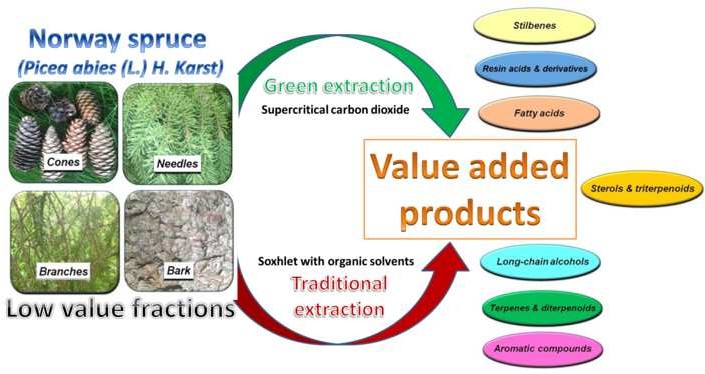
**Graphical Abstract**



**Highlights (for review)**

# Highlights

* + - Forestry wastes have significant potential for exploitation as a chemical feedstock
    - Waste Norway spruce cones, bark, needles and branches were valorised by extraction
    - Waste biomass yielded valuable organic compounds such as sterols and nonacosanol
    - Supercritical CO2 extraction was determined to be a green method for lipid recovery
    - Soxhlet extraction was most effective at recovering lipids from cones and bark

**\*Manuscript**

[**Click here to view linked References**](http://ees.elsevier.com/indcro/viewRCResults.aspx?pdf=1&docID=49536&rev=0&fileID=909889&msid=%7B83198322-5797-43AE-994A-CAB6433DC06B%7D)

**Extraction of lower-value fractions of Norway spruce (*Picea abies*) by supercritical carbon dioxide and soxhlet extractions techniques**

Natalia Bukhanko1, Thomas Attard2, Mehrdad Arshadi1\*, Daniel Eriksson1, Vitaliy Budarin2, Andrew J. Hunt3, Paul Geladi1, Urban Bergsten1, James Clark2

*1Department of Forest Biomaterials and Technology, Swedish University of Agricultural Sciences, SE-901 83, Umeå, Sweden*

*2Green Chemistry Centre of Excellence, Department of Chemistry, University of York, Heslington, York, UK Y010 5DD*

*3Materials Chemistry Research Center, Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Khon Kaen University, Khon Kaen, 40002, Thailand*

*\*- Corresponding author. E-mail:* [*mehrdad.arshadi@slu.se*](mailto:mehrdad.arshadi@slu.se)

# Abstract

Four low-value Norway spruce tree fractions - cones, branches, needles and bark, were separated from the main tree constituents for investigation of their lipophilic extractives; in order to identify potential sources of important chemicals for future biorefinery applications. For the first time, conventional soxhlet and supercritical carbon dioxide (scCO2) extraction techniques were used for extraction of organic compounds from these waste fractions. Soxhlet extraction led to total yields of 4-5% for all fractions except for cones, where the extraction yield was approximately 2%. With scCO2 extraction, the highest yield of extractives was obtained from the branches (*ca.* 5%), whereas for needles, bark and cones the yield was approximately 3%, 2% and 1%, respectively. Extracts from all four tree fractions contain fatty/resin acids, terpenes, stilbenes, sterols and some long chain alcohols. The components composition was different for each of the four fractions depending on the extraction technique, for example, stilbenes and sterols from branches are effectively obtained only with scCO2 extraction, whereas soxhlet extraction was more efficient for isolating terpenes, sterols and resin derivatives from

cones and bark. Needles extractives, e.g. biologically active sterols and nonacosan-10-ol, important for hydrophobic coatings, can be efficiently obtained by both extraction techniques. These results show that these tree fractions, often discarded or combusted, could generate a potentially important source of nutraceutical, pharmaceutical and commodity chemicals.

***Keywords:*** Biorefinery, cones, needles, branches, bark, waste fractions

# Highlights

* + - Forestry wastes have significant potential for exploitation as a chemical feedstock
    - Waste Norway spruce cones, bark, needles and branches were valorised by extraction
    - Waste biomass yielded valuable organic compounds such as sterols and nonacosanol
    - Supercritical CO2 extraction was determined to be a green method for lipid recovery
    - Soxhlet extraction was most effective at recovering lipids from cones and bark

# Introduction

Norway spruce is a common coniferous wood species in Nordic countries and constitutes 42% of total wood resources on productive forest land in Sweden (Christiansen, 2014). The major products of the forest industry, in Nordic countries, are timber from sawmills and the generation of pulp for the paper industry. Both these processes utilize the stem wood of conifers Norway spruce and Scots pine. Typically, the large amounts of bark, cones, needles and branches are left in the forest during harvesting which could also increase the risk of fire.

Extractives from forest residues are a minor part in volume/mass, however the extractives are complex and have high economical potential in biorefinery applications. Extractive chemicals are known to have a negative influence on pulp/paper making process (Valto et al., 2012) but have a diversity of applications elsewhere, e.g. production of biodiesel, glue and paints additives (www.sunpine.se).

The bark of trees, *ca*. 10% of the total biomass, is removed and part of this is often burnt for onsite industrial energy production. The bark of coniferous trees contains high amounts of lipophilic extractives (Anäs et al., 1983; Arshadi et al., 2013; Backlund et al., 2014; Burčová et al., 2018; Jablonsky et al., 2017; Jablonský et al., 2015). In pine and spruce, fatty/resin acids, sterols and triterpenoids are most abundant extractives in the inner and outer bark (Anäs et al., 1983). Spruce bark is also known as a rich source of phenolic extractives, terpenes, resin acids, flavonoids, stilbenes and stilbene glucosides, lignin and holocellulose (Co et al., 2012; Pietarinen et al., 2006; Salem et al., 2016a) (Latva-Mäenpää et al., 2013; Miranda et al., 2012). Spruce bark is a source of β-sitosterol and methyl dehydroabietate, which possess antibacterial and antioxidant properties (Burčová et al., 2018). Stilbenes from spruce bark extracts are also known for their strong antioxidant properties (Pietarinen et al., 2006). Tannins from coniferous woods bark are used in the production of foams, in medicine, cosmetics and wastewater treatment (Feng et al., 2013). Moreover, raw or extracted bark can be used as absorbents (Brás et al., 2004) and spruce bark was suggested as a potential feedstock for the production of lignocellulosic ethanol (Kemppainen et al., 2012).

Needles of conifers contain mono- and sesquiterpenes, fatty acids, waxes and carbohydrates (Backlund et al., 2014; Isidorov et al., 2010; Johansson, 1995) and long chain alcohols, e.g. nonacosan-10-ol, which possesses superhydrophobic properties (McElroy et al., 2018).

Cones of coniferous trees could be utilized as a prospective, widespread and low-cost material in the production of wood-based composites and reinforcing fillers (Ayrilmis et al., 2010), bio-sorbents for heavy metal ions from water (Pholosi et al., 2013) and also for pharmaceutical purposes due to high content of resin acids and alcohols (Backlund et al., 2014; Sahin and Yalcin, 2017).

The spruce branches include knots which contain resin acids and lignans, however the content of lignans in branches is essentially lower than in knots (Willför et al., 2003b). This creates a potential for use in production of surfactants, varnishes, personal care products, cosmetics and adhesives as well as a potential feedstock for production of antioxidant compounds (Pietarinen et al., 2006; Willför et al., 2003b).

Conventional soxhlet extraction is well-known as a standard and reference method for solid-liquid extraction of organic compounds from forest materials (Backlund et al., 2014; Schwanninger and Hinterstoisser, 2002; Soon and Chiang, 2012).

Supercritical CO2 extraction (scCO2) was found to be an environmentally safe and efficient method for semi-scale isolation of lipophilic components from pine sawdust as well as an advanced pre-treatment technology for wood pellets (Arshadi et al., 2016), for isolation of waxes, long-chain aldehydes (e.g. octacosanal) and policosanols from sugarcane wastes (Attard et al., 2015b). Numerous studies have reported that scCO2 extraction is a sustainable and suitable alternative to solvent extraction for extensive range of cellulose biorefinery tasks (Arshadi et al., 2012; Attard et al., 2016a; Attard et al., 2018; Attard et al., 2015c; McElroy et al., 2018).

In the present work, four low-valued fractions of Norway spruce – cones, branches, needles, and bark were collected. Soxhlet and supercritical carbon dioxide fluid extraction techniques were utilized for isolation of organic compounds from these fractions of Norway spruce. Earlier comparison of several extraction techniques (accelerated solvent extraction (ASE), classic soxhlet and scCO2) was performed only for isolation of one group of extractives from the same wood species (Bertaud et al., 2017). The goal of this work was to obtain a qualitative and quantitative description of the different extractives obtained from the four fractions. Since the investigated group of compounds have different properties, in some cases, an adjustment of samples preparations (e.g. derivatisations, hydrolysations) were needed prior to detection by GC- MS. Comparison of the two tested extraction techniques was performed. The yield and composition of the extractives was determined. An optimal extraction of high value chemicals from low value forest biomaterials integrated with pulp and paper production may create a new income sources for industry.

# Experimental part

## Materials and methods

The material was collected from one old Norway spruce (*Picea abies L.*) as a typical representative tree. This tree belonged to a spruce-dominated even aged stand in northern Sweden (66°11N, 19°44E). This tree of intermediate size within the stand had 128 annual rings in sum at stump height. Four tree fractions were sampled. Three fractions were isolated from the complete branches, which were sampled from all parts of the tree, including dead branches. Table 1 summarizes characteristics of Norway spruce fractions.

