istraživanju kora europske crne johe (Alnus*

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glutinosa (L.) Gaertn.) zasebno podvrgnuta liofilizaciji i sušenju u sušioniku prije ekstrakcije oregonina dvama različitim otapalima. Prema dobivenim rezultatima, pokazalo se da je za ekstrakciju oregonina maceracijom iz matrice kore crne johe prikladnije otapalo metanol nego deionizirana voda, bez obzira na primijenjenu dehidracijsku metodu. U skladu s navedenim, upotrebom metanola ostvareni su mnogo viši prinosi oregonina nakon liofilizacije nego nakon sušenja u sušioniku. Za razliku od metanola, pri ekstrakciji deioniziranom vodom prinosi oregonina nešto su veći nakon sušenja u sušioniku nego poslije liofilizacije, ali su njegove koncentracije znatno niže. Među ostalim, sklonost oregonina degradaciji pri povišenoj temperaturi vjerojatno bi se mogla iskoristiti za poboljšanje i olakšanje hidrotermičke obrade crne johe minimalizacijom neravnomjerne promjene boje.

Ključne riječi: *europska crna joha, kora, oregonin, hidrotermička obrada, liofilizacija, sušenje u sušioniku, prinos ekstrakcije, metanol, deionizirana voda*

1 INTRODUCTION
1. UVOD

Oregonin ((5*S*)-1,7-bis(3,4-dihydroxyphenyl)-5-(*β*-D-xylopyranosyloxy)-heptan-3-one) is the first discovered and reported naturally occurring diarylheptanoid glycoside (Karchesy *et al.*, 1974), and afterwards its "S" absolute configuration was determined combining ^{13}C NMR spectroscopy and the X-ray crystallography (Suga *et al.*, 1982). Considering its structure, oregonin belongs to a class of linear 1,7-diarylheptanoids, natural phenolic compounds, which constitute a separate small group of plant metabolites characterized by two aromatic rings connected with seven carbon chain (C_6–C_7–C_6).

In the previous papers of other researchers, oregonin has been isolated and reported from the bark of several alder species (*Alnus* spp.) (Table 1). Its biological activity was reported together with its anti-oxidant, anti-inflammatory, anti-microbial, anti-atopic dermatitis, and anti-cancer properties (Kuo *et al.*, 2008; Choi *et al.*, 2010; Tung *et al.*, 2010; Sati *et al.*, 2011; Telysheva *et al.*, 2011). Therefore, black alder bark could be used as a resource of bioactive compounds, and not only as fuel in wood processing companies as currently used. Besides exhibiting high biological activity, oregonin is also the compound of interest because of its ability to form reddish-orange and reddish-brown colours, which particularly affects alder wood hydrothermal processing.

Among other factors, heat can have negative influence on bioactive compounds during their acquisition and chemical analysis. On the other hand, however, heat could be used for inactivation of chemical compounds (if prone to thermal degradation) that impede hydrothermal wood processing. As for the lyophilisation process, there is an unwarranted and unexamined assumption that it properly and optimally preserves the plant constituents, but these assumptions may be erroneous in some cases (Abascal *et al.*, 2005). In this research, the influence of heat and dehydration procedure on oregonin extraction yield from black alder bark was investigated and reported. Bark was used instead of wood because it contains larger quantities of oregonin than wood (Klarić, 2015). The stated research results will help understanding the influence of lyophilisation on oregonin extraction yield as compared to oven-drying. Furthermore, methanol and deionised water were compared as solvents for the oregonin extraction.

