Apparatus and Selective Solvents for Extraction of Triterpenes from Silver Birch (*Betula pendula* Roth.) Outer Bark

AIGARS PAŽE, JĀNIS ZANDERSONS, JĀNIS RIŽIKOVS, GALINA DOBELE, VILHELMĪNE JURKJĀNE, BAIBA SPINCE AND AUSMA TARDENAKA

Department of Technological Research, Latvian State Institute of Wood Chemistry,
27 Dzerbenes Str., LV-1006, Riga, Latvia
**Corresponding author: Aigars Pazhe, tel: +371 6 755155; Fax: +371 6 7550635;*
e-mail address: aigars.paze@gmail.com

Paže, A., Zandersons, J., Rižikovs, J., Dobele, G., Jurkjāne, V., Spince, B. and Tardenaka, A. 2014. Apparatus and Selective Solvents for Extraction of Triterpenes from Silver Birch (*Betula pendula* Roth.) Outer Bark. *Baltic Forestry* 20(1): 88–97.

Abstract

A method and apparatus for obtaining triterpene extracts and betulin from pelletized birch bark (fraction 0.4-2.0 mm), extracting with petroleum ether (PE140) (boiling temperature range 100-140ºC) at a boiling temperature was developed. In the extraction unit, the process was carried out in a flowing through type reactor in the flow of distilled solvent in the intensive mass exchange regime. The change in the solubility of betulin and other triterpenes of outer birch bark (OBB) in PE140 at varying temperature was used to realise the process. During the process, betulin in the purified crystal form precipitates in the intermediate crystalliser making up 40–50% of the total amount of extractives, with the total betulin, lupeol concentration of 90–93 wt.% o.d. crystals. Decreasing the temperature of the solution remaining in the unit's evaporator, betulin – lupeol crystals with slightly decreased content of betulin, but increased content of lupeol settle. The intensive mass exchange extraction unit can be used also for OBB extraction with polar solvents (alcohols, acetone). The extraction time of pelletized (0.4–2.0 mm) OBB in polar solvents did not exceed 3–5 h. However, the action of the intermediate crystalliser was less efficient, therefore required cooling jacket temperature should be lower than 10ºC. The mother liquor, remaining after isolation of triterpenes from the extract, was collected in the evaporator. It contained about 38-40% of lupeol (wt.% o.d. mother liquor extractives) and can be used for obtaining of pure lupeol.

Key words: outer birch bark, triterpenes, betulin, lupeol, petroleum ether, extraction

Introduction

Birch is one of the most widespread deciduous tree species in Latvia, which, in terms of growing stock in forests, occupies the second place after pine (28.2% from total), with an annual stand growth of 6.1 m^3/ha (Saliņš 2002), which points to this species' importance in the national economy. Plywood production, whose volume is characterised by 220,000 m^3 of plywood exported in 2012, is one of the main fields of birch wood use (http://www.finieris.lv/). For producing 1 m^3 of plywood, on the average 2.75 solid m^3 of birch veneer logs are consumed. Alongside plywood, 0.3 m^3 of wood by-products is obtained from this amount of wood, but the rest of the wood remains in remainders as veneer shorts, cut-off ends, plywood cuttings, cores and bark. On the average, veneer log bark makes up 12.5% of their mass, but outer birch bark (OBB) 2.0 to 3.4% of the veneer log's mass. The remaining part of wood is used for preparing particle-boards, for chemical pulp production or as a fuel. In its turn, bark, jointly with OBB, is used only as a fuel in boiler houses.

Such a way of using OBB is not rational. This applies especially to the birch species growing in Latvia and North Europe, namely, common silver birch (*Betula pendula* Roth.) and downy birch (*Betula pubescens* Ehrh.), the outer bark of which has a very high content of biologically active lupane type pentacyclic triterpenes. The main representative of lupane type triterpenes is betulin (betulinol; lup-20(29)-en-3β,28-diol), the content of which is 16.7-34.0% of the mentioned birch species' OBB from oven dry mass. Total amount of lupane type triterpenes in the OBB of those species is 21-40% of the OBB mass (Ekman 1983). The chemical formulas of the main representatives of OBB lupane type triterpenes are shown in Figure 1.

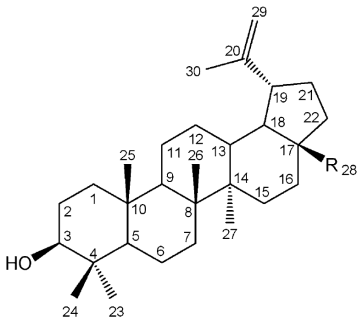

Figure 1. Chemical structure of lupane type pentacyclic triterpenes

R= CH_3	Lupeol
R = CH_2OH	Betulin
R = COOH	Betulinic acid

In recent decades, the synthesis and application of betulin and betulin derivatives in medicine has been investigated especially intensively. For example, betulinic acid acts against melanoma. Betulinic acid selectively kills human melanoma cells while leaving healthy cells intact and is found to delay the progress of the HIV 1 infection which eventually leads to AIDS, by preventing the formation of syncytia (Alakurtti et al. 2006, Kessler et al. 2007). The antiviral activity of betulin and betulinic acid has been demonstrated against the herpes simplex virus (Pavlova et al. 2003).