# Table 1. Structural characteristics of Norway spruce fractions used in work

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| ***Structural characteristics*** | ***Cones*** | ***Branches*** | ***Needles*** | ***Bark*** |
| Log diameter (mm) | - | - | - | 200 |
| Bark proportion (%) | - | 33.8 | - | 100 |
| Inner to outer bark ratio | - | 0.541 | - | 0.410 |
| Mean branch diameter (mm) | - | 16.3 | - | - |
| Green needle proportion (%) | - | - | 80 | - |
| Closed cone proportion (%) | 100 | - | - | - |

The cone fraction was the first to be isolated from the complete branches, followed by the needles after they dropped from the branches (dried) at 30 °C to constant weight, and the third fraction branches consisted of the remaining material. The fraction of needles included fascicles and both green and non-green needles. The fourth tree fraction was bark sampled by debarking the stem between stump height and 5 meters up in the tree, this stem part corresponds to a typical first log to be harvested in trees during commercial forestry operations. All fractions were dried separately to constant mass at 30 °C close to the time of their isolation. The proportion of green needles was measured by visual examination of colour in 100 randomly sampled needles since there are significant differences between green and non-green needles. Structural characteristics in branches were measured by chopping these into one-decimetre long pieces and with subsequent measurements on 15 randomly sampled pieces. Additional information about the feedstock preparation details is presented in the Supporting information.

The determination of water content was performed according to SIS-CEN /TS 14774- 3:2004, ash content according to SIS-CEN/TS 14775:2004 analysis protocols of Swedish Standards Institute.

## Soxhlet extraction

Conventional soxhlet extraction was used for the extraction of lipophilic fractions from the four tree fractions. Three replicates of samples with mass around 3 g were placed in the soxhlet apparatus (Universal Extraction System B-811 from Büchi Labortechnik AG, Flawil, Switzerland) and the lipophilic fractions were extracted using a mixture of petroleum ether (Merck, b.p. 40 – 60 C) and acetone (90:10 v/v) (AnalaR Normapur, VWR Chemicals, 100%) as the solvent for *ca.* 1-2 h (12 cycles optimized) (Arshadi and Gref, 2005; Attard et al., 2016a; Backlund et al., 2014). The extraction yield values were calculated for dry wood masses (Table 2).

## Supercritical CO2 extraction

Supercritical fluid extraction using carbon dioxide was performed using a Thar SCF500 CO2 extraction system. Dried and milled material, with a mass of about 50 g, was loaded into the extraction vessel and extracted in a dynamic regime for 2 h under the following conditions: p = 400 bar, T = 60 C (for needles fraction); p = 300 bar, T = 50 C (for other fractions - cones, branches and bark); CO2 flow rate 40 g/min. After extraction, the system was depressurized to atmospheric pressure and extracts were collected into the vessels using 2\*50 ml dichloromethane washings. The solvent was removed *in vacuo* and the samples were weighed and % yield recorded.

## Non-silylated samples

Approximately 60 mg of crude extracts were dissolved in 10 ml of dichloromethane and 1 ml of the internal standard, heptadecanoic acid (1 mg ml-1) was added to the solution. Then one ml aliquot was placed into vials and GC-MS analysis was performed.

## Alkaline hydrolysis

After extraction, the dried extracts were weighed carefully. 1.0 mg of heptadecanoic acid (Sigma, ≥98%) in hexane (Scharlau analytical grade, 96%, p.a.) (1.0 mg mL−1) was added as an internal standard (Arshadi and Gref, 2005). To perform alkaline hydrolysis (saponification), 10 mL of an ethanolic (90%) 0.4 M KOH solution was added to the extract and the solution was heated to 70 °C for 4 hours, according to a previously reported method (Ekman and Holmbom, 1989). The solution, now containing the potassium salts of fatty and resin acids, was acidified with 1.5 M HCl. The saponification residues were extracted with petroleum ether (two times, 25 mL). 1 mL of the combined extract was transferred to a 14 mL bottle. The organic layer, containing the lipophilic

extractives fraction, was dried over anhydrous MgSO4 overnight and after filtration, the solvent was evaporated by slow nitrogen flow.

## Silylation procedure

The derivatization (silylation) was performed as a final step of sample preparation before GC-MS analysis (Arshadi et al., 2013; Arshadi and Gref, 2005; Ekman and Holmbom, 1989; Örså and Holmbom, 1994). For this step, one ml of extracts in petroleum ether was placed in the vial and then solvent was evaporated to dry by slow flow of nitrogen. After evaporation the silylating agents - 80 l of bis-(trimethylsilyl)-trifluoroacetamide (Fluka,

≥99%, Sigma – Aldrich, Buchs SG, Schweiz) and 40 l of trimethylchlorosilane (TMCS) (Fluka, ≥99%, Sigma – Aldrich, Buchs SG, Schweiz) were added to the samples and then the samples were heated to 70 C for 45 min. The remains of silylating agents were evaporated by N2 flow and finally the samples were dissolved in 1 ml of dichloromethane (Sigma Aldrich, ≥99.9%) prior to GC-MS analysis.

## GC-MS analysis

GC-MS analysis was used to identify components in all extracts, both in non-silylated and silylated samples. The samples were analysed by two GC–MS instruments Shimadzu QP2010 Plus and Agilent Technologies 6890N GC system coupled with MSD 5973. The instruments were operated in electron-impact mode at EI 70 ev. The MS detector was operated at 270 C, mass scanning range was selected as 46 – 800 amu, solvent cutting time was 3 min. The device was fitted with a HP-5MS capillary column (30m  0.25mm

* 0.25m). The column temperature program was set based on previously reported method (Arshadi et al., 2013) as follows: initial T = 100 C isothermal for 0 min, ramp to 220 C (rate 10 C/min), heating to 235C (rate 1 C/min) and finally heating to 260
* C (rate 10 C/min) then maintained at 260C for 5.5 min. Helium was used as a carrier gas. The injection was performed by auto-injection system and the injection volume was 1 l, according to the previous work (Arshadi and Gref, 2005). The injector was operated at 250 C, split ratio 40:1.

The temperature settings for analysis of needles non-silylated extracts were different. The column temperature program: initial T = 100 C isothermal for 0 min, ramp to 220 C (rate 10 C/min), heating to 235 C (rate 1 C/min) and finally heating to 290 C (rate 10

* C/min) then maintained at 290C for 5.5 min. The injector was operated at 300 C, split ratio 40:1. The MS detector was operated at 300 C.

The data were collected with Lab Solution 4.11 SU2 (for Shimadzu QP2010 Plus instrument) and MSD Chem. Station E 02.01 1177 (for Agilent Technologies 6890N GC/MSD 5973 instrument) software packages. The compounds were identified by NIST mass spectral library (version 2.0).

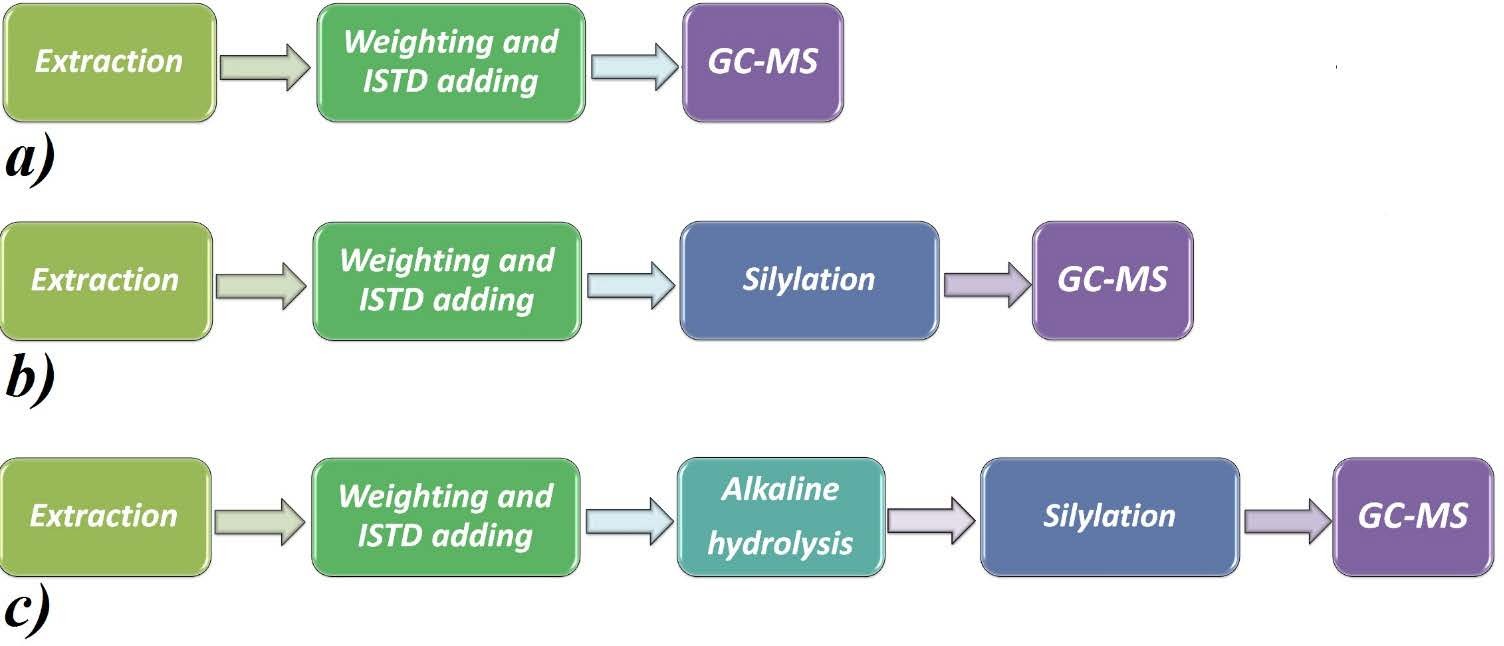
# Results and discussion

Since the extraction was performed with dry materials, it was important to know the content of dry wood in the samples. The determination of dry wood and ash content are given in Table 2.

# Table 2. The average moisture (dry wood, dw.%) and ash content in Norway spruce fractions

|  |  |  |
| --- | --- | --- |
| ***Fraction*** | ***dw content, %*** | ***Ash content, %*** |
| Cones | 91.20.3 | 0.90.1 |
| Branches | 90.70.4 | 1.80.1 |
| Needles | 92.60.5 | 3.60.1 |
| Bark | 90.90.3 | 2.50.1 |

After extraction by soxhlet and scCO2 techniques, the extracts were analysed according to three procedures as shown in Figure 1:



*Figure 1. Scheme of wood extractives analysis: a) direct GC-MS analysis after*

*extraction; b) extraction, silylation and determination by GC-MS; c) extraction, saponification, silylation and determination by GC-MS*

Fatty acids exist in free form and as triacylglycerols in the extracts. In order to release all fatty acids prior to GC-MS analyses a saponification step of the fatty acids was performed.