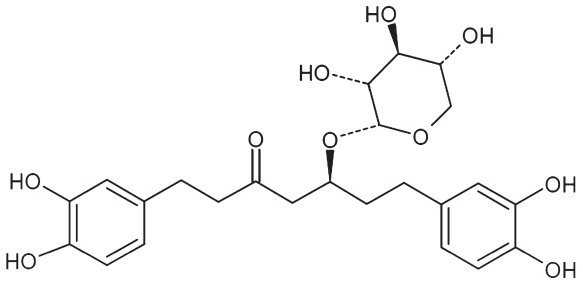

Figure 1 Chemical structure of Oregonin
Slika 1. Kemijska struktura oregonina

Table 1 Alder species (*Alnus* spp.) in which oregonin presence in bark was reported
Tablica 1. Vrste johe (*Alnus* spp.) u kojima je utvrđeno postojanje oregonina u kori

Species / *Vrsta*	**Source** / *Izvor*
Alnus glutinosa (L.) Gaertn.	Guz *et al.*, 2002; Roze *et al.*, 2011; Novaković *et al.*, 2013
Alnus incana (L.) Moench	Guz *et al.*, 2002; Roze *et al.*, 2011; Telysheva *et al.*, 2011
Alnus rubra Bong.	Karchesy *et al.*, 1974
Alnus viridis (Chaix) D.C.	Guz *et al.*, 2002
Alnus cordata (Loisel.) Duby	Guz *et al.*, 2002
Alnus hirsuita var. *sibirica*	Lee *et al.*, 2000
Alnus japonica (Thunb.) Steud.	Baek *et al.*, 2011
Alnus pendula Matsum.	Choi, 2013
Alnus tinctoria Sarg.	Ko *et al.*, 2015

2 MATERIAL AND METHODS
2. MATERIJAL I METODE

2.1 Chemicals
2.1. Kemikalije

High purity (≥95 %) analytical standard of oregonin was obtained from Sigma-Aldrich (Germany). For the extraction process, methanol (MeOH) of HPLC grade was supplied by J. T. Baker (USA), while deionized water (dH_2O) (ASTM Type II) was prepared on TKA/Thermo Scientific MicroMed Pure system (Thermo Fisher Scientific, USA). Formic acid of analytical grade (Orka Lab, Croatia) and deionised water (ASTM Type I, (MiliQ)) prepared on Millipore Simplicity Purification system (Millipore Corporation, USA) were used for mobile phase in high performance liquid chromatography (HPLC) analysis.

2.2 Bark acquisition and preparation
2.2. Dobivanje i priprema kore

Bark (inner & outer bark included) was collected from five-centimetre thick cross-section segment (disk) sawn at the height of 1.5 meters (from the ground) from black alder tree (30 cm breast height diameter). The tree was sampled during July 2014 in thirty-year old forest department "98" section "b" within the management unit "Đurđevačke nizinske šume" owned and governed by the Croatian state forest enterprise Hrvatske šume Ltd. The bark sample was milled and homogenized according to the previously described procedure (Klarić *et al.*, 2016). A certain amount of frozen homogenized raw bark was oven-dried at 103 ± 2 °C till constant mass has been reached. Another amount of frozen homogenized raw bark was lyophilised (Christ alpha 1-2 LD, Germany) by the main drying phase (-55 °C, 0.021 bar, 24 hours) and final drying phase (-50 °C, 0.040 bar, 4 hours).

2.3 Extraction procedure
2.3. Ekstrakcija

Extraction by maceration was conducted on magnetic stirrer (IKA C-MAG HS 7, Germany) for 24 hours (mot 1.5) at 20 ± 1 °C. Two grams of oven-dried and lyophilized bark were separately extracted in 250 mL of MeOH and dH_2O, in triplicates with regard to solvent type and dehydration procedure. The obtained extracts were filtered through grade 388 quantitative ashless filter paper (Munktell, Sweden), and stored in amber glass jars and kept in refrigerator till further analysis. Before HPLC analysis, extracts were additionally filtered through syringe nylon filters (0.22 μm) in amber glass vials with rubber/FEP sept. The amount of extracted oregonin from bark was determined by HPLC-DAD method.

