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Chemical characterization of high-molar-mass fractions in a Norway spruce knotwood ethanol extract

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ABSTRACT

The low-molar-mass (LMM) fraction, only, i.e., the GC-eluting compounds, which are mainly lignans, has been characterized in Norway spruce knotwood hydrophilic extracts previously. Of this fraction, many lignans and sesquilignans and all GC peaks supposedly representing dilignans remain unidentified. In this work, dilignans and the GC non-eluting compounds (the high-molar mass fractions, HMM) were characterized in a 7-hydroxymatairesinol-reduced knotwood ethanol extract of Norway spruce by using several fractionation and analytical techniques. A methyl *tert*-butyl ether (MTBE) insoluble fraction of the extract contained mainly HMM material, of which the main part was shown to consist of lignan oligomers. The oligolignans (with a molar mass up to approximately 3700 Da) seemed to be linked by 5–5′ bonds, some of them containing one or two guaiacylglycerol ether units linked to the lignan by β–O–4 or β–5 bonds. Several oligolignans were identified or tentatively identified. The MTBE soluble fraction, which accounted for the major part (81%) of the extract, contained mainly LMM material (lignans, sesqui- and dilignans). The part of the HMM material in the MTBE soluble fraction that was easily isolable (2%) seemed to contain polymers of fatty acids and alcohols, resin acids, and sterols.

1. Introduction

Lignans are natural polyphenols with health-promoting effects, and they are widely occurring in the plant kingdom. By definition, they consist of two phenylpropanoid structures linked together by β–β bonds (Ayres and Loike, 1990). In softwood species, the lignans consist of guaiacyl units, and knotwood of Norway spruce [*Picea abies* (L.) H. Karst], especially, has been shown to contain a substantial amount of lignans – up to 20% (of dry wood) of the dominating lignan, 7-hydroxymatairesinol (HMR) (Willför et al., 2003). In addition, Norway spruce knots have been found to contain lignan oligomers (oligolignans) (Willför et al., 2004). The lignans, to which a β–O–4-linked guaiacylglycerol unit was attached, we called sesquilignans (according to IUPAC, lignan oligomers are named analogously with terpenoids, and lignans with three C_6C_3 units should be called sesquineolignans; IUPAC Recommendations, 2000). Several sesquilignans were characterized, and dilignans were tentatively identified as two lignans linked by 5–5′ bonds (Willför et al., 2004). The 5–5′-linked dilignans are similar to those derived by DPPH (2,2-diphenyl-1-picrylhydrazyl) coupling of pure lignans (Eklund et al., 2005; Smeds et al., 2012a). Besides a small amount of lipophilic extractives, the total proportion of GC-eluting compounds in Norway spruce knots, here called low-molar-mass (LMM) material, i.e., lignans, sesqui-, and dilignans, was determined to be at most 29% of dry wood (Willför et al., 2003). This means that at least 70% of the compounds are GC-non-eluting compounds, here called high-molar-mass material (HMM) (molar mass > ca. 1000 Da).

Previously, some oligolignans have been identified in stems of the hardwood species *Cerbera* (Abe et al., 1988, 1989). The dilignans, the sesterlignans (dilignans containing a β–O–4-linked guaiacylglycerol unit), and the trilignans were coupled by 5–5′ bonds. Furthermore, some sesqui- and dilignans have been identified in the herb *Campylotropis hirtella* (Han et al., 2008). The dilignans consisted of syringaresinol-type lignans coupled to two guaiacylglycerol units by β–O–4 bonds. Similar dilignans were also identified in the hardwood shrubs *Hedyotis lawsoniae* (Matsuda et al., 1984) and *Tarenna attenuate* (Yang et al., 2007), and in rye bran (Hanhineva et al., 2012). 5–5′-linked and a 5–O–4′ linked dilignans were identified in roots of the hardwood shrub *Wikstroemia indica*

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(Wang et al., 2012). In the softwood species *Larix leptolepis*, five phenylpropane trimers and one tetramer were identified (Sakakibara et al., 1987). Those oligolignans differ from the previously mentioned ones by consisting of neolignans or two or three guaiacylglycerol units linked to each other.

Brauns' native lignin is derived from wood meal by extraction at room temperature in neutral alcoholic solvents (Brauns, 1939). It was suggested that Brauns' lignin from softwoods consists partly or entirely of oligolignans because, among other things, the degradation products of isolated protolignins are very similar to the lignans identified in *L. leptolepis* (Sakakibara et al., 1987; Hiltunen et al., 2006). Brauns' lignin from hardwood (birch), was shown to consist of guaiacyl- and syringylpropane units mainly with β–O–4 and β–β side chain structures (Hiltunen et al., 2006). The oligolignan material isolated in the present study from a Norway spruce knotwood ethanol extract may also be classified as some kind of Brauns' lignin.

The HMM material was isolated from an HMR-reduced Norway spruce knotwood ethanol extract and characterized by several advanced methods, i.e., high-performance size-exclusion chromatography coupled to an evaporative light-scattering detector (HPSEC-ELSD), electrospray ionization-ion trap-mass spectrometry (ESI-IT-MS), introducing the samples using syringe infusion or high-performance liquid chromatography (HPLC), syringe infusion ESI-quadrupole time-of-flight (QTOF)-high-resolution (HR)-MS, pyrolysis (py)-GC-MS, thermally assisted hydrolysis and methylation (THM)-GC-MS, and NMR. We find it important to characterize the HMM material in hydrophilic Norway spruce knotwood extracts because these compounds may account for up to 70% of the extractives, and because the possible use of these knot extracts in human health applications calls for identifying 100% of the material, not just 30%.