Silylation has great importance in analyses of organic compounds by gas chromatography. Silylation will increase volatility and thermal stability of polar compounds in high temperature in GC-MS system.

## 3.1. The total yield of extractives obtained from soxhlet and ScCO2 extraction techniques

Soxhlet extraction led to 4-5% yield for all fractions except for cones, where the yield was only 2.2% while scCO2 extraction resulted in the highest yield from the branches (5.3%), followed by needles (3.3%) and bark (2.4%) as shown in Figure 2. The yield of extractives obtained from needles by both extraction techniques is essentially higher than in previous work where ethanol was used as a co-solvent to scCO2 (Orav et al., 1998). Importantly, no co-solvent was used in this current study. Despite the overall yields of supercritical extraction being generally lower the selectivity of extraction can be enhanced by using the green extraction method as highlighted in section 3.2.

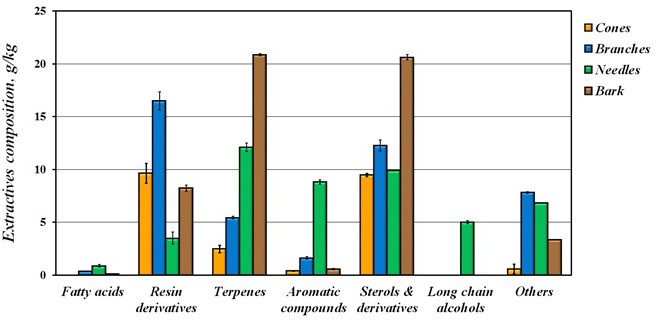


*Figure 2. Total yield of extractives (percentage dry weight) for four Norway spruce fractions obtained by soxhlet and scCO2 extraction techniques*

## Extractives profiles of Norway spruce fractions

* + 1. ***Soxhlet extraction***

The non-silylated soxhlet extract (crude) was analysed by GC-MS to determine all naturally volatile compounds (e.g. terpenes and aromatic compounds) in the extract. The total amounts of major compound groups in extractives, obtained from all fractions of the tree are shown in Figure 3, with the main compounds presented in Table 3. The numerical values (% of total extractives) and the extractives profiles for all fractions (% of total) are summarized in Tables S1 and S2 of Supplementary materials.



*Figure 3. Extractive profile of Norway spruce fractions, obtained for non-hydrolysed non-silylated samples after soxhlet extraction*

# Table 3. Main representatives of extractives, derived from all spruce fractions by soxhlet technique

|  |  |  |
| --- | --- | --- |
| ***Extractives group*** | ***Compound*** | ***C, g kg-1*** |
| ***Cones*** | | |
| **Terpenes and diterpenoids** | Thunbergol | 0.60.0 |
| 13-Epimanool | 1.20.4 |
| **Resin derivatives** | 7-Isopropyl-1,1,4a-trimethyl-1,2,3,4,4a,9,10,10a-  octahydrophenanthrene | 0.60.1 |
| Methyl dehydroabietate | 1.70.1 |

|  |  |  |
| --- | --- | --- |
|  | 4-Dehydroepiabietol | 2.00.1 |
| Dehydroabietic acid | 2.70.5 |
| Methyl 6-dehydrodehydroabietate | 0.80.0 |
| **Sterols** | Androst-5,16-diene-3β-ol | 0.40.0 |
| Androst-5,7-dien-3-ol-17-one | 0.60.0 |
| 5,7,9(11)-Androstatriene, 3-hydroxy-17-oxo- | 1.30.1 |
| Pregna-5,17(20)-dien-3-ol, (3β,17E)- | 1.00.0 |
| Preg-4-en-3-one, 12,17-dihydroxy-20-nitrilo- | 0.50.1 |
| β-Sitosterol | 0.40.2 |
| ***Branches*** | | |
| **Terpenes and diterpenoids** | Longifolene | 1.20.1 |
| Sclaral (sclareolide lactol) | 0.60.1 |
| Manoyl oxide | 0.40.0 |
| Epimanoyl oxide // Manoyl oxide | 0.20.0 |
| Thunbergol | 0.60.0 |
| **Resin derivatives** | 4b,8-Dimethyl-2-isopropylphenanthrene, 4b,5,6,7,8,8a,9,10-  octahydro- | 0.50.0 |
| Norambreinolide | 0.90.0 |
| Dehydroabietal | 0.50.1 |
| Methyl isopimarate | 1.80.1 |
| Methyl dehydroabietate | 2.30.1 |
| 4-Dehydroepiabietol | 1.40.2 |
| Dehydroabietic acid | 3.80.7 |
| 15-Hydroxydehydroabietic acid, methyl ester | 1.30.0 |
| 7-Oxodehydroabietic acid, methyl ester | 0.70.0 |
| **Sterols** | Androst-5,16-diene-3β-ol | 1.40.0 |
| Cholestan-3-ol, 2-methylene-, (3β,5)- | 0.70.0 |
| Stigmasterol acetate | 1.00.5 |
| Androst-5-en-3-one, 17,19-bis(acetyloxy)-4,4-dimethyl-, (17β)- | 0.70.1 |
| 11-Hydroxy-17-methyl testosterone | 1.60.4 |
| β-Sitosterol | 4.00.6 |
| **Others** | 4,8,13-Cyclotetradecatriene-1,3-diol, 1,5,9-trimethyl-12-(1-  methylethyl)- | 2.10.1 |
| ***Needles*** | | |

|  |  |  |
| --- | --- | --- |
| **Terpenes and diterpenoids** | Bicyclo[3.1.0]hexan-2-ol, 2-methyl-5-(1-methylethyl)-,  (1,2,5)- | 0.40.0 |
| Spathulenol | 0.70.1 |
| Caryophyllene oxide | 0.40.1 |
| 13-Epimanool | 3.30.1 |
| Phytol | 2.70.1 |
| 2-allyl-4a,8-dimethyl-1,2,3,4,4a,5,6,8a-octahydro-1-  naphthalenol | 2.30.1 |
| **Fatty acids** | Tetradecanoic acid | 0.10.0 |
| Hexadecanoic acid | 0.10.0 |
| Heptadecanoic acid | 0.40.1 |
| Oleic acid | 0.30.0 |
| **Resin derivatives** | Agathadiol | 2.60.4 |
| Dehydroabietic acid | 0.90.2 |
| **Sterols** | 5,14β-Androstane, 16,17-epoxy- | 0.50.0 |
| β-Sitosterol | 3.60.1 |
| Pregn-16-en-20-one,3-(acetyloxy)-5,6-epoxy-, (3β,5,6) | 1.80.1 |
| 9,19-Cyclolanostan-3-ol, 24-methylene-, (3β)- | 2.40.3 |
| (5β)Pregnane-3,20β-diol, 14,18-[4-methyl-3-oxo-(1-oxa-4-  azabutane-1,4-diyl)]-, diacetate | 0.50.0 |
| **Long-chain**  **alcohols** | Nonacosan-10-ol | 5.00.1 |
| **Aromatic compounds** | Piceol (4'-hydroxyacetophenone) | 6.90.0 |
| -Tocopherol (Vitamin E) | 0.50.4 |
| Bergenin | 0.60.1 |
| **Others** | 1-Cyclohexanone, 2-methyl-2-(3-methyl-2-oxobutyl) | 2.80.1 |
| ***Bark*** | | |
| **Terpenes and diterpenoids** | -Terpineol | 1.10.0 |
| -Muurolol // -Cadinol | 0.90.0 |
| Tricyclo[5.2.2.0(1,6)]undecan-3-ol, 2-methylene-6,8,8-  trimethyl- | 0.50.0 |
| Thunbergen | 0.50.0 |
| Manoyl oxide | 1.30.0 |
| Thunbergol | 4.90.2 |

|  |  |  |
| --- | --- | --- |
|  | 13-Epimanool | 0.60.4 |
| Spiro[4-oxatricyclo[5.3.0.0(2.6)]decan-3-one-5,2'-  cyclohexane], 1'-isopropyl-4'-methyl- | 1.70.0 |
| 4,8,13-Cyclotetradecatriene-1,3-diol, 1,5,9-trimethyl-12-(1-  methylethyl)- | 1.00.3 |
| Sclareol | 2.50.1 |
| Torulosol | 0.40.1 |
| **Resin derivatives** | Dehydroabietal | 0.60.0 |
| Methyl dehydroabietate | 1.80.1 |
| 4-Dehydroepiabietol | 0.60.0 |
| Podocarpa-8,11,13-trien-15-oic acid, 13-isopropyl- | 0.80.0 |
| Methyl 7,13,15-abietatrienoate | 1.00.0 |
| Methyl 15-hydroxyabieta-9(11),8(14),12-trien-18-oate | 1.10.1 |
| **Sterols** | Androst-5,16-diene-3β-ol | 1.40.3 |
| Androst-5,7-dien-3-ol-17-one | 0.40.1 |
| Androst-4-ene-3,20-dione, 11,16,22-triacetoxy- | 0.80.1 |
| Stigmast-4-en-3-one | 1.21.8 |
| Sitosterol | 14.72.4 |
| Cholestan-3-one, cyclic 1,2-ethanediyl acetal, (5)- | 0.40.0 |
| **Other** | 1,6,10,14-Hexadecatetraen-3-ol, 3,7,11,15-tetramethyl-, (E,E)- | 1.80.0 |

The total extractives fraction from the cones is low. The extractives from cones is mainly presented by resins (about 9.5 g kg-1) and sterols (about 9.7 g kg-1). For the resin derivatives, abietane-group of compounds dominated. Dehydroabietic acid was found as a main representative of the resins followed by 4-dehydroepiabietol (methyl dehydroabietate (and other abietane-related compounds (Table 3). There is a difference in the extractives profile of Norway spruce cones when compared to those of other *Abies* species (Kilic et al., 2013) and pine species (Backlund et al., 2014; Ucar and Uçar, 2008), where dehydroabietic acid was not found to dominate in the resins derivatives. The sterols diversity was represented mainly by androstane derivatives, followed by a slightly lower content of pregnane derivatives and -sitosterol (Table 3).