2.4 Liquid chromatography
2.4. Tekućinska kromatografija

The liquid chromatography analysis was conducted using a Varian ProStar 500 (USA) HPLC system consisting of a ProStar 330 diode array detector (DAD), ProStar 410 autosampler, ProStar 230 tertiary pump system and column compartment. Instrument control, data acquisition and evaluation were done with Star Chromatography Work station v5.5 (Varian ProStar 360). Separation was performed on Nucleosil-C18 column 150 × 4.6 mm, particle size 5 μm (Supelco Analytical, USA). The analysis was performed using 0.1 % formic acid in MeOH as eluent A and 0.1 % formic acid in MiliQ water as eluent B in gradient elution mode. The elution started with 90 % of eluent B for 25 min, following 25 min gradient to 0 % of B and then back to initial conditions within 5 min. Flow rate was 0.5 $mL \cdot min^{-1}$ and injection volume was 10 μL. The separation was monitored at absorbance wavelength of 280 nm. Stock standard solution of oregonin was prepared by dissolving accurate quantity of the standard in MeOH and stored in the dark at 4 °C. The working standard solutions of different concentrations were prepared by appropriate dilution of the stock solution. Calibration curve for oregonin was prepared using six working standard solutions in the range 5-100 $mg \cdot L^{-1}$ (5, 20, 40, 60, 80 and 100 $mg \cdot L^{-1}$). The calibration curve was plotted from chromatograms as peak area *vs*. concentration of the standard. For each extract, three separate vials were prepared.

2.5 Statistical analysis
2.5. Statistička analiza

Along with descriptive statistics, Welch's ANOVA compared means test was conducted at the 0.05 significance level. STATISTICA 12 Dell Inc., and MS Excel software were used.

3 RESULTS AND DISCUSSION
3. REZULTATI I RASPRAVA

In this research, the effect of lyophilisation and oven-drying on maceration extraction yield of oregonin with MeOH and dH_2O was investigated. The amount of extracted oregonin was determined by HPLC-DAD analysis. To confirm the presence of the target compound in bark samples, retention time of peak obtained by analysing extracts of bark were compared with retention time of standard compound. Further, confirmation of peak identity was provided by comparison of UV spectra obtained by DAD. Strong agreement of retention time (t_R = 16.50 min) and UV spectra (λ_{max} = 280 nm) from oregonin standard and peak from bark extracts were obtained, thus confirming the identity of target analyte. The chromatogram of oregonin is shown in Figure 2. The quantification of oregonin was carried out on the basis of the calibration curve ($y = 5.8621 \cdot x - 2.322$). Coefficient of determination (R^2) was 0.9993 confirming high degree of correlation and good linearity of the method. Acceptable RSD values were obtained, 0.4 % for repeatability and

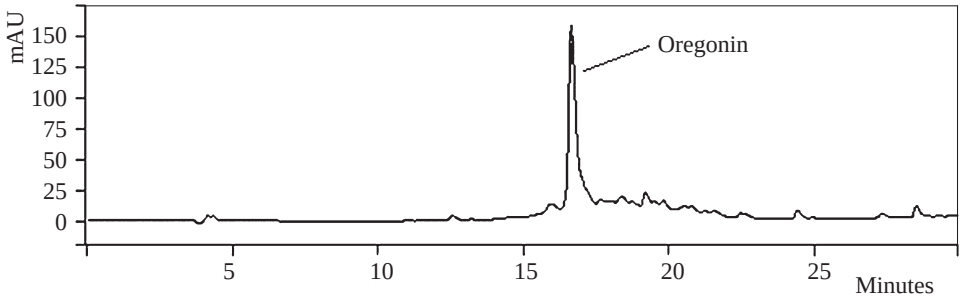

Figure 2 Chromatogram of oregonin obtained with MeOH after lyophilisation
Slika 2. Kromatogram oregonina dobiven uz pomoć MeOH nakon liofilizacije

Table 2 Average content of oregonin, descriptive statistic, $mg \cdot g_{dm}^{-1}$
Tablica 2. Prosječni sadržaj oregonina, deskriptivna statistika, $mg \cdot g_{dm}^{-1}$

