Chemical Composition of Lipophilic Extractives from Grey Alder (*Alnus incana*)

Oskars Bikovens,[a] Liga Roze,[a,b] Andrey Pranovich,[c] Markku Reunanen,[c] and Galina Telysheva [a,*]

The chemical composition of the lipophilic extractives in the hexane extracts from grey alder bark, knotwood, and cones has been investigated by gas chromatography and gas chromatography-mass spectrometry. The efficiency of two extraction methods was compared. The highest amount of lipophilic extractives (about 9% of o.d. material) was observed in grey alder cone, while the lowest (about 3%) was found in knotwood. The three different morphological parts of alder showed significant differences not only in the content but also in composition of extractives, namely fatty acids, triglycerides, and triterpenes. The main identified compounds were triterpenoids (lupen-3-one, lupeol, betulone, betulinol, and betulinic acid) in bark, and triglycerides in cones. The major group in knotwood was free fatty acids (mainly linoleic acid, 18:2).

Keywords: Grey alder; Bark; Cones; Knotwood; Lipophilic extractives; Lupane triterpenoids

*Contact information: a: Latvian State Institute of Wood Chemistry, Laboratory of Lignin Chemistry, Riga, Latvia; b: University of Latvia, Faculty of Chemistry, Riga, Latvia; c: Åbo Akademi University, Laboratory of Wood and Paper Chemistry, Turku, Finland; *Corresponding author: ligno@edi.lv*

INTRODUCTION

Alnus incana (L.) Moench. (Betulaceae family), commonly known as grey alder, is a deciduous tree that is widely distributed in northern Europe. The stands of grey alder comprise 9.8% of the total forest area of Latvia (Latvian Central Statistical Bureau 2012). In the course of processing grey alder, huge amounts of logging waste in forestry (branches with foliage and cones), and wood mechanical processing wastes (bark, sawdust *etc.*) are obtained. Bark content varies from 2 to 4% up to 10% of tree biomass, depending on tree species and age. A majority of these wastes is used as a low calorific fuel, and only a small part is used for further processing. However, they could be a potential source of valuable green chemicals, including biologically active compounds. For example, it is possible to exploit high-value low molecular weight compounds, such as phytosterols (Fernandes and Cabral 2007) and lignans (Pietarinen *et al.* 2006) from by-products of the industrial processing (bark, knotwood, *etc.*). The importance of biologically active natural compounds and plant potential as a source of green chemicals has received fresh appreciation in recent years. Investigation in this area has become one of the most active research fields (Li and Vederas 2009).

Various parts of some alders, such as bark, flowers, cone, and leaves, have been used in folk medicine as remedies for fever, hemorrhage, and alcoholism (Tung *et al*. 2010). The most characteristic feature of the *Alnus* genus is the occurrence of large quantities of diarylheptanoids and their glycosides in different morphological parts of the tree. Earlier it has already been explored and reported that there is a great potential to use

grey alder bark as a source of green chemicals, namely, hydrophilic polyphenols – diarylheptanoids and condensed tannins (Felfoldi-Gava *et al*. 2012; Telysheva *et al*. 2011). The predominant diarylheptanoid in grey alder bark is oregonin, and its concentration can reach *ca.* 7% based on o.d. matter (Telysheva *et al*. 2011). However, for isolation of the mentioned hydrophilic polyphenols, a substantial amount of lipophilic extractives can also be obtained as a by-product. In the biorefinery context, it is necessary to consider the opportunity for application of lipophilic extractives in order to minimize waste streams.

The composition and amount of the extractives are dependent on tree species, morphological part of the tree, tree age, season, and location of tree in the forest. The extractives consist of mixtures of various components, from relatively low-molar-mass fatty acids to the higher molar-mass sterol esters and triglycerides. Various types of non-polar plant secondary metabolites including free fatty acids, triterpenoids, sterols, and stilbenes have been found in stem wood, bark, flowers, and leaves of different *Alnus* species (Phan *et al*. 2011; Stutz and Burris 1951; Suga *et al*. 1972; Tori *et al*. 1995). It is known that physical properties and chemical composition of tree knots are different from those of stem wood. The composition of lipophilic extractives of grey alder knots has not been reported in detail before. Despite the wide distribution of grey alder, the number of studies of chemical composition of lipophilic extractives in its various morphological parts is low and fragmentary.

