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Characterization of high-molar-mass fractions in a Scots pine (*Pinus sylvestris* L.) knotwood ethanol extract

https://doi.org/10.1515/hf-2017-0079
Received May 17, 2017; accepted September 19, 2017; previously published online October 31, 2017

Abstract: The identification of compounds in Scots pine (*Pinus sylvestris* L.) knotwood (KnW), obtained by extracting with hydrophilic organic solvents, has been performed previously almost exclusively by gas chromatography-mass spectrometry (GS-MS) equipped with long GC columns (≥25 m). That means that the molar mass (MM) of the majority of the identified compounds was below 500–600 Da, and the analytical data accounted for only about half of the dry extract weight. In the present work, high-molar-mass (HMM) fractions in a Scots pine KnW-EtOH extract were isolated and chemically characterized by means of several advanced analytical techniques, such as high performance size-exclusion chromatography-evaporative light scattering detector (HPSEC-ELSD), high performance liquid chromatography (HPLC)-electrospray ionization-ion trap-mass spectrometry [(HPLC)ESI-IT-MS], ESI-quadrupole time of flight-mass spectrometry (QTOF-MS), pyrolysis-gas chromatography-mass spectrometry (Py-GC-MS), thermally assisted hydrolysis and methylation-gas chromatography-mass spectrometry (THM-GC-MS), nuclear magnetic resonance (NMR) and GC-MS. The results indicate that the MM maxima of the HMM fractions ranged from approximately 500 to 2200 Da, and that the compounds consist mainly of oligomers of hydroxylated resin acids (RAs), especially dehydroabietic acid, but also of fatty acids (FAs), stilbenes and sterols. A large number of RA dimers were tentatively identified in the HMM fractions. However, it remains unknown how the monomer units are linked together, as it was not possible to isolate a RA dimer fraction pure enough for NMR characterization. RA dimers in native KnW have not been identified previously.

Keywords: characterization, ESI-IT-MS, ESI-QTOF-MS, GC-MS, HPSEC-ELSD, knotwood, NMR, oligomer fraction, Py-GC-MS, resin acid dimers, Scots pine knots, THM-GC-MS

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Introduction

The content and composition of gas chromatography (GC)-eluting extractives in Scots pine (*Pinus sylvestris* L.) knotwood (KnW) were studied in detail by Willför et al. (2003). In addition, the extractive variations depending on the geographical location and the age of the tree, and knots with a living or dead branch were studied. The total amount of GC-eluting hydrophilic extractives (acetone-water extract, 95:5 v v^{-1}) was at most around 10% of dry wood. All the identified compounds were eluted from a long GC column (25 m), however, a small part of the material (at most 0.7%) eluted only from a short GC column (6–7 m). This material was suggested to consist of oligolignans (sesqui-, di- and sesterlignans). Willför et al. (2004) identified two of the sesquilignans of Scots pine KnW as β-*O*-4-linked guiacylglyceryl ethers of nortrachelogenin and secoisolariciresinol.

Characterization of non-GC-eluting compounds, i.e. the high-molar-mass (HMM) material in hydrophilic extracts of Scots pine KnW is important in view of their medical application potential. An ethanol extract, from which the major lipophilic part had been removed, was effective in a prostate cancer model (Yatkin et al. 2014) and in attenuating adipose tissue inflammation and weight gain (Polari et al. 2015). The compounds responsible for this biological activity are pinosylvin and pinosylvin monomethyl ether (stilbenes) and nortrachelogenin and matairesinol (lignans) (Yatkin et al. 2014). These compounds together accounted for 22% of the extract. Other groups of GC-eluting compounds in the extract were unmodified and oxidized or hydroxylated resin acids (RAs), which accounted for another 22% of the extract (Yatkin et al. 2014). The compounds eluting from a long GC column [with a molar mass (MM) up to around 600 Da as silyl-derivatives] accounted for about half of the extract weight, which indicates that

the other half of the extract consisted of HMM compounds. The HMM material in the same extract was characterized in the present study. Only a few studies have been published in terms of the identification of larger molecules than the GC-eluting compounds in pine extracts. In tall oil, fatty acid (FA) dimers and RA dimers and trimers were separated by gel permeation chromatography (GPC) (Chang 1968). In tall oil pitch, a high percentage of acids were found, which were not eluted by GC (Holmbom and Erä 1978). This fraction was suggested to consist of FA and RA dimers, which are partly linked by ester linkages.

In the present work, a Scots pine KnW-EtOH extract was fractionated, and fractions containing mainly HMM material were characterized by several analytical methods, i.e. by high performance size exclusion chromatography analysis coupled to an evaporative light-scattering detector (HPSEC-ELSD), high performance liquid chromatography (HPLC)- and direct infusion-electrospray ionization-ion trap mass spectrometry (MS) (ESI-IT-MS), direct infusion-ESI-quadrupole-time-of-flight MS (ESI-QTOF-MS), pyrolysis combined with GC-MS (Py-GC-MS), thermally assisted hydrolysis and methylation with GC-MS (THM-GC-MS), nuclear magnetic resonance (NMR) and GC-MS.

Materials and methods

Sample pretreatment: The Scots pine (*P. sylvestris* L.) KnW sample was obtained from an industrial pulp mill. The sample was ground, freeze-dried and extracted by accelerated solvent extraction (ASE) (Figure 1). The extract preparation and analysis of the GC-detectable compounds were outlined by Yatkin et al. (2014). The procedure for fractionation and analysis (only ESI-MS, THM-GC-MS and NMR are