Spruce branches were found to contain mainly resin compounds and sterols (about 16.5 g kg-1 and 12.3 g kg-1 respectively). The resin derivatives are represented mainly by the abietane group, which is different from the resin derivatives detected in branches of

lodgepole pine, where the pimarane group of resins were also detected (Backlund et al., 2014). As was shown for spruce knots, dehydroabietic acid was found to be the most abundant resin acid detected in the spruce branches extracts (Willför et al., 2003a). This makes the branches a potential source of chemicals for ink and glue production, as well as for pharmaceutical purposes because of the high content of dehydroabietic acid, methyl dehydroabietate and β-sitosterol. Other derivatives of resin acids were found in lower amounts, < 3% of total extractives (Table 3). Terpenes and diterpenoids constitute a smaller part of the branch extractives (5.4 g kg-1), represented mainly by equal amounts of sclaral and thunbergol (1.4% of total extractives each) and a number of mono- and sesquiterpenes e.g. myrthenol, verbenone and longifolene.

The needles extractives composition was very complex and was represented mainly by terpenes (about 12.1 g kg-1), sterols (9.9 g kg-1), aromatic compounds (8.8 g kg-1), nonacosan-10-ol (5.0 g kg-1), fatty and resin acids (about 0.9 and 3.5 g kg-1 respectively). The presence of such high amounts of nonacosan-10-ol (10.7% of total extractives) makes spruce needles a potential feedstock for production of super hydrophobic coatings (McElroy et al., 2018). The total content of fatty acids was not high, about 1.9% of total extractives, and was represented by oleic acid, *n-*hexadecanoic acid and tetradecanoic acid. The content of resin acid derivatives in needles was constituted mainly of agathadiol (5.5% of total extractives) and dehydroabietic acid (2.0% of total extractives). Therefore, the needles, often left on the forest floor, can be collected and used for pharmaceutical and cosmetic purposes due to high content of sterols, represented majorly -sitosterol (7.7% of total extractives) and terpenes. Moreover, high content of sterols makes spruce needles a potential food source in chicken diet for improvement of poultry meat and eggs quality (Vītiņa et al., 2012).

Terpenes are the major extractives group detected in spruce needles, with a total content

12.1 g kg-1, which is essentially higher than in previous work (2 g kg-1), derived by simultaneous distillation extraction (SDE) technique (Orav et al., 1998). The terpenes profile was represented mainly by several monoterpenes and numerous sesquiterpenes, diterpenoids and their derivatives. This is different from literature data, where the terpenes profile in hexane extract of spruce needles was represented majorly by monoterpenes (Isidorov et al., 2010). However, studies have shown the efficient isolation of monoterpenes and diterpenoids from pine and spruce needles by both scCO2 and simultaneous distillation extraction techniques (Orav et al., 1998). Diterpenoid 13- epimanool (7.0% of total extractives), 2-allyl-4a,8-dimethyl-1,2,3,4,4a,5,6,8a- octahydro-1-naphthalenol (4.8% of total extractives) and phytol (5.8% of total

extractives) were detected in needles extract as a major group representatives. This makes spruce needles a valuable source of 13-epimanool, which possesses cytotoxic and anti- leukemic activity (Dimas et al., 1998). It was previously reported that 13-epimanool was found in methanol extracts of spruce heartwood (12%) (Salem et al., 2016b). Spathulenol in spruce needles was detected in low amount (1.5% of total extractives) in the current study.

Aromatic compounds constituted a large part of the needles extractives (8.8 g kg-1), represented mainly by piceol (4'-hydroxyacetophenone), 14.7% of total extractives. The content of vitamin E (-tocopherol) in needles was much less and constituted 1.2% of total extractives.

Spruce bark was also found to be a valuable source of extractives, having a large set of well-known phenolic compounds. Thus, soxhlet extraction technique was found to be preferred for successful delivering of terpenes and sterols (where β-sitosterol is the major compound), derived from bark compared to scCO2 (see Tables 3 and 4). However, the soxhlet technique is energy intensive and large volume of organic solvents will be consumed.

The spruce bark was found to give a very high yield of sterols (20.6 g kg-1), dominated by β-sitosterol (27% of total extractives), which makes it a potential source of chemicals for medicine and nutraceutical purposes, for example, because of its bacteriostatic activity (Burčová et al., 2018). Sterols can lead to a significant decrease in common cancer risks, namely colon, breast, lung and prostate cancers (De Stefani et al., 2000; MacKay and Jones, 2011; McCann et al., 2003; Mendilaharsu et al., 1998; Ronco et al., 1999; Shimizu et al., 1991). Additionally, β-sitosterol possesses anti-inflammatory, antiplatelet effects, reduces plasma LDL-cholesterol levels and could be used in cortisone

– derivative hormones synthesis. (De Stefani et al., 2000; Fernandez and Vega-Lopez, 2005; Jesch and Carr, 2017)). These results are higher than earlier reported data for inner and outer spruce bark, obtained by soxhlet extraction with pure solvents, hexane and acetone, where maximum values reached about 15 g kg-1 (outer bark, acetone) (Krogell et al., 2012).

Terpenes and diterpenoids (20.9 g kg-1) were found as other major extractives group derived from spruce bark, where diterpenoids constituted the major part (thunbergol, sclareol and 13-epimanool). In the previous work different amount of diterpenoids from inner and outer bark (7 and 32 g kg-1 respectively) isolated by hexane was obtained (Krogell et al., 2012). Sclareol, the labdane-type diterpenoid, is a natural compound with

a wide range of biological activities such as antifungal, antibacterial, antioxidant, anti- inflammatory and anticancer (tumor growth inhibiting) properties (Gao et al., 2017). It is also used as a cosmetic and anti-photoaging (wrinkle smoothing) additive (Park et al., 2016). Previously, sclareol was extracted predominantly from sage (Caissard et al., 2012) and the extraction of sclareol from Norway spruce bark can be a good alternative. The content of resin derivatives, derived from bark by soxhlet extraction, was less (8.2 g kg-

1) and the major components were methyl dehydroabietate (3.3% of total extractives) and equal content of dehydroabietal and 4-dehydroepiabietol (1.1% of total extractives each). It was recently reported that the content of lipophilic extractives in coniferous bark fractions, pulpwood and timber, is not high (Arshadi et al., 2018). Recent data showed that resin derivatives content, obtained by soxhlet extraction was close to those derived by ASE (Jablonsky et al., 2017) the same type of most important components – resin of abietan group. Moreover, the application of hexane as an extraction solvent in ASE leads to the isolation of high amounts of resin acids from inner and outer spruce bark (28 and 100 g kg-1 respectively) whereas acetone extraction gives much lower yields of resin group of extractives (2 and 12 g kg-1 respectively) (Krogell et al., 2012).

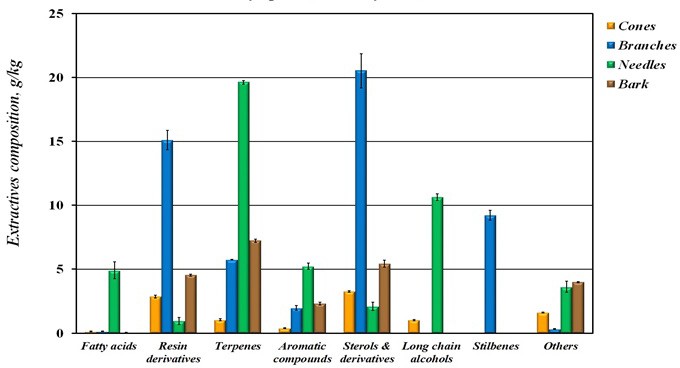
## 3.2.2. Supercritical CO2 extraction

The results of the scCO2 extractives profiles of spruce fractions for crude (non-hydrolysed and non-silylated) samples are presented in Figure 4, Table 4 (major representatives of extractives in all tree fractions) and Table S3 of Supplementary material (% of total extractives). The detailed extractives profiles for all fractions (% of total) are summarized in Table S4 of Supplementary material.

Total crude yield of extractives obtained by scCO2 technique is lower than for soxhlet extraction for cones, needles and bark fractions; however, the technique is more efficient to isolate extractives from branches (Figure 2) and demonstrates enhances selectivity for a number of compounds (Table 4). Spruce cones were not found to contain high amounts of lipophilic extractives. However, the scCO2 method can be used for isolation of androstane and pregnane derivatives and 4-dehydroabietol whereas the soxhlet technique is suitable to deliver 13-epimanool, dehydroabietic acid and other representatives of abietane resins. Supercritical CO2 extraction leads to the isolation of lower amounts of resin acids from cones compared to soxhlet extraction (Figures 3 and 4). Resin compounds in cones constituted 2.9 g kg-1 of total extractives and constituted mainly abietane derivatives (4-dehydroepiabietol was main representative). The results are in agreement with previous work done for southern spruce species (Kilic et al., 2013).

Sterols were a major group of cones extractives (about 3.3 g kg-1) and represented mainly by androstane and pregnane derivatives.

Smaller amounts of terpenes and diterpenoids group (1.0 g kg-1) were found, where 13- epimanool was a main component (6.0% of total extractives). Nonacosan-10-ol was detected in cones in low amounts 0.9 g kg-1. Long chain hydrocarbons and waxes constitute big part of spruce cones extractives.