The aims of the present work are to analyze and compare the amount and chemical composition of lipophilic extractives in different morphological parts of grey alder, *i.e*. bark, cones, and living knotwood, using gas-chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) before and after alkaline hydrolysis, in order to detect free and esterified components.

EXPERIMENTAL

Materials

Four healthy grey alder (*Alnus incana*) trees were felled in the central part of Latvia, Ogre district (N latitude 56° 49′, E longitude 24° 36′) in October 2010, at the age of approximately 30 years. The alder cones were randomly handpicked from the branches felled. The knotwood of living branches were sawn out from stems at the height of 3 to 6 m. Average branches diameter at the stem base was 3 to 4 cm. Stem bark was sampled from the same trees as knotwood.

All samples were air-dried at ambient temperature and ground before extraction using a Wiley mill to pass a 420 μm sieve.

Methods

Extraction

All powdered samples (5 g) were extracted with *n*-hexane using two different extraction methods: Soxhlet and accelerated solvent extraction (ASE). The Soxhlet extraction was performed at ambient pressure for 8 h. ASE300 extractor (Dionex Corp.) was used for extraction with *n*-hexane at temperature 90°C, pressure 13.8 MPa, 3x5 min static cycles under nitrogen atmosphere. The hexane extracts were evaporated to dryness,

and the extracts were weighed. Gravimetrical amounts were reported as weight percent (%) and calculated on an oven-dried matter (o.d.m.) basis.

Alkaline hydrolysis and derivatization

Alkaline hydrolysis of evaporated lipophilic extractives was performed using 0.5 M KOH solution in 90% aqueous ethanol. The solutions were allowed to stand for 5 h at 70°C. After this, distilled water was added. The solutions were acidified to pH *ca*. 3 with 30% H_3PO_4. The acid and neutral components were extracted three times with methyl *tert*-butyl ether. The organic fractions were combined and evaporated (Ekman 1983).

After evaporation, aliquots of hydrolyzed and non-hydrolyzed hexane extracts (*ca.* 0.5 mg) were silylated with 120 µL of pyridine:N,O-bis-trimethylsilyl trifluoroacetamide: trimethyl chlorosilane mixture (1:4:1 v/v/v) and kept at 70°C for 45 minutes. Then the sample was cooled down to room temperature before GC-MS analysis. The silylated derivatives were analyzed using GC-FID and GC-MS before and after hydrolysis as described below.

GC-FID and GC-MS analysis

Silylated fatty acids, fatty alcohols, sterols, and fatty acid monoglycerides were analyzed with a Perkin Elmer AutoSystem XL Gas Chromatograph with a flame ionization detector (GC-FID) using a 25 m x 0.20 mm i.d. column coated with cross-linked methyl polysiloxane (HP-1) with a film thickness of 0.11 µm (Ekman and Holmbom 1989). Cholesterol, 1,3-dihexadecanoyl-2-(*cis*-9-octadecenoyl)glycerol, heneicosanoic acid, and cholesteryl heptadecanoate were used as internal standards. No FID correction factors were used. Sterol esters, di-, and tri-glycerides were analyzed on a Perkin Elmer Clarus 500 Gas Chromatograph using a 6 m x 0.53 mm i.d. DB-1 column covered with a film of 0.15 µm thickness (Örså and Holmbom 1994). The limit of quantification was about 0.01 mg/g. Therefore, compounds present in samples at lower amounts could be detected only qualitatively, and their presence is designed as "trace" in the Table 2. Identification of individual components was performed using GC with a mass spectrometric detector (HP 6890-5973 GC-MSD instrument) and a similar 25 m HP-1 GC column as described above for analysis of the silylated components. The compounds were identified as silylated derivatives, by comparing their retention times and mass spectra with the GC-MS spectral library, and data from the literature (Ekman 1983). All results, given in mg/g, are calculated on an oven-dried matter (o.d.m.) basis.

All quantitative analysis were performed in triplicate, and the variability between analyses was lower than 5%.