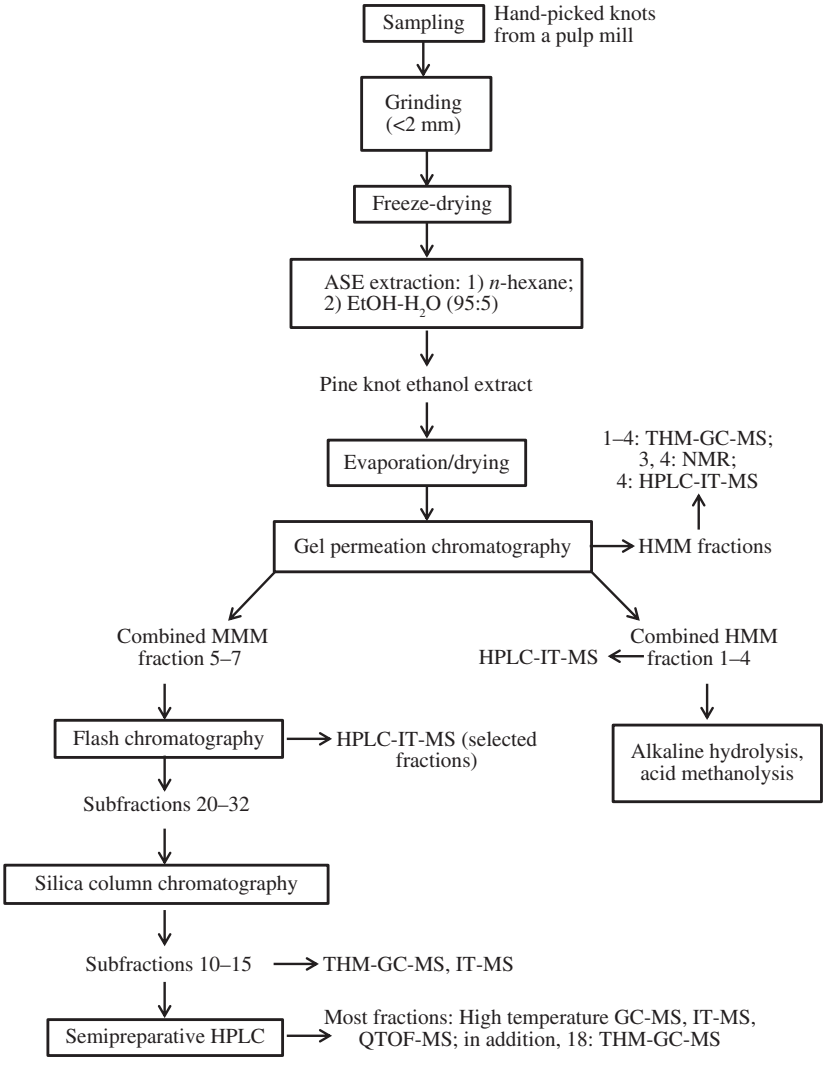

Figure 1: Protocol for treatment of pine knots and fractionation and analysis of the pine KnW-EtOH extract.
Standard analytical methods: GC-FID, GC-MS, and HPSEC-ELSD. MMM, medium-molar-mass Fr., classified as a Fr. containing a mixture of HMM and GC-detectable compounds. The numbers denote Fr. number.

mentioned) of the KnW-EtOH extract (from which lipophilics were removed by *n*-hexane extraction) is presented in Figure 1. The extract was analyzed by GC-FID, GC-MS and HPSEC-ELSD, as most of the obtained fractions (standard methods). Acetylation prior to HPSEC-ELSD analysis was performed by adding 0.25 ml each of pyridine (LabScan Ltd, Dublin, Ireland) and acetic anhydride (Alfa Aesar GmbH & Co KG, Karlsruhe, Germany) to a few mg of the extract and allowing to react for 3 days at room temperature in the dark, after which 0.5 ml EtOH (Altia plc, Helsinki, Finland) was added and the solution was evaporated to dryness and dissolved in THF (inhibitor-free, Chromasolv Plus, Riedel-de Haen/Sigma-Aldrich, Steinheim, Germany).

An aliquot (2 g) of the KnW-EtOH extract was fractionated by GPC on Sephadex LH-40 gel (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), as described by Smeds et al. (2012a). The obtained 22 fractions were weighed and then analyzed by thin-layer chromatography (TLC) and HPSEC-ELSD, and fractions with similar elution profiles by TLC and molar mass distributions (MMD) by HPSEC were pooled. The TLC plates (Merck KGaA, Darmstadt, Germany) were pre-coated with 0.25 mm silica gel and contained a fluorescence indicator. Cyclohexane-acetone (45:55 v v^{-1}) (VWR Chemicals, France) served as the eluent. Another, separate, GPC fractionation from 4 g of extract was made in the same way as described above to collect larger amounts of HMM fractions (Frs. 1–4) for NMR analyses.

GPC Frs. 1–4 (from the first fractionation) were pooled to an HMM fraction, which was treated by alkaline hydrolysis and acid methanolysis. For the first one, 15 mg of the dried fraction was weighed and 2 ml of 0.3 M NaOH (Merck KGaA, Darmstadt, Germany) in 70% MeOH (VWR Chemicals, France) was added. The sample was heated at 70°C for 1 h, and then extracted three times (totally with ca. 5 ml) with ethyl acetate (Merck KGaA, Darmstadt, Germany) after the addition of a small amount of distilled water and NaCl (Merck KGaA, Darmstadt, Germany). To 500 µl (ca. 1.5 mg) of this solution, an internal standard (IS) solution (see GC methods below) was added. The sample was evaporated to dryness and analyzed by HPSEC-ELSD and by GC-flame ionization detector (FID) and GC-MS after silylation. For acid methanolysis, 15 mg of the HMM material was treated with 2 ml of 2 M HCl (Riedel de Haen/Sigma-Aldrich, Steinheim, Germany) in MeOH at 105°C for 3 h, and the sample was then extracted, further treated, and analyzed as the alkaline-hydrolyzed extract.

Some of the combined GPC fractions (5–7), i.e. "medium MM fractions (MMM)" (defined as fractions containing a mixture of HMM and GC-eluting compounds), were analyzed by GC-FID and GC-MS, Py-GC-MS and HPSEC-ELSD. After that, they were further fractionated by flash chromatography on normal-phase silica gel flash cartridges (40 mm×15 cm) (Biotage AB, Uppsala, Sweden). Elution: beginning with 10% acetone in cyclohexane, then increasing the acetone proportion with 10% steps to 70% acetone (300 ml for each step). Thirty-eight fractions of 50 ml each were collected, and fractions with similar elution profiles by TLC were pooled. After this, they were evaporated in a rotating evaporator, dried in a vacuum oven, and weighed. All fractions were analyzed by GC-FID, and selected fractions were further analyzed by HPSEC-ELSD, THM-GC-MS and HPLC-IT-MS.