*Figure 4. Extractive profile of Norway spruce fractions, obtained for non-hydrolysed non-silylated samples after scCO2 extraction*

# Table 4. Main representatives of extractives, derived from all spruce fractions by scCO2 technique

|  |  |  |
| --- | --- | --- |
| ***Extractives group*** | ***Compound*** | ***C, g kg-1*** |
| ***Cones*** | | |
| **Terpenes and**  **diterpenoids** | 13-Epimanool | 0.60.0 |
| **Resin derivatives** | 4-Dehydroepiabietol | 0.50.0 |
| Methyl dehydroabietate | 0.20.0 |
| 4b,8-Dimethyl-2-isopropylphenanthrene,  4b,5,6,7,8,8a,9,10-octahydro- | 0.20.0 |
| Podocarpa-8,11,13-triene-7á,13-diol, 14-  isopropyl- | 0.20.0 |

|  |  |  |
| --- | --- | --- |
|  | 15-Hydroxydehydroabietic acid, methyl ester | 0.10.0 |
| 7-Oxodehydroabietic acid, methyl ester | 0.10.0 |
| **Sterols** | Pregn-4-en-17,20-diol-3-one | 0.10.0 |
| Pregna-5,17(20)-dien-3-ol, (3β,17E)- | 0.40.0 |
| Androst-5,16-diene-3β-ol | 0.40.0 |
| 5,7,9(11)-Androstatriene, 3-hydroxy-17-oxo- | 0.40.0 |
| 4,9(11)-Androstadien-17β-ol-3-one | 0.40.1 |
| **Long chain alcohols** | Nonacosan-10-ol | 0.90.0 |
| ***Branches*** | | |
| **Terpenes and**  **diterpenoids** | Longifolene | 0.70.0 |
| Biformen | 0.50.0 |
| **Resin derivatives** | Dehydroabietal | 1.30.1 |
| Methyl dehydroabietate | 2.10.1 |
| 4-Dehydroepiabietol | 1.10.1 |
| Methyl 15-hydroxyabieta-9(11),8(14),12-trien-  18-oate | 1.80.0 |
| **Stilbenes** | 4'-Methoxy-2-hydroxystilbene | 9.20.4 |
| **Sterols** | Androstane-3,11-diol, (3β,5,11β)- | 1.60.0 |
| Androst-5,16-diene-3β-ol | 2.70.1 |
| 19-Hydroxy-3,5-cyclo-5-androstan-17-one | 3.60.1 |
| Stigmastane-3,6-dione, (5)- | 1.80.0 |
| β-Sitosterol | 5.41.7 |
| ***Needles*** | | |
| **Fatty acids** | Hexadecanoic acid | 1.20.2 |
| Linoleic acid | 0.30.1 |
| Oleic acid | 2.60.3 |
| **Resin derivatives** | Dehydroabietal | 0.10.0 |
| Agathadiol | 0.20.0 |
| 4-Dehydroepiabeitol | 0.10.0 |
| **Sterols** | Stigmastan-3,5-diene | 0.50.1 |
| **Terpenes and diterpenoids** | Borneol, acetate, (1S,2R,4S)-(-)- | 1.70.0 |
| 7-Acetyl-2-hydroxy-2-methyl-5-  isopropylbicyclo[4.3.0]nonane | 2.30.1 |

|  |  |  |
| --- | --- | --- |
|  | 13-Epimanool | 3.50.1 |
| Phytol | 3.90.2 |
| Biformen | 0.50.0 |
| Torulosol | 2.10.0 |
| **Aromatic compounds** | Piceol | 2.20.0 |
| -Tocopherol (Vitamin E) | 1.90.5 |
| **Long chain alcohols** | Nonacosan-10-ol | 10.70.2 |
| ***Bark*** | | |
| **Terpenes and diterpenoids** | -Terpieol | 0.50.0 |
| Manoyl oxide | 0.90.0 |
| Thunbergol | 1.00.1 |
| 13-Epimanool | 0.10.0 |
| Norambreinolide | 0.40.0 |
| 7a-Isopropenyl-4,5-dimethyloctahydroinden-4-  yl)methanol | 1.60.0 |
| Spiro[4.5]decan-7-one, 1,8-dimethyl-8,9-epoxy-  4-isopropyl- | 0.80.3 |
| Sclareol | 1.10.1 |
| **Resin derivatives** | Dehydroabietal | 0.50.0 |
| Methyl isopimarate | 0.40.1 |
| Methyl dehydroabietate | 1.00.0 |
| 4-Dehydroepiabietol | 0.40.0 |
| Methyl 12,13-dihydroxyabiet-8(14)-en-18-oate | 0.30.1 |
| Methyl 6-dehydrodehydroabietate | 0.40.0 |
| Dehydroabietic acid | 0.10.0 |
| 15-Hydroxydehydroabietic acid, methyl ester | 0.60.0 |
| **Sterols** | Androst-5,16-diene-3β-ol | 0.60.0 |
| Cholest-4-en-26-oic acid, 3-oxo- | 0.30.1 |
| β-Sitosterol | 2.60.5 |
| **Other** | 4,8,13-Cyclotetradecatriene-1,3-diol, 1,5,9-  trimethyl-12-(1-methylethyl)- | 0.40.0 |
| 2-Methyl-cis-7,8-epoxynonadecane | 0.40.1 |
| Tetracosanal | 0.70.1 |

ScCO2 extraction as a technique is very efficient for isolation of resin derivatives, terpenes, stilbenes and sterols from spruce branches. The yield of resin derivatives obtained from branch extractives constituted *ca* 15 g kg-1; therefore, scCO2 was able to extract a greater number of these components (Figure 4). Abietane group of resin derivatives is represented mainly by methyl dehydroabietate, 15-hydroxydehydroabietic acid, methyl ester and cryptopinon.

Sterols were a main group of branches extractives (20.5 g kg-1) represented mainly by - sitosterol, androstane and stigmastane derivatives.

ScCO2 extraction is an ideal method for isolation of stilbenes from spruce branches, which did not occur when using soxhlet extraction, as seen from Tables 3 and 4. Stilbenes compounds constituted a high percentage of extractives and were presented by 4'- methoxy-2-hydroxystilbene (9.2 g kg-1). Despite the fact that biological role of stilbenes is not fully studied it is known that some of stilbenes possess anti-carcinogenic and cancer preventive activity (Sirerol et al., 2016). Azo-dyes, derived from stilbene derivatives possess also antioxidant and antibacterial properties (Rezaei-Seresht et al., 2018). It was previously shown that spruce knot wood is a rich source of valuable phenolic compounds

– lignans and lipophilic extractives (Willför et al., 2003a). Herein, we showed the value of branches, which contain many resins, sterols and stilbenes, which can be successfully isolated by scCO2 extraction. 4-Methoxy-2-hydroxystilbene and β-sitosterol were found as main extractives components in spruce branches, using scCO2 extraction (Table 4).

The terpenes content in the extract from branches was lower (5.7 g kg-1) and components were represented mainly bylongifolene and biformen.

Supercritical CO2 extraction is a good method to isolate terpenes (19.6 g kg-1), aromatic compounds (5.2 g kg-1), sterols (2.1 g kg-1) and nonacosan-10-ol (10.6 g kg-1) from spruce needles. Resin derivatives constituted a minor part of needles extractives (*ca* 1.0 g kg-1) and were presented by agathadiol and abietane group compounds- 4-dehydroepiabietol, dehydroabietal and methyl dehydroabietate. Sterols group of needles extractives were represented by wide range of stigmastan and pregnane derivatives. The terpenes and diterpenoids content in spruce needles is very high (19.7 g kg-1) which is higher than in previous work, where the values of terpenes content varied from 6 to 11 g kg-1, depending on the use of ethanol as a co-solvent (Orav et al., 1998). The terpenes profile was represented by a diverse set of mono- and sesquiterpenes compounds. Diterpenoid 13- epimanool (7.4% of total extractives) was detected as a major group. Similar amounts of spathulenol, biformen and borneol in needles were detected. The diverse aromatic

compounds (5.2 g kg-1), detected in needles was represented by piceol (4'- hydroxyacetophenone, 4.6% of total extractives) and vitamin E, -tocopherol (4.1% of total extractives). As seen from Tables 3 and 4 the amount of -tocopherol derived from spruce needles by scCO2 extraction technique is *ca* 4 times higher than by soxhlet.

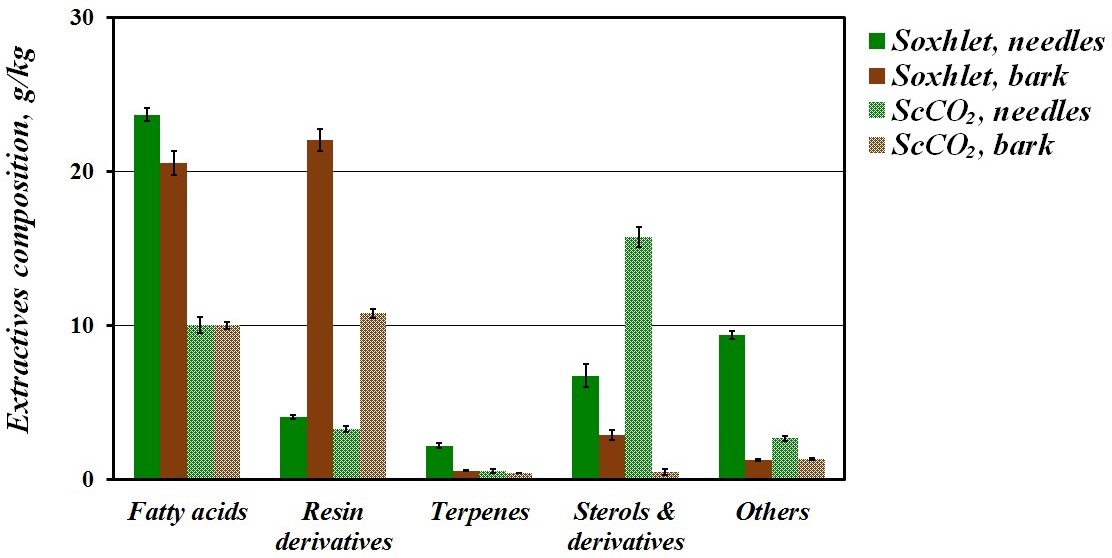
ScCO2 extraction gives lower yields of lipophilic extractives from spruce, fir and pine logs wood (Bertaud et al., 2017). Soxhlet extraction was found to be the most efficient technique for isolation of terpenes from spruce logs compare to ASE, scCO2 and Clevenger techniques. Total yield of lipophilic fractions derived by ASE and soxhlet techniques is around 3.5 and 3.3 g/kg respectively, whereas for scCO2 was only 1.9 g/kg and 0.08 g/kg. Thus, content of terpenes, isolated from spruce logs by using of soxhlet extraction was 1.15 g/kg but by scCO2 0.066-0.085 g/kg (Bertaud et al., 2017).