Lately, promising therapeutic properties of betulin have been revealed against obesity in *in vivo* experiments with mice (Tang et al. 2011). Despite the promising pharmacological properties, low toxicity and high concentration in natural raw materials, neither betulin nor lupeol, nor their derivatives have yet found wider application in clinic or cosmetic practice. One of the reasons is that lupane type triterpenes – betulin, lupeol and their derivatives are very hardly soluble or insoluble in water and in the biological medium in the body. Recently, the solubility of betulin and betulinic acids, and their biopharmaceutical properties have been improved by synthesising inclusion complexes with β-cyclodextrine (Soica et al. 2010), synthesising betulinic acid glucuronides as anticancer prodrugs for application in monotherapy (Gauthier et al. 2009) or also preparing mechanoactivated complexes, for example, from betulin diacetate and arabinogalactan (Кузнецова et al. 2013). Unfortunately, triterpenes extraction with non-polar and polar solvents, and obtaining of individual compounds, namely, betulin and lupeol are rather laborious and expensive.

The anatomical structure of OBB cells, their walls' chemical composition and the limited solubility of the triterpenes themselves in organic solvents complicate their extraction from the OBB material. The extractives obtained from OBB, alongside triterpenes, contain also polyphenols, tannins, carbohydrates and other undesirable admixtures.

Choosing the solvent for preparing pharmaceuticals, the standards on the solvent residues allowed in pharmaceuticals, accepted in the European Union, which are limited by the EU legislation (European Medicines Agency 2009), should be followed. In this respect, hydrocarbon solvents (extraction benzenes, petroleum ethers) and lower alcohols (ethanol, isopropanol) are advantageous, because their use has no restrictions that are defined for their residues in medicinal preparations. Therefore, in this study, to determine optimal methods and solvents for obtaining betulin and also triterpenes, the chosen solvents were alcohols and hydrocarbons. Triterpenes' solubility in alcohols at their boiling temperature is rather good, but is equally good also for tannins, polyphenols and carbohydrates already at room temperature.

The solvents often used for triterpenes extraction are alcohols and hydrocarbons. The solubility of betulin therein differs dramatically. At 5ºC and 25ºC, in 95% ethanol, 4.52 and 6.99 g/L, respectively, are dissolved, while in isopropanol – 6.57 and 8.49 g/L, but, for example, in cyclohexane – 0.07 and 0.25 g/L, respectively (Cao et al. 2007). This manifests itself well after the 11 h extraction in a Soxhlet apparatus, when the yield of extractives in our experiment with 95% ethanol was 35.5%, with acetone – 33-39%, with chloroform – 33%, with cyclohexane – 23-25%, and with n-heptane only 3.8-4.1% from oven dry OBB mass (wt% o.d. OBB). However, the summary content of betulin and lupeol in the cyclohexane extract was 92.7%, in the n-heptane extract – 91.6%, but in the 95% ethanol extract only 70-85% from the extract mass (Pazhe et al. 2012, a, b).

In the case of OBB, the solvent's absorption and extraction are hampered by its lamellar structure, which is formed by compressed lentil-type cells. Those are located in concentric layers. The spring cells have thin walls and in the radial direction the cells lumena measure are thicker. White betulin grains are located in these lumina. Further, in the stem periphery direction, 3-5 rows of strongly flattened cells with thick walls (autumn cells) are located, whose lumina are filled with polyphenols and tannins. The cell walls' main components are C_{16}-C_{22} polysaturated oxy fatty acids polyesters – called suberin, which is insoluble in any solvents (Комаров 1941). Therefore, the solvents' penetration in OBB and cell lumina is burdensome. To reach faster dissolving of extractives, mainly triterpenes, the OBB cell structure should be physically destructed. After shredding, milling and pelletising, the bulk density of OBB increases, and mass exchange is better, because the diffusion is less hampered. For at least partial disintegration of the material's solid matrix, namely, the cell system, crushing and grinding are

used, but the bulk mass is further increased by pelletising (Krasutsky et al. 2007).

To intensify extraction, it is recommended to use the effect of the ultrasound and the high intensity pulsing electric field. Ultrasound destructs the cell walls and favours the mass transfer of their content (Schinor et al. 2004). In the extractor, the zone of the ultrasound action is located only in the immediate vicinity to the emitter. Therefore, the solid phase concentration should be decreased to the solid-solution ratio 1:42.

By intensifying the extraction process, the temperature can be increased above the boiling temperature, for example, up to 140-160°C (Scheffler 2007). It is more convenient to use the good solubility of betulin and other triterpenes, for example, in white spirit (boiling temperature range 150-185°C) at its boiling temperature, but very poor solubility at room and lower temperatures. It is recommended to wash the admixtures, the content of which is not high, from the hot extract with a small volume of the concentrated solution of alkali in water (Кислицын 1998). Unfortunately, the aromatic components of white spirit contaminate the end-product with hard-to-separate polymerisates.

A method for using liquefied carbon dioxide for OBB extraction has become popular for the research purposes. However, also using this method, polar admixtures such as phenols, polyphenols, tannins and sugars, alongside triterpene components, get into the extractives (Krasutsky 1999).

The objective of the study was to elaborate apparatus and a method, as well as to choose suitable solvents to intensify and shorten the triterpene extraction process. Also, in present study during the extraction process an attempt was undertaken to obtain the main component of the OBB triterpenes – betulin, in sufficiently high yield and purity in order for it to be further used for the synthesis of it derivatives. The process should be intensified so that to reach more intensive extraction of betulin from OBB; the process should be carried out at as high as possible temperature. As the solubility is rather limited also at elevated temperature, the maximally high betulin concentration gradient between its concentrations in the solid phase and the liquid phase should be maintained. This can be reached, continuously removing from the extraction chamber the extract that reached a known saturation degree and to substitute it with a fresh solvent. From the heat economy viewpoint, it is expedient to introduce the fresh solvent in the extraction chamber in the form of regenerated solvent condensate with a temperature that is close to the solvent's condensation temperature and corresponds to the temperature in the extraction chamber.