Flash fractions 20–32 were combined (54 g) and separated at a smaller scale on a glass column packed with 12 g silica gel (Merck KGaA, Darmstadt, Germany). The material was eluted with 25-ml portions starting from 20% acetone in cyclohexane to 70% acetone, similarly as was done in the previous flash fractionation. A light N_2 pressure was applied occasionally. Thirty-five fractions of 3 ml each were collected and evaporated under a gentle stream of N_2, dried in a vacuum oven and weighed. The fractions were analyzed by HPSEC-ELSD, and fractions supposedly containing mainly RA dimers (Frs. 10–15) were pooled, evaporated to dryness and weighed (23 mg), and analyzed on GC-FID (short-column), GC-MS and HPLC-IT-MS. The combined fraction was then further separated by semipreparative HPLC, after dissolving in 0.18 ml of 2-propanol (for HPLC, Mallinckrodt Baker, Deventer, The Netherlands). For details of the preparative HPLC system and the semipreparative column see Smeds et al. (2012b). The eluents Milli-Q water (A) and 2-propanol (B) were used as follows: 0–2 min 30% B, 2–15 min 30–80% B, 15–19 min 80–95% B, 19–29 min 95% B, 29–31 min 95–30% B, 31–38 min 30% B. Further details: 5 ml min^{-1} flow rate, column oven 30°C, the injection volume 150 µl, UV detection at 230 and 280 nm; 3-ml fractions were collected. The preparative HPLC fractions were evaporated to dryness and weighed.

NMR: Two HMM GPC fractions were characterized by NMR (500 MHz Bruker Avance Instrument; Bruker Corp., Billerica, MA, USA). The samples were dissolved in DMSO-d_6 (Sigma-Aldrich, Steinheim, Germany); standard Bruker pulse sequences (^{1}H, ^{13}C, COSY and HSQC) at 25°C were applied. NMR reference substances: pure palmitic acid (Sigma-Aldrich, Steinheim, Germany), dehydroabietic acid (DeAbA) (prepared in our laboratory), and methyl 15-hydroxy-7-oxodehydroabietate (Hercules Inc., Wilmington, DE, USA).

GC-MS methods according to Smeds et al. (2012a): IS solution was added only to the alkaline hydrolyzed and acid methanolyzed samples. Details: 2 ml of a solution containing 40 µg each of heneicosanoic acid (Sigma-Aldrich, Steinheim, Germany) (IS for FAs, stilbenes and diterpenes, assuming a response factor of 1.00), and betulinol, prepared in our laboratory as IS for lignans, with the previously determined response factor of 1.2. The individual compounds detected by GC-FID were identified via the GC-MS library (Wiley 10th/NIST12, John Wiley & Sons, Inc., Hoboken, NJ, USA) and based on the laboratory's own spectral databases. Some mass spectra were also manually interpreted. Some semipreparative HPLC fractions were analyzed by GC-MS equipped with a high temperature, medium-length column (DB-1HT, 15 m×0.25 mm, 0.11 µm film thickness), with splitless injection and the following program: 60°C (0.5 min) → 290°C (16°C min^{-1}) → 370°C (8°C min^{-1}), 370°C (20 min). The Py-GC-MS analyses were performed according to Smeds et al. (2012a). THM-GC-MS was performed in a manner of the Py-GC-MS analyses, except for the *in situ* methylation, which was done with tetramethylammonium hydroxide (TMAH, 25% in methanol) (Sigma-Aldrich, St. Louis, MO, USA) at 380°C for 2 s, after adding ca. 10 µl of TMAH containing ca. 100 µg of sample onto the Pt filament.

HPSEC-ELSD according to Smeds et al. (2012a): The MMD of the diterpenes and their oligomers was determined via a calibration curve based on the analysis of pure DeAbA (prepared in our laboratory) and polystyrene standards (Toyo Soda Manufacturing Co., Ltd, Tokyo, Japan and Perkin-Elmer, Norwalk, CT, USA) with MMs ranging from 370 to 4000 Da. The R^2 value of the calibration curve was 0.9992. The retention times (RTs) of stearic acid (Merck KGaA, Darmstadt, Germany), methyl 15-hydroxy-7-oxodehydroabietate (Hercules Inc., Wilmington, DE, USA), and of pure DeAbA, a lignan (7-hydroxymatairesinol), and stilbenes (pinosylvin and pinosylvin monomethylether) (all prepared in our laboratory) were determined. Only the unfractionated KnW-EtOH extract was acetylated before analysis.

ESI-IT-MS based on Eklund et al. (2008): The samples were dissolved in 2-propanol-Milli-Q water 8:2 (v v^{-1}) to a concentration of

0.1–4.6 mg ml^{-1} and introduced by HPLC or direct infusion. The HPLC details: column Zorbax SB-C8 2.1×100 mm, particle size 3.5 µm (Agilent Technologies, Santa Clara, CA, USA); eluent A 10 mM ammonium acetate (Merck KGaA, Darmstadt, Germany) in Milli-Q water; eluent B 2-propanol; flow rate 0.20 ml min^{-1}; column temp. 40°C; $UV_{230\,nm}$ detection; injection volume 20 or 50 µl; gradient 20–50% B from 0 to 8 min, 50–70% B from 8 to 12 min, 70–95% B from 12 to 17 min, held at 95% for 3 min, then 95–20% B from 20 to 21 min, and isocratic at 20% from 21 to 26 min. The MS parameters: range *m/z* 100–2200, 40 psi, gas flow 9 l min^{-1}, target mass was set at *m/z* 600, and a fragmentation amplitude of 1.0 V. Positive ionization was tried for some samples. Most of the analyses were made by direct infusion. In those analyses, the target mass was set at the ion recorded, and the "rolling averaging" was on.

Direct infusion-ESI-QTOF-MS was done according to Smeds et al. (2016) with the following modifications: range 100–1500 *m/z*, the collision cell energy and RF were 10.0 eV and 170.0 Vpp, respectively, transfer time 50.0 µs, pre-pulse storage 5.0 µs, and "rolling averaging" was 3×10.000 summation. Funnel 1 and 2 RF were both 200.0 Vpp and the hexapole RF was 90.0 Vpp; ISCID energy was 0.0 eV.