The extractives profile of bark was mainly represented by terpenes (7.2 g kg-1), sterols (5.4 g kg-1) and resin derivatives (4.5 g kg-1) (Figure 3). The resin group was represented mainly by abietan-group compounds such as methyl dehydroabietate, 15- hydroxydehydroabietic acid, methyl ester, dehydroabietal, methyl 6- dehydrodehydroabietate and 4-dehydroepiabietol. Methyl esters of isopimaric and 7- oxodehydroabietic acid as well as free dehydroabietic acid were detected as minor resin compounds in spruce bark. The sterols group was represented mainly by -sitosterol. The terpenes profile of spruce bark was presented mainly by equal amounts of thunbergol, sclareol and manoyl oxide. Tetracosanal was detected as a long chain aldehyde representative in bark.

## 3.3 Comparison of hydrolysed and silylated extracts from soxhlet and scCO2 techniques

GC-MS analysis is proven as an effective technique for the analysis of waxes and lipids. However, a significant number of non-volatile and large molecules cannot be analysed by this method without derivatisation. For this reason, a new series of extractions followed by hydrolysis and derivatisation steps were performed for two spruce fractions

- needles and bark. Needles and bark were chosen as model fractions for comparison of two extraction techniques since they contain high amounts of valuable compounds (fatty/resin acids, terpenes, sterols, long chain alcohols). These groups of compounds are also existing in the other spruce tree fractions, i.e. branches and cones.



*Figure 5. Extractive profile of Norway spruce needles and bark, obtained for hydrolysed silylated samples after soxhlet and ScCO2 extraction. For needles (soxhlet extraction) the average value for 3 injections is presented, for all other species these data are for 4 injections.*

The results are presented in Figure 5, Table 5 and Table S5 of Supplementary material. The total amount of fatty acids was highest in needles obtained by the soxhlet extraction (23.7 g kg-1), which was almost three times higher compared to that of the scCO2 extraction (10 g kg-1). The amounts of resin acids derived from needles by both soxhlet and scCO2 methods were very low (about 4 g kg-1). The amounts of fatty and resin acids extracted from the bark by the soxhlet method were similar and constituted about 20.6 and 22.1 g kg-1 respectively, which was twice as high as those from the scCO2 method (10 g kg-1 both for fatty and resin acids). The scCO2 method was found to be the best for isolation of the sterol fraction from needles compared to the soxhlet technique (about

15.8 g kg-1 of sterol fraction from the scCO2 vs. ca 6.8 g kg-1 from the soxhlet method).

To summarize, industrial valorization of high amount of lipophilic extractives - free fatty acid/sterols and free fatty/resin acids from needles and bark respectively by soxhlet technique are of great interest due to high efficiency compared to scCO2 extraction.

This shows prospective for commercialization of effective application of these two spruce tree fractions, e.g. for pharmaceuticals, cosmetic and nutrition components production as well as production of biodiesel, glue and varnishes.

# Table 5. Main representatives of lipophilic extractives (% of total) in spruce needles and bark obtained by soxhlet and scCO2 extraction for hydrolysed silylated series

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **№** | **Components** | **Needles** | | **Bark** | |
| ***soxhlet*** | ***scCO2*** | ***soxhlet*** | ***scCO2*** |
| ***Fatty acids*** | | | | | |
| 1 | Tetradecanoic acid | 4.0±0.1 | 0.4±0.0 | 0.04±0.01 | 0.04±0.02 |
| 2 | Hexadecanoic acid | 6.70.1 | 5.50.5 | 2.70.3 | 2.90.2 |
| 3 | -Linolenic acid | 1.00.0 | nd | 6.30.4 | 5.60.3 |
| 4 | Heptadecanoic acid | 0.80.1 | 0.40.0 | 0.80.2 | 1.00.1 |
| 5 | Linoleic acid | 3.60.0 | 3.70.2 | 9.30.2 | 12.30.4 |
| 6 | Oleic acid | 12.30.3 | 16.40.7 | 11.40.6 | 12.70.4 |
| 7 | Octadecanoic acid | 0.30.0 | 0.20.0 | 0.80.1 | 0.80.2 |
| 8 | Docosanoic acid | nd | nd | 5.60.7 | 2.40.3 |
| 9 | Dodecanedioic acid, bis(tert-  butyldimethylsilyl) ester | 8.80.3 | 0.70.1 | nd | nd |
| 10 | Tricosanoic acid | nd | nd | 3.11.3 | 2.60.3 |
| 11 | Tetracosanoic acid | nd | nd | 2.60.5 | 2.30.3 |
| ***Resin acids*** | | | | | |
| 12 | Pimaric acid | nd | nd | nd | 3.00.1 |
| 13 | Isopimaric acid | 0.30.0 | nd | 9.00.4 | 10.40.4 |
| 14 | Dehydroabietic acid | 10.32.3 | 10.00.6 | 26.61.7 | 28.71.1 |
| 15 | Methyl abietate | 0.70.2 | nd | nd | nd |
| 16 | Abietic acid | nd | nd | 3.30.1 | 1.80.0 |
| 17 | 15-Hydroxy-7-oxodehydroabietic  acid, methyl ester,15-trimethylsilyl ether | nd | nd | 0.30.1 | 0.20.1 |
| 18 | 15-Trimethylsilyloxydehydroabietic  acid | nd | nd | 0.30.1 | 0.40.1 |
| 19 | 7-Oxodehydroabietic acid | nd | nd | 0.80.2 | 1.10.1 |
| ***Sterols*** | | | | | |
| 20 | β-sitosterol | 10.80.9 | 48.12.1 | 5.10.7 | 0.90.1 |
| 21 | 9,19-Cyclolanostan-3-ol, 24-  methylene-, acetate, (3β)- | 2.80.8 | nd | nd | nd |

nd – not detected

The results indicate that scCO2 extraction is a facile and robust method for the isolation of sterols from spruce needles and resin acids from bark. An important aspect of this extraction technique is that no solvent residues remain at the end of the extraction, therefore making them ideal for the recovery of nutraceuticals or food products. Previous work has demonstrated that the use of fractional separators in the isolation phase of extraction, can enhance the collection of sterols and thus lead to the production of sterol rich fractions (Attard et al., 2015c). The manipulation of supercritical properties to tune the collection could reduce the downstream purification or separations needed for an extract. In contrast, the isolation of fatty acids fraction from needles is more effective by a soxhlet process. However, such extractions on an industrial scale would require large volumes of traditional petrochemical solvents such as hexane, which are both highly flammable, toxic may lead to atmospheric pollution (Sin et al., 2014). Furthermore, the soxhlet extraction technique cannot be consider as scalable due to high energy consuming and long extraction time so industry uses conventional extraction in a batch reactor instead. Unlike scCO2 extraction, the resulting biomass would contain significant amounts of solvent, which requires an energy intensive remove step prior to further processing or could be burnt for power generation. The added advantage that scCO2 extraction has is the resulting biomass has enhanced downstream processing leading to higher sugar yields on hydrolysis, which is of vital importance as part of a holistic second- generation biorefinery (Attard et al., 2016b; Attard et al., 2015a; Attard et al., 2015c; Yu et al., 2019).

Spruce needles were found to contain also high amounts of fatty acids and sterols whereas bark contains high amounts both of fatty and resin acids. Fatty and resin acids were isolated by using of saponification step for both extraction techniques from spruce needles and bark. High content of fatty and resin acids in spruce needless and bark make these tree fractions potentially valuable for pharmaceutical, cosmetic and dietary purposes, biofuel production. Thus, essential unsaturated fatty acids, oleic (C18:1), linoleic (C18:2) and -linolenic (C18:3) are dominated fatty acids representatives, detected in spruce needles and bark (Table 5). They are well-known due to their biological activity and play a vital role for human body as metabolism regulators, used to decrease a body weight, prevent cardiovascular diseases and possess immune-regulating and anti-inflammatory properties, e.g. used for of dry eye syndrome treatment (Al- Khudairy et al., 2015; Jing et al., 2013; Liu and Ji, 2014). Oleic acid, presented as a major group representative both in needles and bark, is a major unsaturated fatty acid important in human diet because of its cancer preventive and hypotensive activities (Carrillo et al., 2012; Terés et al., 2008). Linoleic acid widely used in cosmetic due to its moisture-

retentive and antioxidant properties (Jandacek, 2017; Rodrigues et al., 2015), may be utilized as a feedstock for biofuel production via hydrothermal process (Besse et al., 2016). *n*-Hexadecanoic and docosanoic acids are the main representative of saturated fatty acids, derived from spruce needles by scCO2 and soxhlet technique respectively (Table 5) which makes them a potentially interesting feedstock for soaps and lubricants production (Stirton, 1952).

The resin acids derivatives are successfully used for paint, printing inks, gum, varnishes and glue production. Beside this, resin acids derivatives, especially dehydroabietic acid, are interesting and perspective compounds for pharmaceutical purposes, e.g. for hyperexcitability diseases treatment (Ottosson et al., 2015), as anti-inflammatory, antimicrobial and antifungal agents (Savluchinske-Feio et al., 2006).

## Hydrolysed and silylated samples after soxhlet extraction

The results from samples analyses as hydrolysed and silylated shows large amount of fatty and resin acids in all 4 fractions, the results are shown on Figure S1 and Tables S6 and S7 of Supplementary material since we not comparing this data with supercritical extraction.

## Non-hydrolysed silylated samples after scCO2 extraction.

Figure S2, Tables S8 and S9 of Supplementary material present extractives profiles data, obtained for all tree fraction for non-hydrolysed silylated samples derived by supercritical CO2 extraction.

# Conclusions

The results support the hypotheses that by adoption of appropriate methods for biomass selection and through the optimisation of green extraction processes forestry biomass with low economic value can be transformed into valuable bio-based chemicals. Such strategies are vital for the long-term success of biorefineries as part of a circular bio- economy. The amount and composition of the extracts from different tree fractions varies. Both extraction techniques can deliver optimal yield of certain desired compounds. ScCO2 extraction technique is more efficient for isolation of sterols and resin derivatives from spruce branches compare to soxhlet; moreover, stilbenes from branches may be extracted only by scCO2 method. On the contrary, the soxhlet technique is much more effective for delivering sterols and terpenes from spruce bark compared to scCO2. Furthermore, the soxhlet technique is more convenient for spruce cones extractives isolation. Spruce needles extractives, fatty acids, terpenes, sterols, aromatic compounds

and nonacosan-10-ol, can be efficiently isolated by both of these extraction techniques. These compounds are precursors for manufacturing of numerous products mainly in cosmetic and pharmaceutical industries. Further investigations may be needed since the logistics and primary production of the low value forest feedstock should be estimated and added to the assessment of the extractives value.