The inclusion of an intermediate crystalliser in the technological flow already during the extraction process makes it possible to obtain crystalline betulin with a content of the basestock of 90–95%. Decreasing the extract concentration prior to getting in the evaporator, the amount of energy needed for the solvent's evaporation decreases. The mother liquor that gets from the intermediate crystalliser to the evaporator, regenerating the solvent, is evaporated and the concentration of lupeol in the remaining triterpene mixture gradually increases; this, after the separation of betulin and other triterpenes' crystals from the evaporator solution in its mother liquor, can be used for obtaining crystalline lupeol.

The laboratory equipment corresponding to those principles is produced, completed and patented (Zandersons et al. 2013), and used in the experimental work.

Materials and Methods

Apparatus

The apparatus for realising the extraction of triterpenes consists of a flowing through type extractor, an evaporator, a heater and a condenser for feeding the recovered solvent in the extractor; in addition, it contains a crystalliser that is connected to the extractor and an additional heater, adjusted between the crystalliser and the evaporator.

The equipment for processing of OBB is shown in the flow diagram (Figure 2).

It includes a through flow type extractor (1) (geometrical volume 3.5 L), connected to an intermediate crystalliser (2) (geometrical volume 0.65 L), with a cooling jacket. The intermediate crystalliser is connected to an evaporator (4) (geometrical volume 3.5 L) with the help of tube with a steam jacket (3) for drainage and heating up of crystalliser and mother liquor. The evaporator (4) is connected to a condenser (6) with the help of a solvent vapour exhaust pipe with a heating jacket (5).

In the extractor's (1) removable lid, a propeller type mixer (7) with an electrical drive (8) is installed, but inside the extractor (1), a wire net basket (9), covered with a filter material, is installed, in which the ground OBB is poured. The extractor (1) has a steam jacket. The extractor (1) is connected with the atmosphere through a reflux cooler (10).

The desired size of milled OBB pellets is 0.4-2.0 mm, which ensures an active mass exchange in the solvent-solid system. Because a known degradation of the solid phase occurs during the process, the solution, which leaves the extractor, is continuously filtered through a filter material, with which the steel wire

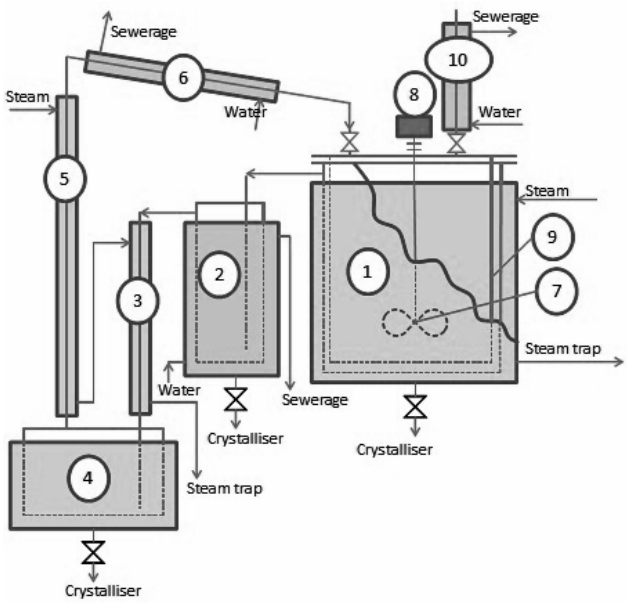

Figure 2. Intense mass exchange extraction unit. 1 – extractor with heating jacket; 2 – intermediate crystalliser with cooling jacket; 3 – mother liquor heater; 4 – evaporator; 5 – vapour exhaust pipe with heating jacket; 6 – descent condenser; 7 – mixer; 8 – electromotor of the mixer; 9 – wire net basket; 10 – reflux cooler

net sieve basket (9) is covered, for placing and transport of the material within the laboratory.

Raw material and solvents

The birch bark from a plywood factory was dried at room temperature up to moisture 10-15 wt.% and the dry bark was ground in a cutting mill "Retsch GmbH SM100". The OBB from bast, with the content of 65% on the average, was separated by the flotation method, mixing the shredded and milled bark in water at a weight ratio of 1:10 for 24 h and collecting the floating OBB, drying at room temperature and at 50ºC in a drying cabinet till a moisture of 10%. The bast admixture in OBB was determined from the combustion heat of oven dry ground OBB, using a calibration curve, or evaluated from the content of easily hydrolysable substances in the OBB.

The dried OBB was pelletised on a laboratory-scale "Amandus KAHL" flat die type pelletiser with holes of diameter 6 mm. OBB pellets was ground in a "Retsch GmbH SM100" mill and sieved to prepare the particles with a size of 0.4-2.0 mm and a volume mass of 363 g/L for extraction.

A list of the solvents used for the extraction of OBB is given in Table 1. All the solvents were used without any prior treatment.

For determination of phenolic compounds content extra pure grade standard of tannin was used, which is purchased from "Sigma Aldrich" supplier.