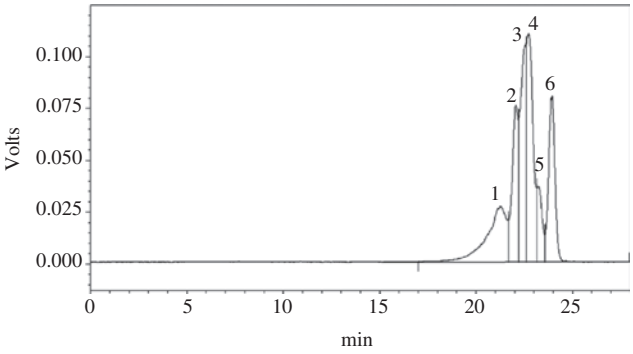

Figure 2: HPSEC-ELSD chromatogram of the whole pine KnW-EtOH extract.
Peak 1: mainly HMM material, consisting of unmodified and hydroxylated RA and FA dimers and oligomers; retention time (t_R) 21.7 min, 17% of total peak area; peak 2: t_R 22.6 min – lignans, 15%; peak 3: t_R 23.0 min – di- and polyhydroxylated/oxidized RA (tentatively), 19%; peak 4: t_R 23.3 min – unmodified and monohydroxylated/oxidized RAs (tentatively), 25%; peak 5: t_R 23.7 min – pinosylvin, 6.6%; peak 6: t_R 24.8 min – pinosylvin monomethyl ether, 17%. The results are an average of three determinations.

Results and discussion

The whole pine knot ethanol extract

GC-FID and GC-MS analyses show that RAs account for 22% of the dry extract weight with the dominating compounds: unmodified RAs 12% with DeAbA (4%) and oxidized/hydroxylated (OH)-RAs (10%); stilbene yields 14% (pinosylvin monomethyl ether 10% and pinosylvin 4%), and lignans occur with 9% (of which nortrachelogenin 7%) (Yatkin et al. 2014). Thus, in total, the GC-eluting compounds accounted for 45% of the dry extract weight, i.e. as much as 55% of the extract may consist of HMM material.

The HPSEC-ELSD analysis indicates that the HMM material did not elute to any significant degree from the HPSEC column (Figure 2), as peak 1 (mainly HMM material) accounted for only 17% of the total peak area. A similar observation was made in case of Norway spruce KnW extract (Smeds et al. 2016). Acetylation of the extract did not increase peak 1, but only slightly increased the lignan peak and made the pinosylvin peak disappear. This is the reason why the fractions were not acetylated.

GPC fractions

In total, 22 fractions were collected in the first GPC fractionation of the total KnW-EtOH extract. The material eluted quantitatively from the GPC column. The first six fractions did not contain material, but Frs. 1–4 contained almost exclusively HMM compounds. According to HPSEC-ELSD, the mass range of the compounds was between 0.3 and 13.4 kDa with MM maxima at 500, 650, 800, 1000, 1200 and 2200 Da (the two last ones in the smallest fraction weighing only 6 mg). The combined amount of these fractions account for 7%, while the expected amount of HMM material is 55%. The combined fraction was analyzed by HPLC-IT-MS and treated by alkaline hydrolysis and acid methanolysis (discussed below). The HPLC-MS analysis showed that a complex mixture of compounds in the mass range 620–790 *m/z* eluted at the end of the chromatogram. The combined Frs. 5–7 (20% of the extract weight), which seemed to contain mainly compounds with a MM of 500–600 Da and monomeric RAs, was separated by flash chromatography. Frs. 8–22 contained mainly GC-eluting compounds and were not further fractionated or analyzed. Hence, the rest of the HMM material had to be present in GPC Frs. 5–22, in a complex mixture together with low-molar mass (LMM) material.

The four fractions collected in the second GPC fractionation, which was done to obtain more HMM material (especially for NMR analysis), were analyzed by THM-GC-MS. The results are presented in Table 1 and the pyrogram of Fr. 4 is in Figure 3. The results indicate that RAs and other diterpenes, mainly as dimers and possibly trimers according to the MM maxima of around 600 and 800 Da (Table 1), were dominating (53% of the total peak area) followed by FAs. A large part of this Fr. contained unidentified compounds (34%). Fr. 3 showed similar results, although the FA part was larger and the RA part smaller than in Fr. 4. The MM maximum was around 950 Da (Table 1), which may correspond to the mass of RA trimers. In Fr. 2, unidentified compounds were dominating, while RAs and FAs were in similar amounts among the identified compounds. The dominating masses

Table 1: Yield (gravimetric), approximate molar mass of dominating fraction (as RA oligomers) and dominating units as determined by THM-GC-MS, in HMM GPC fractions of the pine KnW-EtOH extract.

Fr. #	Yield (%)	Approx. MM maxima (Da)	Dominating compound groups (%) of total peak area
1	1.2	2000	FAs 41% (of which hydroxy and dioic acids 6%); RAs 18%; sterols 3%; unknown 38%
2	2.3	1500, 1000	RAs and other diterpenes 27%; FAs 27% (of which hydroxy and dioic acids 3%); sterols 4%; unknown 42%
3	3.0	950	RAs and other diterpenes 39%; FAs 22% (of which hydroxy and dioic acids 5%); aromatics 2%; monoterpenes 2%; unknown 34%
4	4.6	800, 600	RAs and other diterpenes 53%; FAs 6%; sterols 1%; monoterpenes 1%; unknown 34%

RAs, resin acids; FAs, fatty acids.