Table 6 summarizes the most suitable techniques (indicated by\*) for isolation of various type of organic compounds from spruce waste fractions.

# Table 6. Groups of extractives, efficiently derived from spruce low value fractions

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| ***Compound type*** | ***Cones*** | ***Branches*** | ***Needles*** | ***Bark*** |
| Fatty acids |  |  | \*Soxhlet | Soxhlet |
| Resin acids and derivatives | \*ScCO2 |  |  |  |
| Terpenes and diterpenoids |  |  | \*ScCO2 | Soxhlet |
| Aromatic compounds |  |  | \*Soxhlet |  |
| Sterols and triterpenoids |  | \*ScCO2 |  | Soxhlet  ScCO2 |
| Stilbenes |  | \*ScCO2 |  |  |
| Long chain alcohols |  |  | \*ScCO2  Soxhlet |  |

**Funding**

The Swedish Research Councils FORMAS (CETEX project-grant № 2014-12813- 27337-64) supported this work. Andrew Hunt would like to thank the financial support of the Thailand Research Fund (RSA6280031) and Khon Kaen University.

# Conflict of interest statement

None declared.

# Acknowledgements

The authors wish to thank Carina Jonsson at SLU for technical assistance at the laboratory and Svartberget Experimental Forest division at SLU for providing samples. The authors are grateful to Calle Nilsson for useful advice and fruitful discussions regarding data interpretation.

# References

Al-Khudairy, L., Hartley, L., Clar, C., Flowers, N., Hooper, L., Rees, K., 2015. Omega 6 fatty acids for the primary prevention of cardiovascular disease. Cochrane Database Syst Rev, Cd011094.

Anäs, E., Ekman, R., Holmbom, B., 1983. Composition of Nonpolar Extractives in Bark of Norway Spruce and Scots Pine. Journal of Wood Chemistry and Technology 3, 119-130.

Arshadi, M., Attard, T.M., Lukasik, R.M., Brncic, M., da Costa Lopes, A.M., Finell, M., Geladi, P., Gerschenson, L.N., Gogus, F., Herrero, M., Hunt, A.J., Ibanez, E., Kamm, B., Mateos-Aparicio, I., Matias, A., Mavroudis, N.E., Montoneri, E., Morais, A.R.C., Nilsson, C., Papaioannou, E.H., Richel, A., Ruperez, P., Skrbic, B., Bodroza Solarov, M., Svarc-Gajic, J., Waldron, K.W., Yuste-Cordoba, F.J., 2016. Pre-treatment and extraction techniques for recovery of added value compounds from wastes throughout the agri-food chain. Green Chemistry 18, 6160-6204.

Arshadi, M., Backlund, I., Geladi, P., Bergsten, U., 2013. Comparison of fatty and resin acid composition in boreal lodgepole pine and Scots pine for biorefinery applications. Industrial Crops and Products 49, 535-541.

Arshadi, M., Eriksson, D., Isacsson, P., Bergsten, U., 2018. Bark Assortments of Scots Pine and Norway Spruce as Industrial Feedstock for Tall Oil Production. Forests 9, 332.

Arshadi, M., Gref, R., 2005. Emission of volatile organic compounds from softwood pellets during storage. Forest Products Journal 55, 132-135.

Arshadi, M., Hunt, A.J., Clark, J.H., 2012. Supercritical fluid extraction (SFE) as an effective tool in reducing auto-oxidation of dried pine sawdust for power generation. RSC Advances 2, 1806-1809.

Attard, T.M., Arshadi, M., Nilsson, C., Budarin, V.L., Valencia-Reyes, E., Clark, J.H., Hunt, A.J., 2016a. Impact of supercritical extraction on solid fuel wood pellet properties and off-gassing during storage. Green Chemistry 18, 2682-2690.

Attard, T.M., Bukhanko, N., Eriksson, D., Arshadi, M., Geladi, P., Bergsten, U., Budarin, V.L., Clark, J.H., Hunt, A.J., 2018. Supercritical extraction of waxes and lipids from biomass: A valuable first step towards an integrated biorefinery. Journal of Cleaner Production 177, 684-698.

Attard, T.M., McElroy, C.R., Gammons, R.J., Slattery, J.M., Supanchaiyamat, N., Alvim Kamei, C.L., Dolstra, O., Trindade, L.M., Bruce, N.C., McQueen-Mason, S.J., Shimizu, S., Hunt, A.J., 2016b. Supercritical CO2 Extraction as an Effective Pretreatment Step for Wax Extraction in a Miscanthus Biorefinery. ACS Sustainable Chemistry Engineering 4, 5979−5988.

Attard, T.M., McElroy, C.R., Hunt, A.J., 2015a. Economic Assessment of Supercritical CO2 Extraction of Waxes as Part of a Maize Stover Biorefinery. International Journal of Molecular Sciences 16, 17546-17564.

Attard, T.M., McElroy, C.R., Rezende, C.A., Polikarpov, I., Clark, J.H., Hunt, A.J., 2015b. Sugarcane waste as a valuable source of lipophilic molecules. Industrial Crops and Products 76, 95-103.

Attard, T.M., Theeuwes, E., Gomez, L.D., Johansson, E., Dimitriou, I., Wright, P.C., Clark, J.H., McQueen-Mason, S.J., Hunt, A.J., 2015c. Supercritical extraction as an effective first-step in a maize stover biorefinery. RSC Advance 5, 43831-43838.

Ayrilmis, N., Buyuksari, U., Dundar, T., 2010. Waste pine cones as a source of reinforcing fillers for thermoplastic composites. Journal of Applied Polymer Science 117, 2324-2330.

Backlund, I., Arshadi, M., Hunt, A.J., McElroy, C.R., Attard, T.M., Bergsten, U., 2014.

Extractive profiles of different lodgepole pine (Pinus contorta) fractions grown under a direct seeding-based silvicultural regime. Industrial Crops and Products 58, 220-229.

Bertaud, F., Crampon, C., Badens, E., 2017. Volatile terpene extraction of spruce, fir and maritime pine wood: supercritical CO2 extraction compared to classical solvent extractions and steam distillation. Holzforschung 71, 667-673.

Besse, X., Schuurman, Y., Guilhaume, N., 2016. Hydrothermal conversion of linoleic acid and ethanol for biofuel production. Applied Catalysis A: General 524, 139-148.

Brás, I., Teixeira Lemos, L., Alves, A., Fernando R. Pereira, M., 2004. Application of pine bark as a sorbent for organic pollutants in effluents. Management of Environmental Quality: An International Journal 15, 491-501.

Burčová, Z., Kreps, F., Greifova, M., Jablonský, M., Ház, A., Schmidt, Š., Šurina, I., 2018. Antibacterial and antifungal activity of phytosterols and methyl dehydroabietate of Norway spruce bark extracts. Journal of Biotechnology 282, 18- 24.

Caissard, J.-C., Olivier, T., Delbecque, C., Palle, S., Garry, P.-P., Audran, A., Valot, N., Moja, S., Nicolé, F., Legrand, S., Baudino, S., Jullien, F., 2012. Extracellular Localization of the Diterpene Sclareol in Clary Sage (*Salvia sclarea* L., *Lamiaceae*). PLoS ONE 7, e48253.

Carrillo, C., del M. Cavia, M., Alonso-Torre, S.R., 2012. Antitumor effect of oleic acid; mechanisms of action. A review. Nutricion Hospitalaria 27, 1860-1865.

Christiansen, L., 2014. Swedish Statistical Yearbook of Forestry, in: Christiansen, L. (Ed.). Swedish Forest Agency, Jönköping, Sweden, p. 370 pp.

Co, M., Fagerlund, A., Engman, L., Sunnerheim, K., Sjoberg, P.J., Turner, C., 2012.

Extraction of antioxidants from spruce (Picea abies) bark using eco-friendly solvents. Phytochem Anal 23, 1-11.

De Stefani, E., Boffetta, P., Ronco, A.L., Brennan, P., Deneo-Pellegrini, H., Carzoglio, J.C., Mendilaharsu, M., 2000. Plant Sterols and Risk of Stomach Cancer: A Case- Control Study in Uruguay. Nutrition and Cancer 37, 140-144.

Dimas, K., Demetzos, C., Marsellos, M., Sotiriadou, R., Malamas, M., Kokkinopoulos, D., 1998. Cytotoxic Activity of Labdane Type Diterpenes Against Human Leukemic Cell Lines in vitro. Planta Medica 64, 208-211.

Ekman, R., Holmbom, B., 1989. Analysis by gas chromatography of the wood extractives in pulp and water samples from mechanical pulping of spruce. Nordic Pulp and Paper Research Journal 04, 16-24.

Feng, S., Cheng, S., Yuan, Z., Leitch, M., Xu, C., 2013. Valorization of bark for chemicals and materials: A review. Renewable and Sustainable Energy Reviews 26, 560-578.

Fernandez, J.L., Vega-Lopez, S., 2005. Efficacy and safety of sitosterol in the management of blood cholesterol levels. Cardiovascular Drug Reviews 23, 57-70.

Gao, J., Wang, D., Jing, S., Hao, S., Huang, X., Miao, Q., 2017. The study of sclareol in inhibiting proliferation of osteosarcoma cells by apoptotic induction and loss of mitochondrial membrane potential. Journal of Medical Oncology and Therapeutics 2, 40-44.

Isidorov, V.A., Smolewska, M., Purzyńska-Pugacewicz, A., Tyszkiewicz, Z., 2010.

Chemical composition of volatile and extractive compounds of pine and spruce leaf litter in the initial stages of decomposition. Biogeosciences 7, 2785-2794.

Jablonsky, M., Nosalova, J., Haz, A., Skulcova, A., Kreps, F., Malvis, A., Surina, I., 2017. Phytochemical analysis of *Picea abies* bark obtained by Accelerated Solvent Extraction. International Journal of Recent Scientific Research 8, 16075-16079.