Table 1. Solvents used for the OBB extraction

Solvents	Source	Description
Ethanol	JSC "Latvijas Balzāms", Latvia	>95%, b.p. 78.4ºC
Isopropanol	Acros Organics, Belgium	> 99%, b.p. 82.5ºC
Acetone	Acros Organics, Belgium	> 99.8%, b.p. 56.5ºC
Cyclohexane (CH)	Acros Organics, Belgium	> 99%, b.p. 80.7ºC
Petroleum ether (PE140)	Acros Organics, Belgium	Extra pure, boiling range 100-140ºC

Obtaining of betulin and lupeol enriched concentrate of extractives

In the stainless steel cylindrical extractor (1), a cylindrical stainless steel wire net basket (9) with a geometrical volume of 2.65 L was placed. For extraction 200 g (in terms of the oven dry mass) of crashed pelletised OBB was fed in net basket. The extractor was covered with a lid, in which a mixer (7) and its driver (8) were installed. Reflux (10) and discharge (6) coolers were connected. The extractor was filled till the overflow with 2.3 L of the solvent heated till boiling temperature. The intermediate crystalliser (2) was attached to the extractor (1) and filled with 500 mL of solvent. The cooling jacket of the intermediate crystallisers (2) was connected for cooling to water supply. The cooling water temperature was maintained at no higher than 10ºC. In the evaporator (4), 2 L of the solvent was filled, and with a heated vapour pipe (5) and a descent condenser (6), was connected to the extractor (1). The evaporation intensity was regulated so that the vapour supply rate would be approximately 2 L of the condensate per hour. Thereby, the extract was gradually replaced from the extractor, in which the turbulent regime and a temperature of 3 to 5ºC under boiling temperature were permanently maintained. The extract was continuously discharged from the extractor (1) to the intermediate crystalliser (2) through the extractor's removable steel wire sieve basket (9) and the filter material. In the intermediate crystalliser (2), with decreasing extract's temperature from that close to the solvents' boiling temperature to 15-20ºC, triterpene extractives in the white crystal form settled in 7 h.

At the end of process, cooling the solution collected in the evaporator (4) till room temperature, triterpene crystals with decreased betulin content and increased lupeol concentration were obtained. Evaporating the remaining mother liquor till a dry matter, a substance was isolated, which contained a small amount of betulin and a noteworthy quantity of lupeol. The solution remaining in the extractor (1) was discharged through a valve installed at the bottom of the extractor and, for emptying, with the help of a small air overpressure, about 90% of this solution, in which a small amount of triterpenes was present, was collected.

Taking of raw material samples in the course of the intensive extraction process

The samples containing outer bark, jointly with the boiling solvent from the intensive extraction reactor (1), using a specific pipette, were sucked out through a reflux cooler (10) using a vacuum pump. The hot filtered OBB samples were dried in a fume hood at room temperature on Petri dishes. Samples were taken each hour during the whole period.

Analyses of the amount of extractives, triterpenes and polyphenols in the course of the intensive extraction process

Air-dry outer bark samples, taken in the course of the intensive extraction process, were weighed, with determining moisture, and extracted in a Soxhlet apparatus for 11 h with 95% ethanol. Extractives of the obtained samples were evaporated on a rotation vacuum distillation unit "Laborota 4003", but the extracted OBB, jointly with thimble, was dried in a drying cabinet at a temperature of 105ºC till a constant mass. From the OBB mass loss, the percentage of the extractives remaining in the raw material was calculated. The content of triterpenes and phenolic compounds in the extractives' dry matter was determined.

Determination of the equilibrium concentration of OBB extractives at the PE140 boiling temperature

250 g of crashed (0.4-2.0 mm) OBB pellets was placed in the reactor (the flowing through type extractor) (1). The extractor (1) with superheated steam was heated till 125ºC. Separately, 2 L of PE140 was warmed up in a bath till 90ºC. The heated PE140, through the reflux cooler (10) connected to the reactor, was poured in the reactor and, intensively mixing, counting down of time was started. After 1 min, through the extractor's lower part, which was equipped with a ball-valve, about 25 mL of hot (~103ºC) OBB extractive solution was let run out, which was collected in a 50 mL pear-shaped flask with a ground stopper that had been preliminarily dried till a constant mass and weighed. Such operations were repeated also after 2, 3, 4, 5, 6, 7, 10 and 13 min. The retorts with the obtained extractive solutions were weighed on an analytical balance (accuracy of 0.1 mg) and the solvent was evaporated in a vacuum rotation distillation apparatus. The flasks with extractives were dried in a drying cabinet at 105ºC till a constant mass and, taking into account the PE140 density (d = 0.74 g/cm^3), and the concentrations of the sample solutions were calculated (g/L).

GC-MS analysis of triterpenes

The gas chromatography – mass spectrometry (GC-MS) analysis was performed using "Shimadzu GC/MS-QP 2010" apparatus. GC-MS analysis to determine the content of betulin and lupeol in dry matter of OBB extracts was performed on a 30 m × 0.25 μm × 0.25 μm film HP-5MS capillary column (GC-2010 Shimadzu) with high purity helium as a carrier gas at a constant flow rate of 1 mL/min throughout the run. The inlet was operated in a splitless mode at 300ºC. The injection volume was 1 μL. The oven temperature was maintained at 160ºC for 1 min and then programmed at 10ºC/min to 300ºC, which was maintained for 15 min. Analysis time was 40 min. The temperature of the mass-selective detectors was 300ºC. The components were identified by comparison of the spectra with those of the standards and those in the NIST library of spectra. Two parallel aliquots of each extract were analysed by triple injections. Average values, obtained from aliquots' injections and separate extracts' aliquots, for which the mutual variation is less than 5%.

Determination of phenolic compounds

3–5 mg of the dry extract sample was placed into a 50 mL volumetric flask and dissolved in methanol. The prepared solution was transferred in a 1 cm optical glass cell, and absorbance at a wavelength of 280 nm was measured in a Spectrophotometer "Lambda 25" Perkin Elmer using methanol as a control solution. The phenolic compounds content was calculated using the calibration curve of standard tannin. Coefficient of variations for the values of three parallel measurements did not exceed 1%.

