Triterpene alcohols and sterols of Siberian larch (Larix sibirica Ldb.) needles

D S Mikson, V I Roshchin
St. Petersburg State Forest-Technical University, 5 Institutskiy Lane, St. Petersburg 194021, Russian Federation
*Corresponding email: ms.mikson@mail.ru

Abstract. The composition of triterpene alcohols and sterols in the fraction of Siberian larch (Larix sibirica Ldb.) conifer needles is considered in paper. The needles are extracted with propan-2-ol; resinous substances are extracted with petroleum ether (40–70°C); substances extracted with petroleum ether are divided into free acids and neutral substances. Neutral substances are separated in a silica gel column; eluent - petroleum ether supplemented with diethyl ether. The isolated esters fraction is hydrolyzed with potassium hydroxide in ethanol, and the “bound” acids are separated from unsaponifiable substances. Non-saponified substances are separated by chromatography into 5 fractions of phytosterols, the alcohol components of esters. The fractions are acetylated; the alcohol acetates are separated on silica gel with added silver nitrate. The isolated compounds was identified by NMR and GC-MS methods. Cycloartenol (6.82%, hereafter based on unsaponifiable substances), 24-methylenecycloartenol (6.75%), citrastadienol (7.69%), sitosterol (7.18%), cycloevcalenol (0.09%) are identified, obtussifoliol (0.01%), 24-methyleneophenol (0.10%), methyllophenol (1.84%) and campesterol (0.33%).

1. Introduction
The development of integrated and wasteless technologies for raw material processing is one of the modern society priorities. The synthetic substances are used in most cases for producing medicinal, cosmetic preparations, as well as the products used in various branches of agriculture and animal breeding. They do not always have a positive effect, and sometimes harm a human body, as well as the environment.

The tree crown is the center of the main photo- and biosynthetic nutrient processes, which can be the basis for producing biologically active substances. The technologies of integrated wood biomass application which includes the wood greens processing are developed [1, 2]. Currently, there are only a few operating enterprises located in Russia and Latvia. But the society is increasingly expressing interest in forest products in order to produce environmentally friendly products.

The wood greens of such trees as pine, spruce and fir are used for processing purposes. No technologies of wood green processing are developed for the main tree species in Russia, namely, the Siberian larch (Larix sibirica Ldb.) [3]. Its main feature is the dumping of larch needles in autumn-winter period. It causes variability of raw materials (covered summer shoots, and branches and shoots without needles in autumn-winter period). That is why no interest is paid to process the larch green on an industrial scale, as well as notolaboratory research on the composition of their compounds is carried out.
The purpose of this research is to study the composition of triterpene alcohols and sterols founds esters in the Siberian larch needles.

2. Experimental part
The needles of Siberian larch (Larix sibirica Ldb.) during the summer vegetation season were the object of study [4]. The selected wood green samples were manually divided into the following groups: pine needles, bark and wood parts. The ratio of the parts is 4: 2: 1, respectively.

The shredded needles was characterized by the content of extractives obtained with the help of organic solvents with increasing polarity: petroleum ether (PE), diethyl ether (DE), ethyl acetate (EA) and propan-2-ol (IP), according to the standard method in the Soxhlet extractor [5] (50 ml volume), followed by removal the solvent and drying the extract to the constant weight.

The production of researched extractive substances is carried out in the Soxhlet extractor (1000 ml volume, 8 h extraction time, hydromodule 1: 5) according to the method [5] using propan-2-ol as the solvent. All alcohol is distilled off from the obtained IP extract under vacuum. The IP-extract for isolating the non-polar group of resinous substances was exhaustively extracted with PE (b.p. - 40…70°C). Extraction is carried out with PE portions in a water bath (temperature 55…58°C) in a round-bottomed flask and vigorous stirring. After extraction the content was settled in the flask, the upper layer (PE soluble substances) was decanted, and the lower layer (PE insoluble substances) was again extracted with PE portions. In total the extraction module was 1:15; finally the ether was distilled off from the extract in a rotary evaporator and dried to constant weight upon completing the extraction process.

The application of this extraction method allows to obtain the yield of extractive substances in qualitative and quantitative composition close to those extracted by hydrocarbon extractant in industrial devices of irrigation-reflux type [6].