S	*D*	*E*	*N*	*Mean*	*SD*	*95 CI*	*Med*	*IQR*	*MIN*	*MAX*
MeOH	Lyo.	I	3	60.63	0.20	60.144 – 61.121	60.53	0.35	60.51	60.86
		II	3	58.21	0.34	57.380 – 59.047	58.03	0.59	58.01	58.60
		III	3	60.25	0.58	58.818 – 61.688	60.42	1.12	59.61	60.73
	O.D.	I	3	7.71	0.09	7.489 – 7.929	7.69	0.18	7.63	7.80
		II	3	7.72	0.10	7.479 – 7.954	7.67	0.18	7.65	7.83
		III	3	7.53	0.17	7.122 – 7.946	7.61	0.31	7.34	7.65
dH_2O	Lyo.	I	3	1.26	0.04	1.163 – 1.364	1.26	0.08	1.22	1.30
		II	3	1.25	0.08	1.058 – 1.434	1.28	0.14	1.16	1.30
		III	3	1.21	0.04	1.110 – 1.300	1.22	0.07	1.16	1.23
	O.D.	I	3	4.12	0.04	4.012 – 4.230	4.12	0.09	4.08	4.17
		II	3	4.22	0.06	4.079 – 4.353	4.21	0.11	4.17	4.27
		III	3	4.10	0.04	3.991 – 4.208	4.10	0.09	4.06	4.14

Note: *S* – type of solvent / *vrsta otapala*; *D* – type of dehydration / *vrsta dehidracije*; *Lyo.* – lyophilisation / *liofilizacija*; *O.D.* – oven-drying at 103 ± 2 °C / *sušenje u sušioniku pri 103 ± 2 °C*; *E* – extraction process / *proces ekstrakcije*; *N* – number of measurements / *broj mjerenja*; *Mean* – arithmetic mean / *aritmetička sredina; SD* – standard deviation / *standardna devijacija*; *95 CI* – 95 % confidence interval of the mean / *95 %-tni interval pouzdanosti*; *Med* – median / *medijan*; *IQR* – interquartile range / *interkvartilni raspon*; *MIN* – minimum value / *minimalna vrijednost*; *MAX* – maximum value / *maksimalna vrijednost*

1.4 % for reproducibility. Extracts of oregonin from bark were analysed and expressed as mg of oregonin per g of dry bark mass. The descriptive results of the research are shown in Table 2.

As it is evident from the presented results (Table 2), statistically significant difference for oregonin yields was established between dehydration methods for MeOH ($F(1, 8.220 = 17297.271$, $p < 0.001$), and for dH_2O ($F(1, 15.191) = 10136.376$, $p < 0.001$). A statistically significant difference was also observed in oregonin yields between MeOH and dH_2O after lyophilisation ($F(1, 8.033) = 22078.953$, $p < 0.001$), and after oven-drying ($F(1, 11.646) = 4657.159$, $p < 0.001$), as determined by Welch's ANOVA. With methanol, much higher yields of oregonin from black alder bark were obtained after lyophilisation than after oven-drying at 103 ± 2 °C. However, in case of dH_2O, this trend is reversed. This reversed trend in the case of dH_2O was unexpected, but regardless of dehydration method applied, much higher oregonin yield was obtained with MeOH than with dH_2O as a solvent. During the dehydration process, changes that take place within wood are dependent on time-temperature-moisture-oxygen interrelationship (Navi and Sandberg, 2012). Consequently, in specific cases, the degradation of compounds present in wood and/or formation of newly formed compounds is possible, due to a wide range of chemical reactions, such as oxidation, hydrolysis, deacetylation, depolymerisation, *etc.* (Hon and Minemura, 2001; Fengel and Wegener, 2003; Hill, 2006; Navi and Sandberg, 2012; Németh *et al.*, 2013). Although it is generally considered that the lyophilisation is a preferable dehydration method, which retains higher levels of phenols *i.e.* bioactive compounds in the sample, it may not always be the case (Abascal *et al.*, 2005; Dai and Mumper, 2010; Jiang *et al.*, 2016). As regards VOCs (volatile organic compounds), they are probably lost to a greater extent during lyophilisation, as opposed to other dehydration methods at lower temperatures during which no vacuum is applied, or if fresh plant material is used (Abascal *et al.*, 2005). In addition to low temperatures, another important advantage of lyophilisation during the acquisition of natural bioactive compounds is that the sample oxidation is prevented during dehydration process due to the lack of oxygen, considering bark and wood great internal structural voids and hygroscopicity of structural components of the matrix. On the other hand, if an elevated temperature near 100 °C is applied, catechol oxidases will most likely be inactivated, *i.e.* subjected to denaturation causing the loss of activity. This inactivation of the enzymes can then facilitate the successful implementation of wood hydrothermal processing. As regards methanol, it is often a solvent of choice for ex-