RESULTS AND DISCUSSION

The yields of hexane extracts obtained with Soxhlet and ASE extraction are compared in Table 1. Both showed similar results; however, according to Shen and Shao (2005), it could be expected that the Soxhlet method will give higher extraction efficiency for non-polar compounds. The highest gravimetric amount of lipophilic extractives (> 9% o.d.m.) was obtained from grey alder cones. It was found to be two times higher than other parts (Table 1). The stem wood contained lower amounts of extractives (0.9% o.d.m.) than knotwood, bark, and cones; therefore the chemical

composition of stem wood lipophilic extractives was not analyzed in detail in the present study.

Table 1. Yield of Lipophilic Extractives from *Alnus incana* (% of o.d.m. ± S_n)

	Soxhlet extraction	ASE extraction
Bark	4.5 ± 0.2	4.4 ± 0.1
Knotwood	3.2 ± 0.1	3.7 ± 0.1
Stem wood	0.9 ± 0.1	n.d.*
Cones	9.0 ± 0.5	9.5 ± 0.2

* n.d. – not determined

The chemical composition of the hexane extracts showed significant differences in different parts of the alder. A chromatogram of the hexane extract is presented in Fig. 1, and qualitative and quantitative composition is shown in Table 2. Triterpenoids, sterol esters, fatty acids, and triglycerides were the main groups.

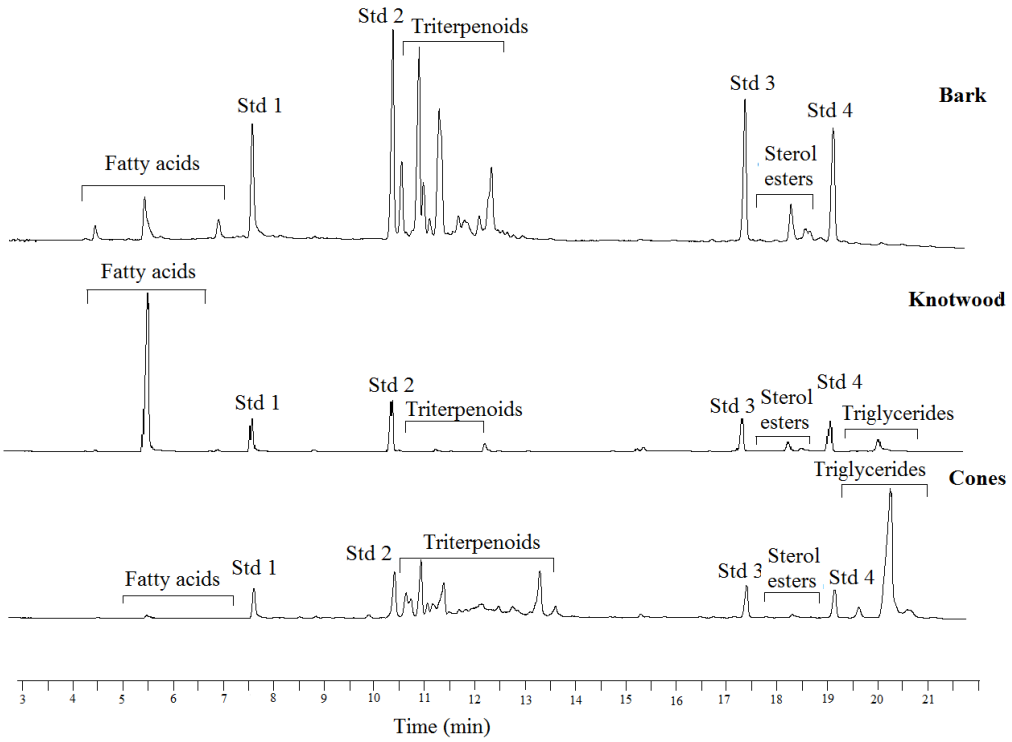

Fig. 1. GC chromatogram of lipophilic extractives of bark, knotwood, and cones of *Alnus incana* (short column, 6m). Internal standards: Std 1 = heneicosanoic acid, Std 2 = cholesterol, Std 3 = cholesterol heptadecanoate, Std 4 = 1,3-dihexadecanoyl-2-(*cis*-9-octadecenoyl)glycerol

The triglycerides are the main storage lipids in plants, and they were accumulated in the cones. Pentacyclic lupane-type triterpenoids predominated in the extract of bark. The amount of free fatty acids was significantly higher in the extract of knotwood. Other compounds such as fatty alcohols and alkanes were present in small amounts in all samples.