2. Results and discussion

2.1. An overview of the fractionation and analysis of the HMR-reduced knotwood extract

An advanced fractionation of the HMR-reduced knotwood ethanol extract, outlined in Fig. 1, was necessary in order to isolate and separate the HMM material, which was shown to occur as a complex mixture in the extract, into purer and less complex fractions.

The extract was analyzed by HPSEC-ELSD in order to determine the molar mass distribution before any fractionation. This method was a key method which was used in all fractionation steps in order to follow changes in the molar mass distribution. Furthermore, the amount or existence of GC-eluting compounds was followed up by short-column (6–7 m) GC-FID in all steps. Also GC-MS analysis (long column, 25 m) was performed on some fractions.

A dry extract was impossible to obtain, even after drying in a vacuum oven overnight, why it was extracted with an organic solvent. The solvent chosen was methyl *tert*-butyl ether (MTBE) because it is a strong medium-polar solvent. It appeared that part of the material was insoluble in MTBE, forming dark-brown "pitch" lumps. These lumps, called the "MTBE insoluble fraction" were isolated and treated separately from the "MTBE soluble fraction", which accounted for most of the material in the HMR-reduced extract. The fractions were dried by evaporation in a rotating vacuum evaporator and in a vacuum oven and analyzed by py- and THM-GC-MS, which are convenient methods for characterization of HMM material.

The two fractions were then further fractionated by gel-permeation chromatography (GPC) in order to separate them into subfractions with different molar mass distributions. Subfractions

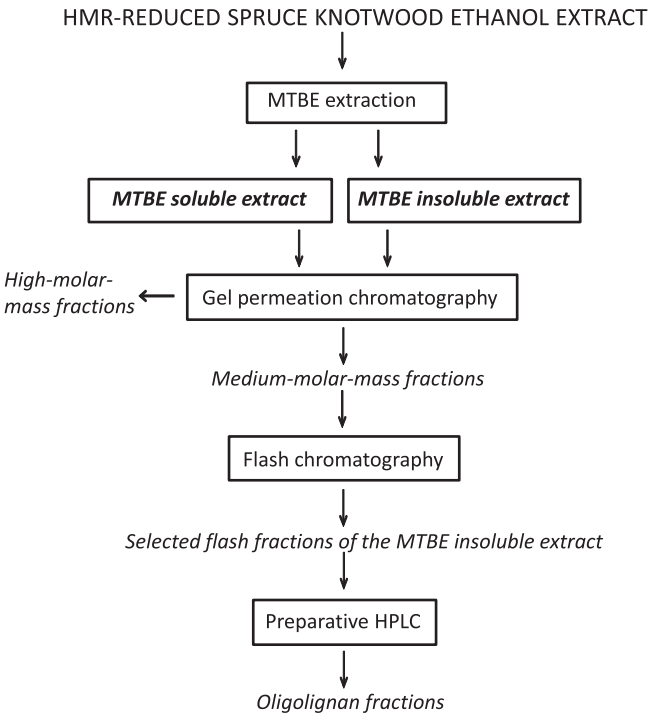

Fig. 1. Flow-chart showing the fractionation of the HMR-reduced knotwood ethanol extract.

of the MTBE insoluble fraction containing only HMM material were shown by py- and THM-GC-MS to consist mainly of guaiacyl units, which are an indication of lignin or oligolignans, and they were therefore characterized by NMR.

The medium-molar-mass (MMM) fractions were further fractionated by flash chromatography in an attempt to separate the HMM material from the rest. MMM material is here defined as fractions containing a mixture of HMM and LMM material.

Some flash fractions of the MTBE insoluble material were further separated by preparative HPLC in order to isolate less complex oligolignan fractions or pure oligolignans for NMR and LC-MS characterization. Unfortunately, the quantity of the isolated fractions was not sufficient for NMR analysis. The LC-MS characterization was performed by ESI-IT-MS introducing the sample using syringe infusion or HPLC and syringe infusion ESI-QTOF-MS. HPLC-ESI-IT-MS was performed in an attempt to separate the compounds and syringe infusion ESI-IT-MS to obtain higher-order MS^n fragmentations, as this method gave a better result than MS^n fragmentations made in the HPLC-ESI-IT-MS analyses. Another reason for using IT-MS for the fragmentations was the possibility to compare with previously published fragmentation data of lignans and oligolignans made with the same instrument. However, the HPLC separations were not very successful and higher-order MS fragmentations were not in all cases possible to obtain because of low concentrations of individual compounds. ESI-QTOF-MS was performed in order to obtain exact masses of the molecular ions for deducing the molecular structures.

2.2. Characterization of the whole HMR-reduced extract

HPSEC-ELSD analysis showed that this extract consisted mainly (94%) of an LMM fraction corresponding to ca. 350 Da (67%, lignans), ca. 550 Da (18%, sesquilignans), and ca. 700 Da (9%, dilignans) (Fig. 2). Of LMM compounds, the extract consisted of lignans, sesqui-, and dilignans, only. We have previously studied

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