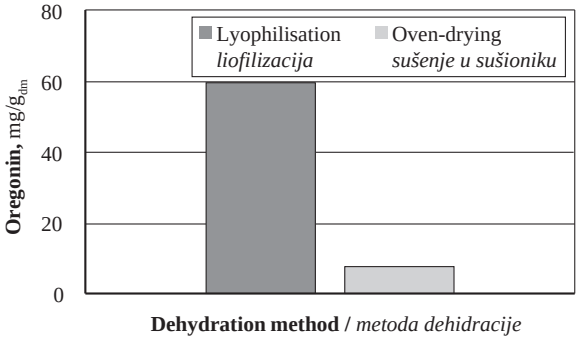

Figure 3 Oregonin average content – MeOH
Slika 3. Prosječni sadržaj oregonina – MeOH

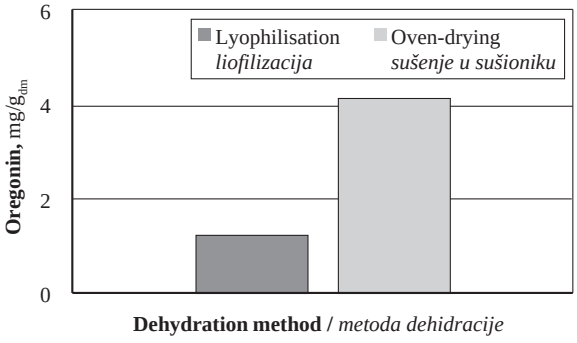

Figure 4 Oregonin average content – dH_2O
Slika 4. Prosječni sadržaj oregonina – dH_2O

traction of phenols from plant material. Comparing several organic solvents and deionised water, Klarić *et al.* (2016) established that MeOH produce highest yields of total soluble extractives (TSEs), phenols (TSPs) and flavonoids (TSFs) from black alder bark and wood. Presumably, additional improvements of oregonin extraction yields with MeOH could most likely be achieved by mixing MeOH with smaller portions of dH_2O (Fang *et al.*, 2013). Also, if slightly higher extraction temperature was employed, the oregonin yield with dH_2O would probably be somewhat higher, due to surface tension and viscosity reduction, thus facilitating dH_2O penetration into the bark matrix (Dai and Mumper, 2010; Fang *et al.*, 2013). Additional advantage of MeOH, compared to dH_2O, is that enzymes (in this case catechol oxidase) present in the plant will be deactivated (Jones and Kinghorn, 2006), and that fungi development will be prevented. Figure 3 and Figure 4 show the combined MeOH and dH_2O oregonin average yields after lyophilisation and oven-drying.

Further research regarding oregonin will be focused on its presence, concentration and influence on black alder wood discolouration during hydrothermal processing.

4 CONCLUSION
4. ZAKLJUČAK

It is more preferable to use MeOH as a solvent for oregonin extraction by maceration from black alder bark than dH_2O, regardless of the applied dehydration method. According to the obtained results, the highest yields of oregonin were achieved by conducting extraction with MeOH after lyophilisation, while significantly lower yields were achieved after oven-drying at 103 ± 2 °C. On the basis of the above mentioned findings, it could be concluded that, among others, oregonin is presumably prone to degradation at elevated temperature (103 ± 2 °C). This finding could probably be implemented in hydrothermal wood processing to minimize the uneven discolouration of wood by conducting pre-steaming or similar high temperature procedures immediately after sawing and before kiln drying. If oregonin is the compound of interest because of its high biological activity, lyophilisation should be a preferred method of dehydration, accompanied by a suitable extraction solvent.

In the case of dH_2O, yields of oregonin obtained after lyophilisation and oven-drying were reversed compared to MeOH as can be seen in Figure 3 and Figure 4. This reversed trend in the case of dH_2O is an interesting and unexpected phenomenon that should be further researched.

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