Table 2. Chemical Composition of Hexane Extracts of Different Morphological Parts of *Alnus incana* (g/kg of o.d.m.)

Rt, min	Compound	Bark	Knotwood	Cones
	Fatty acids (FA)			
	Saturated			
10.3	Tetradecanoic acid	trace	trace	trace
13.2	Hexadecanoic acid	0.12	0.05	trace
14.6	Heptadecanoic acid	0.01	0.01	0.05
16.0	Octadecanoic acid	0.02	-	trace
18.5	Eicosanoic acid	0.01	0.01	0.01
20.9	Docosanoic acid	0.02	0.02	0.02
23.1	Tetracosanoic acid	0.01	0.01	0.04
25.2	Hexacosanoic acid	0.01	trace	0.01
	Unsaturated			
12.8	Hexadecenoic acid	0.01	0.01	trace
15.4	Octadecadienoic acid	0.33	3.97	0.13
15.5	Octadec-9-enoic acid	0.08	0.15	0.04
15.6	Octadec-11-enoic acid	0.03	0.04	0.01
18.1	Eicosenoic acid	trace	0.01	trace
	Other fatty acids			
18.3	Hydroxyoctadecadienoic acid	0.11	0.05	0.01
25.6	1,22-Docosanedioic acid	0.02	0.01	0.03
	Sum FA	**0.78**	**4.34**	**0.35**
	Fatty alcohols (FAl)			
20.0	Docosan-1-ol	0.01	trace	0.02
22.2	Tetracosan-1-ol	0.01	trace	0.03
24.4	Hexacosan-1-ol	trace		0.06
	Sum FAl`s	**0.02**	**trace**	**0.11**
	Glycerides			
20.5	Hexadecanoyl monoglycerol	0.01	trace	0.01
22.3	Octadecadienoyl monoglycerol	0.01	0.03	0.01
*	Diglycerides	0.02	0.02	0.2
*	Triglyceride	-	0.86	4.75
	Sum glycerides	**0.04**	**0.91**	**4.97**
	Other components			
24.7	Tocopherol	trace	trace	0.03
21.6	Heptacosane	trace	trace	0.06
	Triterpenes			
27.6	Lupen-3-one	0.75	-	0.96
28.4	Lupeol	0.34	trace	0.56
29.4	Betulone	0.04	trace	0.01
30.0	Betulinol	0.16	0.01	0.02
30.2	Betulinic Acid	0.24	0.01	0.03
	Sum triterpenes	**1.53**	**0.02**	**1.58**
	Sterols			
28.0	Sitosterol	**0.22**	**0.04**	**0.19**
	Total sum	**2.6**	**5.3**	**7.4**

* - were determined on short (6 m) column. Rt. - diglycerides: 15.6 min, triglycerides: 19.5-21 min.

The major groups of compounds identified in the bark lipophilic extracts were lupane-type triterpenoids (lupeol [lup-20(29)-en-3β-ol], lupen-3-one, betulinol [lup-20(29)-ene-3β,28-diol], betulone, and betulinic acid), sterol esters, and fatty acids,

followed by minor amounts of diglycerides and aliphatic alcohols. The chemical composition profile of grey alder bark lipophilic extract was similar to that previously reported for common alder (*Alnus glutinosa*) (Felfoldi-Gava *et al*. 2009). However, the yield of lipophilic extractives (*ca.* 4.5%, Table 1) found for *A. incana* bark was higher in comparison with values (*ca.* 2.3% and 3.5%) reported for *A. glutinosa* (Felfoldi-Gava *et al*. 2009, 2012).

The knotwood extract contained the highest amounts of free fatty acids: *ca*. 80% of all identified compounds (Table 2). The short-chain fatty acids (C14-C20) predominated over the long-chain fatty acids (C22-C26). Octadecadienoic or linoleic acid was the dominating fatty acid. The profile of fatty acids in grey alder knotwood was similar with the composition in trees of the Betulaceae family relative to fatty acid composition (Freire *et al*. 2006). Various glycerides were the second largest group of compounds identified in lipophilic extract of knotwood.