Results

The process of obtaining triterpenes by extraction is complicated by several problems: first, small OBB bulk density and its cell wall specific structure and chemical composition; second, the low solubility of the triterpenes themselves in organic solvents; and third, the fact that those are present in cells jointly with better soluble substances such as fatty acids and their esters, waxes, phenols, polyphenols, tannins and sugars. The problems of anatomical structure and low bulk mass, at least partially, are prevented by the OBB shredding, pelletising and then repeatedly crushing and sieving. Pelletising and crushing, the cell walls consisting mainly of insoluble suberine are partially destructed, which facilitates the access of the solvent to the triterpenes and other outer bark components located in lumina. The pellets fraction with a size of 0.4-2 mm with the bulk density of 363 g/L is used for extraction.

Choosing the solvent, there is a dilemma between the good solubility and selectivity of triterpenes, when the choice of the solvent should be made, partially

sacrificing for the insolubility of the triterpene solubility by-products – fats, phenols, waxes and carbohydrates. All OBB extractive groups are equally well dissolved by polar solvents (alcohols, acetone), but hydrocarbons demonstrate a considerable selectivity (Кислицын 1994). The results of the tests of our solvents chosen according to (Береговцова et al. 2012, Левданский et al. 2012), carried out in a Soxhlet apparatus, are shown in Figure 3.

The intensity of extractives' solubility in ethanol considerably exceeds that in CH and PE140. Also the absolute yield of extractives obtained by use of ethanol already after 5-6 h are considerably higher (35% from o.d. OBB mass), which is explained by the good solubility in this solvent of not only triterpenes, but also fats, fatty acids, waxes, phenols and tannins. Similar results have been recently published in a special study (Левданский et al. 2012), in which, after 10 h extraction in a Soxhlet apparatus, from *Betula pendula* Roth. OBB, 7.0% and 28.5% of extractives from the o.d. OBB mass were obtained with petroleum ether and cyclohexane, respectively, but 41.0% with ethanol.

Figure 3 shows that the triterpenes extraction by a traditional way is a time and energy consuming operation, but some intensification parameters of this process, as shown in Introduction, are complicated. Therefore, we have developed an extraction unit, in which intensive mass exchange, maintaining the solid phase – solvent medium concentration gradient, high temperature and turbulence, as well as including the selective crystallisation of triterpenes in the process flow, is realised. The unit is shown in Figure 2 (Zandersons et al. 2013). In principle, the unit and method are suitable for polar and non-polar solvents. However, it is fully realised in the case of non-polar solvents, when intermediate crystalliser is connected for the mass exchange intensification and the rationalisation of the product' obtaining in the extraction process. The extraction process is realised at temperatures close to the solvents' boiling temperature. In Table 2 the results of our searching experiments are presented.

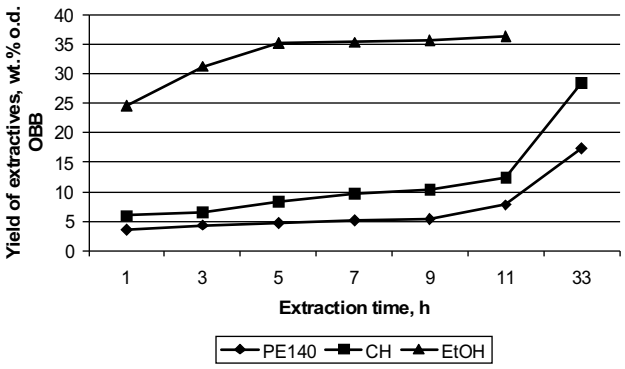

Figure 3. Comparative representation of OBB Soxhlet extraction with ethanol (EtOH), cyclohexane (CH), petroleum ether – boiling range 100-140ºC (PE140)

It can be seen that the extraction process is intensified and, in a short period of time, it is possible to extract a considerable amount of triterpenes. Working with cyclohexane, the basic mass of extractives is composed by triterpenes. This applies especially to crystals, which settle in the intermediate crystalliser. Already during the extraction, a part of triterpenes, mainly betulin and lupeol, can be isolated with a high concentration, which facilitates the release of the end product – betulin from admixtures. The decrease in the dry matter concentration in the solution flow, which, in the form of mother liquor, flows from the intermediate crystalliser to the evaporator, makes it possible to spare heat energy, regenerating the solvent.

Extraction is considerably intensified also in polar solvents, in the case of which, in our experiment's conditions, when the temperature of the intermediate crystalliser solution was no lower than 10ºC, lower amounts of betulin and lupeol crystals were formed or were not formed at all.

Taking into account the practical reasons using the intensive mass exchange extraction unit, in parallel with the Soxhlet apparatus, a method comparison

Table 2. Efficiency of different solvents used in an intensive mass exchange extraction unit equipped with an intermediate crystalliser

Solvents	Duration of extraction, h	Intermediate crystalliser			Evaporator			Total yield of extractives*
		Crystal yield*	Betulin (wt.% dry crystal basis)	Lupeol (wt.% dry crystal basis)	Solid extract yield*	Betulin (wt.% dry extractives basis)	Lupeol (wt.% dry extractives basis)	
Isopropanol	1.0	1.2	88.6	3.2	13.0	48.3	14.9	16.5
	1.5	3.5	82.0	3.4	24.0	78.2	8.6	25.2
Acetone	1.5	-	-	-	26.3	70.1	10.2	26.3
	5.0	-	-	-	30.0	85.1	10.7	30.0
Ethanol	1.5	-	-	-	27.3	71.9	5.1	27.3
	3.0	-	-	-	33.3	75.9	6.2	33.3
CH	1.5	1.6	75.0	7.0	6.7	79.2	6.8	8.3
	3.0	6.1	90.9	5.0	13.2	85.8	6.9	19.3
PE140	3.0	6.2	91.6	6.2	4.4	61.8	18.4	10.6
	5.0	8.7	94.6	3.1	8.4	63.5	15.1	17.1

*wt.% dry OBB basis

test of the intensity and extractives' quality was done working with CH and PE140. The results of the experiments are summarised in Table 3.