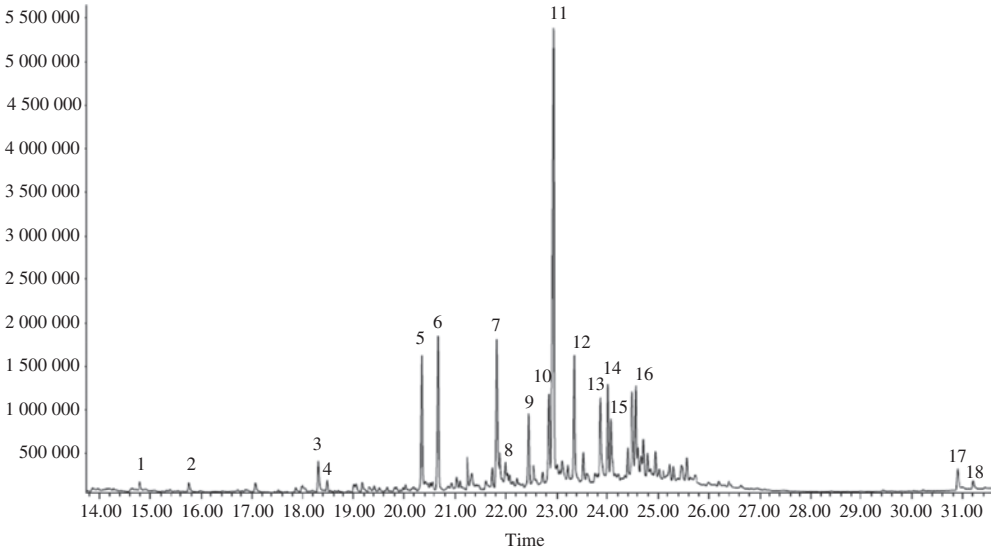

Figure 3: THM-GC-MS pyrogram of a GPC Fr. with MM maxima of approximately 600 and 800 Da.
Peak annotations: (1) alpha-cadinol; (2) myristic acid; (3) palmitic acid; (4) pimaradiene; (5) oleic acid; (6) unknown diterpenoid; (7) sandaracopimaric acid; (8) pimaric acid; (9) isopimaric acid; (10) 6,8,11,13-abietatetraen-18-oic acid; (11) DeAbA; (12) abietic acid (AbA); (13) 8,11,13,15-abietatetraen-18-oic acid; (14) 7-OH-DeAbA; (15) 7-OH-abieta-6,8,11,13-tetraen-18-oic acid; (16) 15-OH-abieta-9(11),8(14),12-trien-18-oic acid; (17) sitosterol and (18) cycloartenol.

of around 1000 and 1500 Da (Table 1) could correspond to RAs tri- and pentamers. In Fr. 1, the smallest fraction with the largest molecules, the FAs were preponderant. The MM maximum of 2000 Da indicates that they could be heptamers of oleic acid or octamers of palmitic acid, which were the most abundant FAs in the pyrogram.

The NMR spectra of Frs. 3 and 4, with MM maxima of ≈950 and ≈600, and ≈800 Da, respectively, were almost identical. The spectra of these complex mixtures with severe overlapping signals could be partly evaluated. In the ^{1}H spectra, signals were detected in the aromatic region, in the double bond region, in oxygenated aliphatic regions, and in the aliphatic region (Figure 4a). For further characterization, ^{1}H NMR spectra of methyl-15-OH-7-oxo-dehydroabietate (Figure 4b), palmitic acid (Figure 4c), and DeAbA were recorded for comparison. Based on the palmitic acid spectrum, it is obvious that the sample contained FA-like aliphatic chains. Based on DeAbA and oxygenated DeAbA spectra and the published NMR data of hydroxylated DeAbAs (van Beek et al. 2007), signal similarities allow the conclusion that the samples contained both AbA-like and oxidized RA structures. In the ^{13}C NMR spectra, signals around 180 ppm indicated the presence of carboxylic acid groups, and signals at 172–175 ppm are indicative for esters. These signals with similar intensities lead to the conclusion that free and esterified carboxyls occur in the oligomers with similar abundancies. Signals at 70–80 ppm and 40–50 ppm are indicative for ethers and esters. The aromatic methoxy group signal at 55 ppm is probably originating from stilbene oligomers. Two

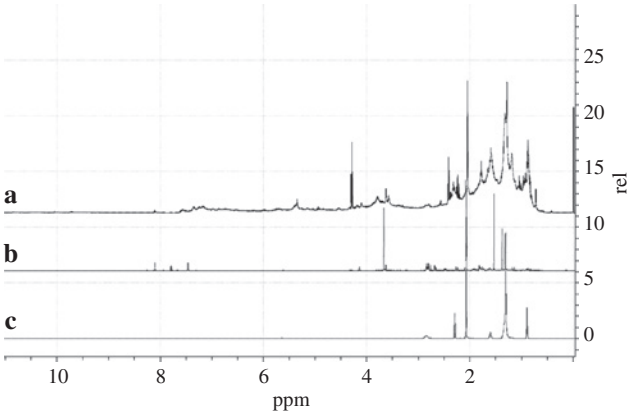

Figure 4: The pine KnW-EtOH Fr. shows signal similarities with the FA and RA reference spectra.
^{1}H NMR spectra of: (a) a GPC Fr. with a MM maximum of approximately 950 Da (Fr. 3); (b) methyl-15-OH-7-oxo-dehydroabietate; (c) palmitic acid.

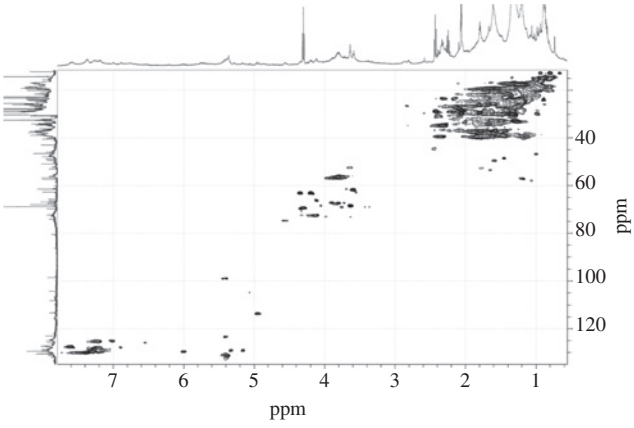

Figure 5: 2D HSQC spectrum of GPC Fr. 3.

dimensional HSQC spectra revealed the same structural functionalities (Figure 5). The carbon spectra showed both broad signals from the complex oligomeric structures and sharp signals from low MM compounds, e.g. butyrolactone, which most probably originates from unstabilized THF as eluent in the GPC fractionation.

Fr. 4 was analyzed by HPLC-IT-MS, which revealed the dominance of unmodified RAs. A complex of several broad overlapping peaks showed MMs corresponding to OH-RAs and RA dimers; analysis of flash fractions (see below) confirmed these masses.

Alkaline hydrolysis

Quantitative GC-FID analysis of the alkaline-hydrolyzed GPC HMM Fr. showed the presence of FAs, mainly oleic acid, but also palmitic acid, linoleic acid and fatty dioic acids, especially 1,9-dioic-nonanoic acid (altogether 4.1% of the Fr. weight). The amount of unmodified and oxidized/OH-RAs was 3.3%, and of sitosterol 0.4%. This supports the THM-GC-MS results presented above, and as the amount of released FAs and RAs was low (altogether 7.4%), it can be assumed that the majority of the polymer bonds are stable under alkaline conditions.

According to HPSEC-ELSD analysis, the material contained more low MM compounds (with maxima around 250–300, 400, 600 and 750 Da) than before hydrolysis, i.e. fractions corresponding to FAs and RA monomers have been released, and the largest molecules disappeared. However, a substantial part of the larger molecules, at least those with masses corresponding to RA dimers (ca. 600 Da) and some unknown compounds (ca. 750–800 Da) were still present, i.e. they are stable under alkaline conditions.