The PI extract substances which are soluble in PE according to the acid-base scheme [6] were divided into neutral substances and free acids. The sum of neutral substances was divided into fractions which differs in the polarity of containing compounds by column chromatography. In the research, the Merck silica gel with 40–60 μm grain size, an eluent — PE with the DE addition (1% to 50%), DE and ethanol are used as the stationary phase. For the control and high-quality separation of fractions, the thin-layer chromatography (TLC) is used applying the following standards: squalene, fat fraction, bornyl acetate, sitosterol. The plates were developed in an iodine chamber. In some cases the plates are additionally sprayed with H2SO4 and developed at temperature of 105±2°C in a drying cabinet.

The ester fraction was treated with alcoholic alkali (0.5 M KOH in ethanol) for 30 minutes. The obtained extract was separated according to the scheme [6] in order to obtain the products of ester hydrolysis - the alcohol component of esters (unsaponifiable substances) and “bound” acids.

The unsaponifiable part of the esters was separated in the silica gel column to isolate individual fractions: the fraction, which does not change the Rf value at TLC after hydrolysis was eluted first, and the fraction of alcohols - the products of ester hydrolysis, was eluted later.

In order to isolate and purify individual compounds, the triterpene alcohol fraction in the form of acetates was re-chromatographed in the silica gel column impregnated with AgNO3. The triterpene alcohol acetates were acetylated with acetic anhydride in pyridine. The isolated fractions were indentified using instrumental methods of analysis [7].

The IR spectra of esters and unsaponifiable substances were recorded with the help of the Shimadzu FTIR-8400Sc IR Fourier spectrophotometer using KBr tablets or on the ATR-impaired total internal reflection diamond attachment.

The isolated fractions and compounds were analyzed by gas chromatography-mass spectrometry using the Agilent Technologies 6850A gas chromatograph and the Agilent Technologies 5973N quadrupole mass spectrometer, the HP-5MS standard quartz capillary column, 30 m long and 0.25 mm internal diameter, the 0.25 μm stationary phase film thickness with 1: 100 flow separation. The
The thermostat temperature was programmed from 150°C to 280°C with the 5°C/min flow rate, exposing for 20 min at 280°C.

The NMR 1H and 13C individual compound spectra were recorded using the “JEOL JNM-ECX400A” device in CDCl3, for 1H – 399.9 MHz, for 13C - 100 MHz.

3. Discussion

The initial needles are subdivided according to their content of extractive substances (ES) obtained by organic solvents (Table 1).

| Substances extracted by: | The ES content, % by weight of dry raw materials (needles) |
|--------------------------|-----------------------------------------------------------|
| petroleum ether          | 2.5                                                       |
| diethyl ether            | 7.2                                                       |
| ethyl acetate            | 12.6                                                      |
| propane-2-ol             | 32.8                                                      |

As it follows from the data obtained, the ES yield is increased with increasing the solvent polarity. The highest ES extraction percentage value is achieved by extracting needles with propan-2-ol. According to the ES content extracted by the hydrocarbon extractant (PE), larch is inferior to spruce (7.8%, hereafter % by dry raw material weight), pine (15.0%) and fir (10.1%) [8]. When the scheme of extracting resinous PE substances from the PI extract [6] is used, the PE part yield is 21.0% by the IP extract weight and 6.9% by the dry raw material weight. The group compositions (Table 2) of the IP extract petroleum-soluble part reveal that the Siberian larch needles contain a lot of waxes, which is a distinctive feature of larch.

| Groups of substances          | Content, % by PE needle extract weight |
|-------------------------------|---------------------------------------|
| Wax                           | 8.0                                   |
| Free acids                    | 31.5                                  |
| Neutral substances, including:|                                       |
| unsaponifiable substances     | 43.1                                  |
| “bound” acids                 | 16.4                                  |

Neutral substances are divided into fractions using the column chromatography method. The fraction of hydrocarbons (2.4% of neutral needle substances) is eluted the first from the chromatographic column. The substances with Rf values greater - or equal to the Rf value of the reference squalene are selected into this fraction.