It can be seen that the extraction intensity, working with an intense mass exchange unit, in the case of both the solvents, grows two- to five-fold, depending on the extraction time. Certainly, this is due to the high temperature; but, however, mainly because turbulence provides an intense mass exchange on the OBB pellets' surface.

Table 3. Yield of extractives and leaching intensity at different OBB extraction methods

Solvent	Extraction time, h	Soxhlet apparatus			Intense mass exchange unit		
		Temp., ºC	Yield*	Betulin / lupeol**	Temp., ºC	Yield*	Betulin / lupeol**
CH	3	52.0	6.6	81.9	79.0	13.2	95.9
	7		9.7	99.5		25.3	96.2
	33		28.4	99.6		-	-
PE140	3	63.0	4.4	74.1	105.0	10.6	97.8
	7		5.2	94.6		26.6	95.5
	33		17.4	99.7		-	-

*extractives yield wt.% o.d. OBB, **wt.% o.d. crystals

Continuously removing the solution from the extractor to the intermediate crystalliser and replenishing with a fresh solvent, comparatively high triterpenes' concentration difference between the OBB pellets' surface and the solvent's mass is permanently maintained. This is well illustrated by the fast setting of the extractives' solubility equilibrium in the intense mass exchange reactor, as shown in Figure 4.

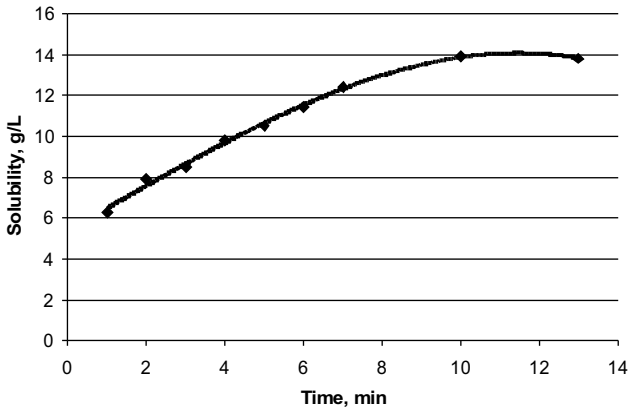

Figure 4. Pace of achieving the OBB extractives' concentration equilibrium in PE140 during extraction in an intense mass exchange unit

Already after 1 min, the extractives' concentration is 6.3 g/L, but the equilibrium concentration of about 14 g/L is reached after 10 min, which agrees well with the data of other authors (Eckerman and Ekman 1985).

The extractive's choice was in favour of PE140, because, as shown by the experimental tests (Table 3), this extractive ensured a high concentration of triterpenes in the extract. Data on the course of extraction and the changes in the extractives' composition in OBB are listed in Table 4.

Samples of OBB, partially extracted with PE140 in an intense mass exchange unit, were extracted with ethanol in a Soxhlet apparatus; it was found that 74% of the extractives mass present in OBB was extracted in 7 h. In the mass of the extractives, non-extracted from the OBB pellets during the OBB extraction, the

Table 4. Course of extraction and change of the triterpenes and phenolic compounds quantity in the leached out OBB during processing in an intense mass exchange unit

Duration of extraction, h	Remainder of extractives, wt.% o.d. OBB basis	Components of extractives' remainder		
		Betulin*	Lupeol*	Phenolic compounds*
0	30.5	75.8	6.9	9.6
1	23.6	50.5	4.6	19.5
2	15.8	38.1	2.2	20.4
3	13.5	22.5	1.0	23.9
4	12.1	21.1	0.8	24.2
5	10.2	11.5	0.6	25.0
6	10.0	11.3	Trace	28.1
7	9.3	8.8	Trace	29.8

*wt.% dry matter on the extractives basis

betulin and lupeol concentration gradually falls, but the amount of phenolic compounds (e.g. polyphenols, tannins) grows, which testifies that, from the selectivity viewpoint, for obtaining triterpenes, PE140 is a suitable solvent, because polar and also undesirable OBB mass components are not dissolved therein. To characterise the relative changes in the amount of phenolic compounds during the extraction, tannin was used as a standard of polyphenols (tannic acid, gallotannic acid). This compound is a weak base – its pK_a is approximately 10 due to many phenol OH-groups. Commercial preparation has a molecular mass 1701.2 g/mol^{-1}, which corresponds to formula $C_{76}H_{52}O_{46}$ – decagalloyl glucose; in fact it is a mixture of polygalloyl glucoses or esters of polygalloyl quinic acid. It can be seen that the phenolic compounds concentration in the extracted OBB grows 3-fold. Judging from

the changes in the lupeol and betulin amounts in OBB during the extraction process, it can be concluded that the solubility of lupeol in PE140 is better than that of betulin. Table 4 shows that the concentration of lupeol in the intense extraction unit within three hours decreases approximately seven-fold, while that of betulin only somewhat higher than three-fold.

The intense mass exchange extraction unit's verification output results are summarised in Table 5.