Acid methanolysis

GC analysis shows that after acid methanolysis mainly RAs are released from oligomers. They accounted for 44% of the pooled GPC Fr. weight, compared to 3% yield after alkaline hydrolysis. DeAbA and methylated DeAbA were dominating with amounts of 12% and 7%, respectively. Large amounts of FAs were also released (17%), about four times more than after alkaline hydrolysis. Furthermore, oxidized/OH-RAs, lignans and stilbenes were released in larger amounts than after alkaline hydrolysis (4.6, 2.6 and 1.9%, respectively). Accordingly, these compounds must have been predominantly linked by bonds less stable under acidic than alkaline conditions. Both the FAs and the RAs were only partly detected as methyl esters, i.e. the carboxyl groups are predominantly in free form. This contradicts to NMR results, according to which there are approximately equal amounts of free and esterified carboxyl groups in the oligomers. It is possible that the esters were not released quantitatively during the acid methanolysis. A part of the FAs can be steryl esters, but as no steryl esters were detected in the original extract by GC short-column analysis, these steryl esters may have larger MMs than those eluting from the short GC column.

HPSEC-ELSD analysis showed MM maxima at around 250 Da (probably palmitic acid) and 300 Da (RAs), which is in accordance with the GC results. However, large and broad peaks at around 500 and 700 Da were also detected (probably RA dimers), which indicates that acid methanolysis, although more effective than alkaline hydrolysis,

failed to cleave all bonds in the oligomers. Consequently, it seems that there are more oligomers unstable under acid than under alkaline conditions. Especially the bonds in molecules with MMs around 500–800 Da (like RA dimers) seem to be linked predominantly by stable C-C bonds.

Flash fractions

Flash chromatography of GPC Frs. 5–7 was performed in an attempt to separate the rest of the HMM material from LMM compounds. In total, 38 Frs. were collected, and Frs. showing similar TLC elution patterns were pooled. GC analysis of the pooled fractions shows that they contain mainly RAs in unmodified and oxidized/OH-forms (in the first and middle fractions), monohydroxylated linoleic acid (in some fractions), lignans (in later fractions) and unknowns.

HPSEC-ELSD analysis revealed that the 19 first eluting Frs. contained mainly LMM material (RAs). Frs. 20–38 with compound masses corresponding to RA dimers and monomers, were combined into six different Frs. and analyzed by THM-GC-MS. In flash Frs. 20–27, RAs (especially DeAbA, abietatetraenoic acid, 7-OH-DeAbA, 7-OH-abietatetraenoic acid and 15-OH-abietatrienoic acid) were preponderant, accounting for 24–40% of the total peak area, whereas pinosylvin (and possibly pinosylvin monomethyl ether) and aliphatic acids were minor compounds. In flash Frs. 28–38, RAs were also relevant with the same dominating compounds as in Frs. 20–27. A small pinosylvin peak was detected. However, unidentified compounds were abundant in all Frs. with 51–70% of the total peak area.

Frs. 20–32, which seemed to contain mainly RA monomers and dimers, were pooled (54 mg) and further fractionated by smaller-scale silica column chromatography. HPSEC-ELSD analysis showed that a Fr. corresponding to RA (and possibly FA) dimers was dominating in Frs. 10–15. These Frs. were combined (14 mg) and analyzed by different methods. Long column GC-MS, by which only the minor Fr. of monomeric compounds is detected, showed DeAbA, 7-oxo-DeAbA, OH-DeAbA and monoglycerides as dominating peaks. In the THM-GC-MS pyrogram, DeAbA and an unidentified compound (probably a OH-RA) were dominating; other identified compounds were abietatetraenoic acid, 7-OH-DeAbA, 7-OH-abietatetraenoic acid, FAs (with the preponderant palmitic and stearic acid), dimethyl pinosylvin and several unhydroxylated RAs. This indicates the presence of several different RA and FA dimers in this Fr. In addition, there were a number of unidentified peaks. By short-column GC, a slight rising of the baseline was observed in the "diglyceride region" of the chromatogram (ca. 600 Da) indicating the presence of a complex mixture of dimers, probably mainly of RAs, as indicated by THM-GC-MS. Direct infusion-ESI-IT-MS confirmed the presence of

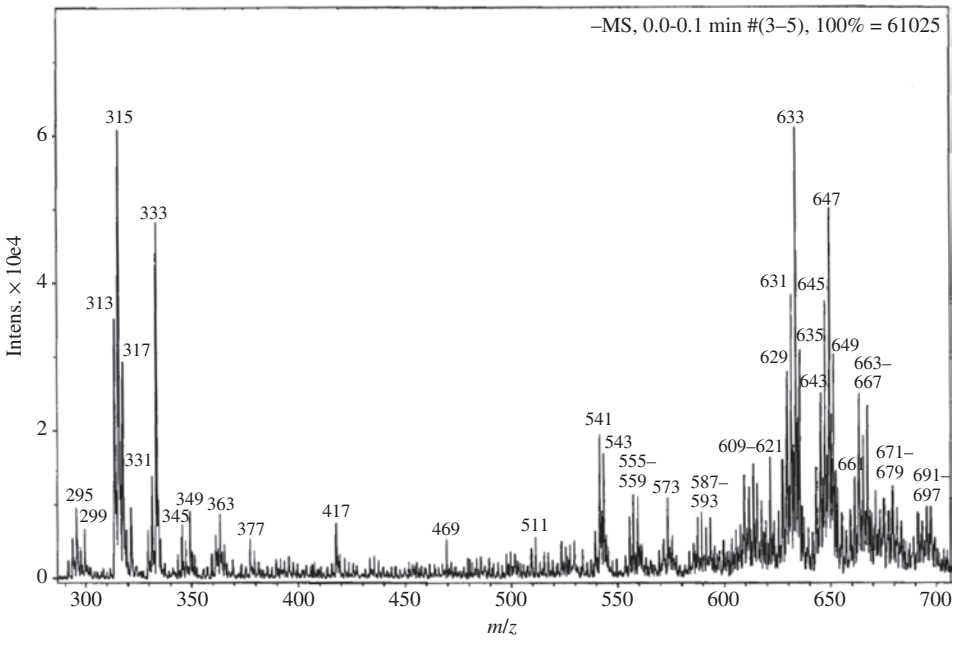

Figure 6: Direct infusion-ESI-IT-MS scan of pooled fractions 10–15 obtained by fractionation of flash Fr. 20-32 by small-scale silica column chromatography.