The quantitative characteristics of lipophilic extractives of grey alder cone showed that triglycerides were the major group of compounds. Triglycerides constituted about 60% of all GC-eluted and identified compounds in the grey alder cones. Triterpenoids was the second most abundant group of compounds, accounting for about one-fifth part of all identified lipophilic compound of grey alder cones. Chemical composition of alder cone and bark triterpenes were similar, but cones contained slightly larger amounts of lupen-3-one and lupane than the bark.

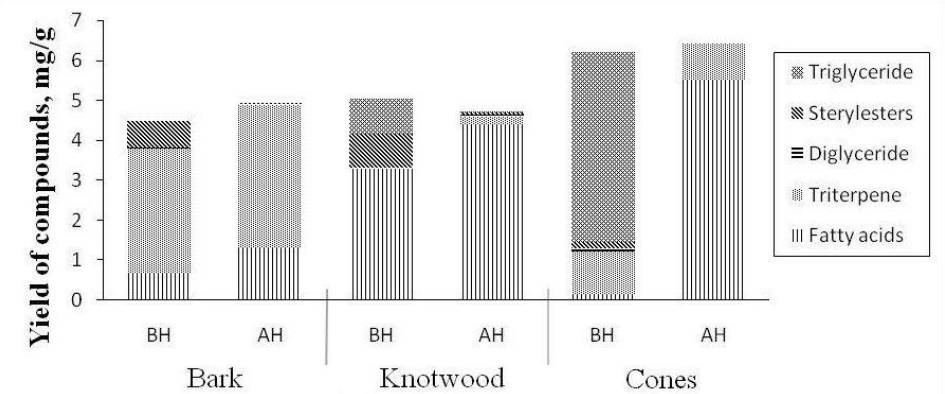

Fig. 2. Major groups of compounds in the hexane extract of *Alnus incana* before (BH) and after (AH) alkaline hydrolysis (mg/g of o.d.m.)

All extracts were hydrolyzed in order to estimate the concentration of esterified compounds. A large increase in the total amount of free fatty acids detected by GC-MS was the result of alkaline hydrolysis of esters. Fatty alcohols represented a small portion of the total extractives analyzed by GC-MS before and after hydrolysis. These results provide evidence of the presence of significant amounts of esterified compounds, such as di- and triglycerides and also sterol esters, in the original extracts.

The total amount of pentacyclic lupane-type triterpenoids in bark and cones were almost the same (1.5 to 1.6 g/kg). They are well known as biologically active compounds (Jagan and Chinthalapally 2012; Wal *et al*. 2011). Triterpenoid content in grey alder bark and cones is lower in comparison with values reported for barks of industrially important *Betula* (up to 92 g/kg of lupeol) (Diouf *et al.* 2009) and *Eucalyptus* species (4.5 to 21.6 g/kg) (Domingues *et al.* 2011), but taking into account that grey alder bark contain significant amounts of valuable diarylheptanoids and tannins, it could be possible to

develop further processing to obtain triterpenoids from grey alder bark in the context of bark biorefinery with extraction of value-added diarylheptanoids and tannins (Telysheva *et al*. 2011).

The obtained results create background for future development of grey alder bark biorefinery with additional usage of isolated extractives for their possible application in medicine, cosmetics, and as bioactive agents in the pharmaceutical industry.

CONCLUSIONS

The yields of the hexane extractives from various morphological parts of *Alnus incana* were found to differ significantly. The highest content of these extractives (about 9% of o.d. material) was observed in the cones, while the lowest (about 3%) was found in knotwood.

The main group of lipophilic extracts found in the cones was triglycerides, whereas the main group in knotwood extract was free fatty acids (mainly, octadeca-dienoic or linoleic acid). The dominant groups identified in the bark lipophilic extract were triterpenoids: lupen-3-one, lupeol, betulone, betulinol, and betulinic acid. Sterol esters, fatty acids, di-, tri-glycerides, and aliphatic alcohols were found in minor amounts.

The bark could represent a prospective raw material for producing valuable chemicals. Pentacyclic lupane-type triterpenoids, known as high potential biological active substances, could be obtained as by-products of diarylheptanoid and/or tannin extraction from alder bark.

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