Table 5. Summary of the intense mass exchange extraction unit's verification output results (solvent: PE140; process duration 7 h; temperature 105ºC)

Trial	Yield of extractives**	Intermediate crystalliser		Evaporator		Extractor	
		Yield of crystals*	Joint betulin, lupeol content***	Yield*	Joint betulin, lupeol content***	Yield*	Joint betulin, lupeol content***
1	27.0	41.8	96.6	53.5	93.8	4.7	82.2
2	26.6	48.9	95.5	46.2	88.6	4.9	81.4
3	28.7	43.9	92.2	47.5	84.5	8.6	81.8
Average	27.4 ±0.9	44.9 ±3.0	94.8 ±1.9	49.1 ±0.9	89.0 ±3.8	6.1 ±1.8	81.8 ±0.3

*wt.% o.d. extractives, **wt.% o.d. OBB, *** wt.% o.d. crystals

It can be seen that 44.9 ± 3.0% of the isolated extractives' mass is collected in the intermediate crystalliser in the form of triterpene crystals. The content of betulin and lupeol in the crystals is 94.8 ± 1.9%. Almost all the rest of extractives are accumulated in the evaporator with a high content of betulin and lupeol. From the total extractives' dry matter mass, those make up 49.1 ± 0.9%. Cooling the evaporator's extractives solution to 15-20ºC, 85-86% of crystals from this solution dry matter is obtained. The crystals have 89.0 ± 3.8% of the betulin and lupeol sum. After the crystals' separtion, the remaining mother liquor dry matter contains 38-40% of lupeol. Although the mother liquor dry matter contains only 13-14% of the total dry matter mass collected in the evaporator, however, taking into account the high concentration of lupeol in this product, it would be advantageous to use them for obtaining crystalline lupeol, for example, by the column chromatography method (Abyshev et al. 2007).

After the seven hour extraction, high quality triterpene extracts are obtained, from which those obtained in the intermediate crystalliser are practically free from fat, wax or polyphenol admixtures. It is most advantageous to use the small amount of extractives, which, jointly with the solution, are removed from the extractor when finishing the process for the next operation as the solvent, because their concentration is only 0.18% from the solution mass.

Conclusions

1. The excellent pelletisation properties of OBB, after its chipping in a cutting mill and pelletisation, provide the feasibility to eliminate its low bulk density and improve the leachability by solvents.

2. Triterpenes' solubility in PE140 in an intense mass exchange extractor at its boiling temperature of 103ºC is 13.9 g/L and, after cooling to room temperature – 2.69 g/L. 81% of the extractives' mass crystallises (forms crystals of betulin and lupeol).

3. The high concentration of lupeol in the mother liquor, which remains after the isolation of triterpenes from the extract collected in the evaporator, indicates the possibility to use it for separating crystalline lupeol.

4. The extraction unit of intense mass exchange, equipped with an intermediate crystalliser, by use of PE140, reduces the process time 2-3-fold and, during the extraction process, produces purified betulin comprising 44.9 ±3.0% of the joint amount of extractives.

5. In an intermediate crystalliser, the yield of crystalline triterpenes with a joint betulin and lupeol concentration of 94.8 ± 1.9%, after 7 h of the process, is 12.3 ± 0.7 wt.% o.d. OBB.

Acknowledgements

The work was financed by the European Regional Development Fund, contract No. 2010/0289/2DP/2.1.1.1.0/10/APIA/VIAA/058.

IEGULDĪJUMS TAVĀ NĀKOTNĒ
EIROPAS SAVIENĪBA
ERAF
EIROPAS REĢIONĀLĀS ATTĪSTĪBAS FONDS

References

Abyshev, A. Z., Agaev, E. M. and Guseinov, A. B. 2007. Studies of the chemical composition of birch bark extracts (*Cortex betula*) from the Betulaceae family. *Pharmaceutical Chemistry Journal*, 41(8): 419-423.

Alakurtti, S., Mäkelä, T., Koskimies, S. and Yli-Kauhaluoma, J. 2006. Pharmacological properties of the ubiquitous natural product betulin. *European Journal of Pharmaceutical Sciences* 29(1): 1-13.

Cao, D., Zhao, G., and Yan, W. 2007. Solubilities of betulin in fourteen organic solvents at different temperatures. *Journal of Chemical & Engineering Data* 52(4): 1366-1368.

Eckerman, C. and Ekman, R. 1985. Comparison of solvents for extraction and crystallisation of betulinol from birch bark waste. *Paperi ja puu* 67(3): 100-106.

Ekman, R. 1983. The Suberin Monomers and Triterpenoids from the Outer Bark of Betula verrucosa Ehrh. *Holzforschung* 37(4): 205-211.

European Medicines Agency. 2009. ICH Topic Q3C (R4) Impurities: Guideline for Residual Solvents. CPMP/ICH 283/95

Gauthier, C., Legault, J., Rondeau, S. and Pichette, A. 2009. Synthesis of betulinic acid acyl glucuronide for application in anticancer prodrug monotherapy. *Tetrahedron Letters* 50(9): 988-991.

Kessler, J. H., Mullauer, F. B., de Roo, G. M. and Medema, J. P. 2007. Broad *in vitro* efficacy of plant-derived betulinic acid against cell lines derived from the most prevalent human cancer types. *Cancer Letters* 251(1): 132-145.

Krasutsky P. A. et al. 2007. Birch bark processing and the isolation of natural products from birch bark. *Patent* US 7264184 B2.

Krasutsky, A. 1999. Birch bark processing and the isolation of natural products from birch bark. *Patent* US 6634575 B2.

Pavlova, N. I., Savinova, O. V., Nikolaeva, S. N., Boreko, E. I. and Flekhter, O. B. 2003. Antiviral activity of betulin, betulinic and betulonic acids against some enveloped and non-enveloped viruses. *Fitoterapia* 74(5): 489-492.

Pazhe, A., Zandersons, J., Rizhikovs, J., Dobele, G., Spince, B., Jurkjane, V. and Tardenaka A. 2012a. Composition of Birch Bark Extracts Depending on the Solvent Type. *Abstracts of the Riga Technical University 53rd International Scientific Conference, section: Material Science and Applied Chemistry*: 92.