RAs; OH-tetraOH-RAs and a large number of different dimers of OH-hexaOH-RAs were tentatively identified. The ESI-IT-MS scan is presented in Figure 6. It should be noted that ESI-MS is very selective for easily ionizable compounds, while FA esters remain undetected, if they are not hydroxylated.

Semipreparative HPLC fractions

The small-scale silica column pooled Frs. 10–15 were further separated by semipreparative HPLC into 25 Frs. One of the largest HPLC Fr. (Fr. 18, 1.2 mg) was analyzed by short-column GC-FID and by GC-MS, HPSEC-ELSD, THM-GC-MS and ESI-MS. The results were very similar to those obtained for the unfractionated pooled Frs. 10–15 (see above mentioned). Obviously, the further fractionation of this material is very difficult.

Direct infusion-ESI-IT-MS and ESI-IT-QTOF-MS

Most semipreparative HPLC Frs. were analyzed by ESI-IT- and -QTOF-MS. Table 2 shows molecular formulas, monoisotopic masses, and ESI-IT-MS fragmentations of DeAbA and AbA and of some chosen tentatively identified OH-RAs and OH-RA dimers. Supposedly, the dominating OH-RAs consist of DeAbA and AbA, as they are the dominating RAs in the THM-GC-MS analyses and after alkaline hydrolysis and acid methanolysis. A large number of masses corresponding to RAs and RA dimers were detected, with deprotonized molecular ions ($[M-H]^-$) ranging from *m/z* around 293 to 699 (Figure 6 and Table 2). All compounds except those with masses at *m/z* 293–301 (unhydroxylated RAs) seemed to be monomers or dimers of OH-RAs. This may be because RAs containing OH groups are more easily ionized than those containing only one or two carboxyl groups. Unhydroxylated dimers linked by ester bonds or containing keto groups instead of OH groups do not dispose of ionizable groups at all. In Figure 6, the signals at *m/z* 293, 295, 297, 345 and 363 could be tetradehydro-, tridehydro- and dideRA, triOH-dideRA and tetraOH-deRA, respectively.

The only fragmentation reactions that occurred for DeAbA and AbA were dehydrogenation, both in MS^2 and in MS^3 (Table 2). Dehydrogenation, dehydration and decarboxylation were the essential fragmentation reactions of the hydroxylated RAs (Figure 7). The main fragments were found at *m/z* 313 and 315 for OH-RAs.

Figure 8 describes the putative fragmentation pathways for a OH-RA dimer showing $[M-H]^-$ at *m/z* 649 (Table 2). The molecule is cleaved into two monomers, losing mainly 318 Da, which corresponds to OH-RA at *m/z* 331 and 334 Da, equivalent to diOH-RA (*m/z* 315). In addition, the latter fragment is dehydrogenated and dehydrated in MS^3.

As visible in Table 2, the tentatively identified hydroxylated RA dimers lost the following fragments in MS^2: H_2 (2 Da), 2 H_2 (4 Da), H_2O (18 Da), $H_2O + H_2$ (20 Da), deRA (300 Da), OH-dideRA (314 Da), OH-deRA (316 Da), OH-RA (318 Da), diOH-dideRA (330 Da), diOH-deRA (332 Da), diOH-RA (334 Da), triOH-deRA (348 Da), triOH-RA (350 Da), tetraOH-RA (366 Da) and pentaOH-RA (382 Da). In MS^3, the predominant fragmentations were loss of H_2 and H_2O. Another example from Table 2 is a compound with a molecular ion at *m/z* 681, which lost mainly 332 Da (diOH-deRA); the rest of the molecule corresponding to 349 Da could be triOH-RA. Consequently, the molecule could correspond to triOH-RA–diOH-deRA. The compound with a molecular ion at *m/z* 633 lost mainly 300 Da (deRA); the rest of the molecule corresponding to 333 Da could be diOH-RA, leading to the suggestion that this molecule would be diOH-RA-deRA. The structures of the other molecules were deduced in the same way.

The ESI-MS results can be interpreted that dimers of some hydroxylated modified RAs may be present in the HPLC Frs. as visible on the signals at *m/z* 541–573 in Figure 6. The masses indicate that the compounds have lost C_3H_6 or C_5H_{10} (possibly the isopropyl group and two methyl groups). The exact masses detected were: 541.2949 Da, giving the molecular formula $C_{35}H_{41}O_5$, which could correspond to a OH-dideRA, dimer which has lost C_5H_{10}; 559.3065 Da, giving the formula $C_{35}H_{43}O_6$, which could correspond to a diOH-dideRA–deRA dimer, which has lost C_5H_{10}; and 573.3585, giving $C_{37}H_{49}O_5$, which could correspond to a OH-deRA dimer, which has lost C_3H_6. The signal at *m/z* 543 in Figure 6 could be a OH-deRA–dideRA dimer, which has lost C_5H_{10}, and *m/z* 557 could be a diOH-dideRA dimer, which has lost C_5H_{10}. The fragmentations of the two compounds with deprotonated molecular ions at *m/z* 541 and 543 were studied by ESI-IT-MS; they differed from those of unmodified OH-RAs by more extensive loss of small molecules like H_2, H_2O and CO. The identity of OH-RAs was confirmed by the presence of abundant fragments at *m/z* 313 (OH-dideRA), 315 (OH-deRA) and 317 (OH-RA), indicating that the alkyl groups had been lost from one of the RAs, only. The exact masses obtained by ESI-QTOF-MS confirmed the molecular formulas of the suggested structures.

Some monoOH-, diOH- and triOH-DeAbAs have been characterized by NMR after treatment of DeAbA with

Table 2: ESI-IT-MS fragmentations (MS^2 and MS^3) of pure DeAbA and AbA and tentatively identified hydroxylated RAs (OH-RAs) and OH-RA dimers.