The carotene fraction is eluted after the hydrocarbon fraction, followed by the ester fraction. There are several types of esters presented in wood greens: methyl esters of resin acids, fats, aliphatic esters, triterpene alcohols and sterols with higher fatty acids, acetates of isoprenoid alcohols. The ester fractions make up 31.3% of the needle neutral substances. After the alkaline hydrolysis was carried out and the unsaponifiable substances (86.1%, here in after % of the ester fraction) and “bound” acids (9.1%) were extracted, the unsaponifiable substances were divided into a series of fractions in a silica gel column. The triterpene alcohol fractions were eluted from the chromatographic column after polyprenols, alongside with aliphatic alcohols. The isolated fractions were re-separated in a chromatographic column using an additive in the AgNO3 sorbent to isolate individual compounds. The fraction containing sitosterol and campesterol were further recrystallized in ethyl acetate in the cold to purify sitosterol from impurities. The solvent is decanted from the crystals; the crystals are dried from
ethyl acetate residue in a rotary evaporator. In total, phytosterols (Table 3) make up 30.81% of the unsaponifiable part of esters.

The hypothetical scheme of formation of basic sterols in Siberian larch needles from cycloartenol is presented in Figure 1.

Figure 1. Hypothetical biosynthesis scheme of main sterols in Siberian larch needles.
Table 3. Extracted triterpene alcohols and sterols from Siberian larch (Larix sibirica Ldb.) needles.

| No. | Component             | Content, % of unsaponifiable substances | Structure establishing method |
|-----|-----------------------|----------------------------------------|-------------------------------|
| 1   | Cycloartenol          | 6.82                                   | GC-MS, NMR                    |
| 2   | Cycloevacalenol       | 0.09                                   | NMR                           |
| 3   | Obtuzifoliol          | 0.01                                   | NMR                           |
| 4   | 24-Methylencycloartanol | 6.75                                 | GC-MS, NMR                    |
| 5   | Citrastadienol        | 7.69                                   | NMR                           |
| 6   | Methylphenol          | 1.84                                   | NMR                           |
| 7   | 24-Methylenolphenol   | 0.1                                    | NMR                           |
| 8   | Campesterol           | 0.33                                   | GC-MS, NMR                    |
| 9   | β-Sitosterol          | 7.18                                   | GC-MS, NMR                    |
|     | **Total:**            | **30.81**                              |                               |

As it follows from the research results of triterpene alcohols and sterols of Siberian larch (Larix sibirica Ldb.) conifer needle esters, the main compounds are usual for conifers [9,10] (pine, spruce, fir) cycloartenol, 24-methylene cycloartanol, citrastadienol, sitosterol and campesterol. In addition to these compounds, cycloevacalenol, obtuzifoliol, 24-methylene lophenol, and methyl lophenol are identified in minor amounts. Also, these compounds are more often identified in deciduous woody plants, shrubs or herbaceous plants.

4. Conclusion

The composition of triterpene alcohols and sterols of Siberian larch (Larix sibirica Ldb.) needles esters has been studied for the first time. The presence of cycloartenol, 24-methylene cycloartanol, cycloevacalenol, obtuzifoliol, citrastadienol, 24-methylene lophenol, and methyl lophenol in the composition of Siberian larch needles is established.

A hypothetical scheme for the formation of sitosterol and campesterol from cycloartenol has been shown.

References

1. Yagodin V I, Vyrodov V A 1996 Technology green wood (SPb.: LTA) p 92
2. Levin E D, Repyakh S M 1984 Processing of green wood (Moscow: Forestry) p 120
3. Milyutin L I 2003 Biodiversity of larches of Russia Coniferous boreal zone 1 pp 6-9
4. Mikson D S and Roshchin V I 2016 Composition of free and “bound” acids from the needles of Siberian larch Forests of Russia. Volume 2 / ed. V.M. Gedyo. (Saint-Petersburg: SPBFTU) pp 35-38
5. Obolenskaya A V, Elnitskaya Z P, Leonovich AA 1991 Laboratory work on chemistry of wood and cellulose (Moscow: Ecology) pp 75-164
6. Roshchin V I, Baranova R A, Beloozerskikh and O A Soloviev V A 1983 The composition of extractive substances of needles and shoots of European spruce Wood Chemistry 4 pp 56-61
7. Prech E, Bulmann F, Affölter K 2006 Determination of the Structure of Organic Compounds (Moscow: Mir) 438
8. Roshchin V I, Kolodynskaya L A, Baranova P A and Nagibina N Y 1985 Composition of extractive substances of wood greens of Siberian fir Chemistry of wood 5 pp 96-105
9. Malcolm J. Thompson, Samson R. Dutky 1972 NMR spectra of C-24 isomeric sterols Phytochemistry 11 pp 1781-179
10. Heftman E. 1972 Biochemistry of steroids (Moscow: Mir) p 175