Pazhe, A., Zandersons, J., Rizhikovs, J., Dobele, G., Spince, B., Jurkjane, V. and Tardenaka, A. 2012b. Obtaining Pentacyclic Triterpenes from Outer Birch Bark. *Latvian Journal of Chemistry* 51(4): 415-420.

Plywood sales at 2012 from www.finieris.lv/docs/file/par_mums_faili/lf_info_en.pdf

Saliņš, Z. 2002. Mežs – Latvijas nacionālā bagātība [Forest-Latvian national treasure]. *Jelgavas tipogrāfija, Jelgava,* 248 pp, (in Latvian).

Scheffler, A. 2007. Emulsion containing a plant extract, method for producing said emulsion and for obtaining said plant extract. *Patent* US 7482383 B2.

Schinor, E. C., Salvador, M. J., Turatti, I. C., Zucchi, O. L. and Dias, D. A. 2004. Comparison of classical and ultrasound-assisted extractions of steroids and triterpenoids from three Chresta spp. *Ultrasonics Sonochemistry* 11(6): 415-421.

Soica, C. M., Peev, C. I., Ciurlea, S., Ambrus, R. and Dehelean, C. 2010. Physico-chemical and toxicological evaluations of betulin and betulinic acid interactions with hydrophilic cyclodextrins. *Farmacia* 58(5): 611-619.

Tang, J. J., Li, J. G., Qi, W., Qiu, W. W., Li, P. S., Li, B. L. and Song, B. L. 2011. Inhibition of SREBP by a small molecule, betulin, improves hyperlipidemia and insulin resistance and reduces atherosclerotic plaques. *Cell Metabolism* 13(1): 44-56.

Zandersons, J., Rizhikovs, J., Pazhe, A., Dobele, G., Tardenaka, A., Spince, B. and Jurkjane, V. 2013. Method for the chemical processing of outer birch bark and apparatus for its realisation. *Patent application number* 13004479.5-1456.

Береговцова, Н. Г., Шарыпов, В. И., and Кузнецов, Б. Н. 2012. Исследование процесса экстракции бересты березы этанолом и изопропанолом при повышенных температурах [Investigation of birch bark extraction process with ethanol and isopropanol at elevated temperatures]. *Химия растительного сырья* 1: 71-75 (in Russian).

Кислицын, А. Н. 1994. Экстрактивные вещества бересты: выделение, состав, свойства, применение [Birch bark extractives: isolation, composition, properties, application]. *Химия древесины* 3: 3-28 (in Russian).

Кислицын, А. Н. 1998. Способ выделения бетулинола [Method of isolating betulinol]. Patent RU 2138508 C1 (in Russian).

Комаров, В.Л. 1941. Практический курс анатомии растений [Practical course of plant anatomy]. *Издательство Академии наук СССР*, 311 pp. (in Russian).

Кузнецова, С. А., Шахтшнейдер, Т. П., Михайленко, М. А., Маляр Ю. Н., Замай А. С. and Болдырев, В. В. 2013. Механокомпозит диацетата бетулина и его противоопухолевая активность [The mechanocomposite of betulin diacetate and its antitumor activity]. *Journal of Siberian Federal University*, 6(2): 192-202 (in Russian).

Левданский, В. А., Королькова, И. В., Левданский, А. В., Иванченко, Н. М. and Кузнецов, Б. Н. 2012. Влияние природы органического растворителя на экстракционное выделение бетулина из бересты коры березы [Influence of the nature of organic solvent on betulin isolation from an external layer of birch bark]. *Journal of Siberian Federal University. Chemistry* 3 (5): 302-310 (in Russian).

Received 05 November 2012
Accepted 15 May 2014

АППАРАТ И СЕЛЕКТИВНЫЕ РАСТВОРИТЕЛИ ДЛЯ ЭКСТРАКЦИИ ТРИТЕРПЕНОВ ИЗ БЕРЕСТЫ БЕРЁЗЫ ПОВИСЛОЙ (*BETULA PENDULA* ROTH.)

А. Паже, Я. Зандерсонс, Я. Рыжиковс, Г. Добеле, В. Юркьяне, Б. Спинце и А. Тарденака

Резюме

Разработаны способ и аппаратура для получения экстрактов тритерпенов и криталлического бетулина из гранулированной бересты (фракция 0.4–2.0 мм), экстрагируя петролейным эфиром (интервал кипения 100–140°C) при температуре кипения. Процесс экстракции проводится в проточном реакторе в режиме интенсивной массопередачи в потоке перегнанного растворителя. В процессе используются изменения растворимости бетулина и других тритерпенов в петролейном эфире, меняя температуру растворителя. В ходе процесса в среде петролейного эфира, в тоже время, в промежуточном кристаллизаторе из потока раствора в виде очищенных кристаллов бетулина накапливается 40–50% от общего количества экстрактивных веществ с общей концетрацией бетулина – лупеола 90–93% от общей массы абсолютно сухих кристаллов. При снижении температуры оставшегося раствора в испарителе экстрактора осаждаются кристаллы бетулина – лупеола с немного уменьшенным количеством бетулина, но с повышенным содержанием лупеола. Оборудование интенсивного массопереноса также может быть использовано для экстракции бересты полярными растворителями (спирты, ацетон). Полная экстракция гранулированной бересты полярными растворителями занимает не более 3–5 часов, но действие промежуточного кристаллизатора менее эффективно и потому требует температуры менее 10ºC для охлаждающей рубашки. В маточном растворе, оставшимся после выделения тритерпенов из экстракта испарителя, обнаружен лупеол – 38–40% от массы абсолютно сухих экстрактивных веществ маточного раствора, поэтому они могут быть использованы для получения кристаллического лупеола.

Ключевые слова: береста, тритерпены, бетулин, лупеол, петролейный эфир, экстракция.