Jablonský, M., Vernarecová, M., Ház, A., Dubinyová, L., Škulcová, A., Sladková, A., Šurina, I., 2015. Extraction of phenolic and lipophilic compounds from spruce (*Picea abies*) bark using accelerated solvent extraction by ethanol. Wood Research 60, 583-590.

Jandacek, R.J., 2017. Linoleic Acid: A Nutritional Quandary. Healthcare 5, 25. Jesch, E.D., Carr, T.P., 2017. Food Ingredients That Inhibit Cholesterol Absorption.

Preventive Nutrition and Food Science 22, 67-80.

Jing, K., Wu, T., Lim, K., 2013. Omega-3 Polyunsaturated Fatty Acids and Cancer.

Anti-Cancer Agents in Medicinal Chemistry 13, 1162-1177.

Johansson, M.-B., 1995. The chemical composition of needle and leaf litter from Scots pine, Norway spruce and white birch in Scandinavian forests. Forestry 68, 49-62.

Kemppainen, K., Inkinen, J., Uusitalo, J., Nakari-Setälä, T., Siika-aho, M., 2012. Hot water extraction and steam explosion as pretreatments for ethanol production from spruce bark. Bioresource Technology 117, 131-139.

Kilic, A., Hafizoglu, H., Donmez, I.E., Tümen, I., Sivrikaya, H., Hemming, J., Reunen, M., 2013. Lipophilic constituents of some coniferous cones. Bartın Orman Fakültesi Dergisi 15, 122-127.

Krogell, J., Holmbom, B., Pranovich, A., Hemming, J., Willför, S., 2012. Extraction and chemical characterization of Norway Soruce inner and outer bark. Nordic Pulp and Paper Research Journal 27, 6-17.

Latva-Mäenpää, H., Laakso, T., Sarjala, T., Wähälä, K., Saranpää, P., 2013. Variation of stilbene glucosides in bark extracts obtained from roots and stumps of Norway spruce (Picea abies [L.] Karst.). Trees 27, 131-139.

Liu, A., Ji, J., 2014. Omega-3 essential fatty acids therapy for dry eye syndrome: a meta-analysis of randomized controlled studies. Med Sci Monit 20, 1583-1589.

MacKay, D.S., Jones, P.J.H., 2011. Phytosterols in human nutrition: Type, formulation, delivery, and physiological function. European Journal of Lipid Science and Technology 113, 1427-1432.

McCann, S.E., Freudenheim, J.L., Marshall, J.R., Graham, S., 2003. Risk of Human Ovarian Cancer Is Related to Dietary Intake of Selected Nutrients, Phytochemicals and Food Groups. Journal of Nutrition 133, 1937−1942.

McElroy, C.R., Attard, T.M., Farmer, T.J., Gaczynski, A., Thornthwaite, D., Clark, J.H., Hunt, A.J., 2018. Valorization of spruce needle waste via supercritical extraction of waxes and facile isolation of nonacosan-10-ol. Journal of Cleaner Production 171, 557-566.

Mendilaharsu, M., De Stefani, E., Deneo-Pellegrini, H., Carzoglio, J.C., Ronco, A., 1998. Phytosterols and risk of lung cancer: A case-control study in Uruguay. Lung Cancer 21, 37-45.

Miranda, I., Gominho, J., Mirra, I., Pereira, H., 2012. Chemical characterization of barks from Picea abies and Pinus sylvestris after fractioning into different particle sizes. Industrial Crops and Products 36, 395-400.

Orav, A., Kailas, T., Koel, M., 1998. Simultaneous Distillation, Extraction and Supercritical Fluid Extraction for Isolating Volatiles and Other Materials from Conifer Needles. Journal of Essential Oil Research 10, 387-393.

Ottosson, N.E., Wu, X., Nolting, A., Karlsson, U., Lund, P.-E., Ruda, K., Svensson, S., Konradsson, P., Elinder, F., 2015. Resin-acid derivatives as potent electrostatic openers of voltage-gated K channels and suppressors of neuronal excitability.

Scientific Reports 5, 13278.

Park, J.-E., Lee, K.-E., Jung, E., Kang, S., Kim, Y.J., 2016. Sclareol isolated from Salvia officinalis improves facial wrinkles via an antiphotoaging mechanism. Journal of Cosmetic Dermatology 15, 475--483.

Pholosi, A., Ofomaja, A.E., Naidoo, E.B., 2013. Effect of chemical extractants on the biosorptive properties of pine cone powder: Influence on lead(II) removal mechanism. Journal of Saudi Chemical Society 17, 77-86.

Pietarinen, S.P., Willför, S.M., Ahotupa, M.O., Hemming, J.E., Holmbom, B.R., 2006. Knotwood and bark extracts: strong antioxidants from waste materials. Journal of Wood Science 52, 436-444.

Rezaei-Seresht, E., Salimi, A., Mahdavi, B., 2018. Synthesis, antioxidant and antibacterial activity of azo dye-stilbene hybrid compounds. Pigment & Resin Technology.

Rodrigues, F., Pimentel, F.B., Oliveira, M.B.P.P., 2015. Olive by-products: Challenge application in cosmetic industry. Industrial Crops and Products 70, 116-124.

Ronco, A., De Stefani, E., Boffetta, P., Deneo-Pellegrini, H., Mendilaharsu, M., Leborgne, F., 1999. Vegetables, Fruits, and Related Nutrients and Risk of Breast Cancer: A Case-Control Study in Uruguay. Nutrition and Cancer 35, 111-119.

Sahin, H.T., Yalcin, O.U., 2017. Conifer Cones: An Alternative Raw Material for Industry. British Journal of Pharmaceutical Research 17, 1-9.

Salem, M.Z.M., Elansary, H.O., Elkelish, A.A., Zeidler, A., Ali, H.M., Yessoufou, K., 2016a. *In vitro* Bioactivity and Antimicrobal Activity of *Picea abies* and *Larix decidua* Wood and Bark Extracts. BioResources 11, 9421-9437.

Salem, M.Z.M., Elansary, H.O., Elkelish, A.A., Zeidler, A., M. Ali, H., Yessoufou, K., 2016b. *In vitro* Bioactivity and Antimicrobial Activity of *Picea abies* and *Larix decidua* Wood and Bark Extracts. BioResources 11, 9421-9437.

Savluchinske-Feio, S., Marcelo Curto, M.J., Gigante, B., Roseiro, J.C., 2006. Antimicrobal activity of resin acid derivatives. Applied Microbiology and Biotechnology 72, 430-436.

Schwanninger, M., Hinterstoisser, B., 2002. Comparison of the classical wood extraction method using a Soxhlet apparatus with an advanced extraction method. Holz als Roh- und Werkstof 60, 343-346.

Shimizu, H., Ross, R.K., Bernstein, L., Yatani, R., Henderson, B.E., Mack, T.M., 1991.

Cancers of the prostate and breast among Japanese and white immigrants in Los Angeles County. British Journal of Cancer 63, 963-966.

Sin, E.H.K., Marriott, R., Hunt, A.J., H., C.J., 2014. Identification, quantification and Chrastil modelling of wheat straw wax extraction using supercritical carbon dioxide. C. R. Chim. 17, 293-300.

Sirerol, J.A., Rodríguez, M.L., Mena, S., Asensi, M.A., Estrela, J.M., Ortega, A.L., 2016. Role of Natural Stilbenes in the Prevention of Cancer. Oxidative Medicine and Cellular Longevity Article ID 3128951, 15 pages.

Soon, L.K., Chiang, L.K., 2012. Influence of Different Extraction Solvents on Lipophilic Extractives of Acacia Hybrid in Different Wood Portions. Asian Journal of Applied Sciences 5, 107-116.

Stirton, A.J., 1952. Raw Materials for Soap. Saturated and Unsaturated Fats. The Journal of American Oil Chemists' Society, 482-485.

Terés, S., Barceló-Coblijn, G., Benet, M., Álvarez, R., Bressani, R., Halver, J.E., Escribá, P.V., 2008. Oleic acid content is responsible for the reduction in blood pressure induced by olive oil. PNAS 105, 13811-13816.

Ucar, M.B., Uçar, G., 2008. Lipophilic extractives and main components of black pine cones. Chemistry of Natural Compounds, 44, 380-383.

Valto, P., Knuutinen, J., Alen, R., 2012. Overview of avalytical procedures for fatty and resin acids in the papermaking process. BioResources 7, 6041-6076.

Willför, S., Hemming, J., Reunanen, M., Eckerman, C., Holmbom, B., 2003a. Lignans and Lipophilic Extractives in Norway Spruce Knots. Holzforschung 57, 27–36.

Willför, S.M., Ahotupa, M.O., Hemming, J.E., Reunanen, M.H., Eklund, P.C., Sjöholm, R.E., Eckerman, C.S., Pohjamo, S.P., Holmbom, B.R., 2003b. Antioxidant activity of knotwood extractives and phenolic compounds of selected tree species. Agric Food Chem 51, 7600-7606.

Vītiņa, I., Krastiņa, V., Jemeļjanovs, A., Ceriņa, S., Mičulis, J., Aņenkova, R., Lujāne, B., Markovs, K., Daugavietis, M., 2012. Effect of extractive substances from spruce needle biomass on egg production and quality. Chemine Technologia 4, 40-44.

[www.sunpine.se.](http://www.sunpine.se/)

Yu, I.K.M., Attard, T.M., Chen, S.S., Tsang, D.C.W., Hunt, A.J., Jérô me , F.o., Ok, Y.S., Poon, C.S., 2019. Supercritical Carbon Dioxide Extraction of Value-Added Products and Thermochemical Synthesis of Platform Chemicals from Food Waste. ACS Sustainable Chemistry Engineering 7, 2821-2829.

Örså, F., Holmbom, B., 1994. A convenient method for the determination of wood extractives in papermaking process waters and effluents. Journal of Pulp Paper Science 20, 361-366.

**Supporting information**

[**Click here to download Supplementary Interactive Plot Data (CSV): Supporting information.pdf**](http://ees.elsevier.com/indcro/download.aspx?id=909887&guid=5380367f-fbeb-4cef-af3d-af17d1d88b3d&scheme=1)