Compound	Molecular formula, $[M-H]^-$	Mono-isotopic mass, $[M-H]^-$	MS^2 and MS^3 fragmentations, *m/z* (relative abundance)[a]
RAs:			
DeAbA	$C_{20}H_{27}O_2$	299.2017	MS^2: 297 (100); MS^3: 293 (100)
AbA	$C_{20}H_{29}O_2$	301.2173	MS^2: 299 (100); MS^3: 297 (100)
OH-RAs:			
OH-dideRA	$C_{20}H_{25}O_3$	313.1804	MS^2: 269 (100), 251 (21); MS^3 of 269: 173 (100), 235 (93), 149 (55), 251 (45)
OH-deRA	$C_{20}H_{27}O_3$	315.1966	MS^2: 313 (100); MS^3: 252 (24), 269 (100)
OH-RA	$C_{20}H_{29}O_3$	317.2122	MS^2: 315 (100); MS^3: 313 (100), 271 (60)
diOH-deRA	$C_{20}H_{27}O_4$	331.1926	MS^2: 313 (98), 329 (100); MS^3 of 329: 267 (100), 273 (52), 285 (70), 313 (74)
diOH-RA	$C_{20}H_{29}O_4$	333.2071	MS^2: 245 (62), 271 (50), 315 (100), 331 (35); MS^3 of 315: 245 (100), 271 (22)
triOH-RA	$C_{20}H_{29}O_5$	349.2015	MS^2: 347 (100), 287 (43), 305 (29); MS^3 of 347: 285 (100)
OH-RA dimers:			
OH-RA-deRA	$C_{40}H_{57}O_5$	617.4206	MS^2: 317 (100), 615 (39); MS^3 of 317: 299 (100), 315 (94)
hydr. RA-OH-RA	$C_{40}H_{61}O_5$	621.4524	MS^2: 301 (29), 317 (100); MS^3 of 317: 299 (100)
OH-deRA-OH-deRA	$C_{40}H_{55}O_6$	631.4004	MS^2: 315 (100); MS^3: 257 (26), 313 (100)
diOH-RA-deRA	$C_{40}H_{57}O_6$	633.4161	MS^2: 315 (55), 317 (27), 333 (100), 629 (25); MS^3 of 333: 315 (58), 331 (100)
OH-hydr. RA-OH-deRA	$C_{40}H_{59}O_6$	635.4317	MS^2: 315 (100), 333 (30); MS^3 of 315: 255 (48), 297 (100), 299 (73), 313 (100)
diOH-RA-RA	$C_{40}H_{59}O_6$	635.4317	MS^2: 315 (56), 317 (42), 333 (100); MS^3 of 333: 315 (100)
diOH-deRA-OH-deRA	$C_{40}H_{55}O_7$	647.3953	MS^2: 315 (62), 331 (100); MS^3 of 331: 329 (100)
OH-deRA-diOH-RA	$C_{40}H_{57}O_7$	649.4110	MS^2: 315 (100), 331 (28); MS^3 of 315: 297 (58), 313 (100)
triOH-RA-OH-dideRA	$C_{40}H_{55}O_8$	663.3902	MS^2: 329 (32), 331 (74), 333 (79), 347 (62), 349 (100), 645 (61), 647 (46), 661 (57); MS^3 of 349: 285 (36), 331 (43), 347 (100)
diOH-RA-diOH-dideRA	$C_{40}H_{55}O_8$	663.3902	MS^2: 329 (35), 331 (51), 333 (100), 335 (45), 345 (46), 347 (88), 349 (84), 351 (31), 605 (33), 645 (97), 647 (75), 659 (49), 661 (75), 663 (32); MS^3 of 645: 327 (100)
triOH-RA-OH-deRA	$C_{40}H_{57}O_8$	665.4059	MS^2: 331 (53), 333 (64), 347 (48), 349 (100), 647 (49), 663 (57); MS^3 of 349: 331 (100), 347 (67)
diOH-deRA-diOH-RA	$C_{40}H_{57}O_8$	665.4059	MS^2: 313 (32), 315 (39), 329 (57), 331 (100), 347 (51), 349 (35), 647 (94), 661 (34); MS^3 of 647: 313 (100), 333 (35)
diOH-RA-diOH-RA	$C_{40}H_{59}O_8$	667.4215	MS^2: 333 (100); MS^3: 137 (78), 305 (44), 331 (100)
triOH-RA-OH-RA	$C_{40}H_{59}O_8$	667.4215	MS^2: 333 (34), 347 (60), 349 (100), 647 (69), 661 (51), 663 (86); MS^3 of 349: 331, 347
diOH-hydr. RA-diOH-deRA	$C_{40}H_{59}O_8$	667.4215	MS^2: 331 (100), 333 (59); MS^3 of 331: 177 (33), 313 (100), 331 (29)
diOH-deRA-triOH-deRA	$C_{40}H_{55}O_9$	679.3846	MS^2: 329 (61), 331 (63), 661 (100); MS^3 of 661: 273 (53), 300 (100), 329 (66), 345 (65), 455 (63), 559 (52), 573 (39)
triOH-RA-diOH-deRA	$C_{40}H_{57}O_9$	681.4003	MS^2: 315 (32), 329 (42), 331 (42), 333 (55), 345 (34), 347 (52), 349 (100), 363 (61), 365 (64), 661 (35), 663 (74), 677 (49), 679 (68); MS^3 of 349: 287 (33), 289 (37), 347 (100)
tetraOH-deRA-OH-RA	$C_{40}H_{57}O_9$	681.4003	MS^2: 315 (38), 331 (53), 333 (62), 335 (46), 347 (49), 349 (39), 363 (78), 661 (63), 663 (100); MS^3 of 663: 255 (86), 301 (62), 313 (66), 329 (100), 345 (71), 605 (82), 617 (76), 627 (74), 661 (80)
triOH-RA-diOH-RA	$C_{40}H_{59}O_9$	683.4159	MS^2: 331 (47), 333 (55), 349 (100), 365 (54), 663 (63), 665 (38); MS^3 of 349: 193 (38), 273 (46), 285 (32), 305 (39), 331 (78), 347 (100)
hydr. triOH-RA-diOH-deRA	$C_{40}H_{59}O_9$	683.4159	MS^2: 331 (100), 347 (35), 349 (57); MS^3 of 331: 313 (100)

Table 2 (continued)

Compound	Molecular formula, $[M-H]^-$	Mono-isotopic mass, $[M-H]^-$	MS^2 and MS^3 fragmentations, *m/z* (relative abundance)[a]
tetraOH-deRA-diOH-RA	$C_{40}H_{57}O_{10}$	697.3952	MS^2: 347 (79), 349 (57), 363 (100), 365 (56), 653 (67), 677 (70); MS^3 of 363: 267 (42), 345 (89), 361 (100)
pentaOH-RA-OH-deRA	$C_{40}H_{57}O_{10}$	697.3952	MS^2: 315 (100), 347 (91), 677 (84), 693 (65); MS^3 of 315: 297 (79), 313 (100)
triOH-RA-triOH-RA	$C_{40}H_{59}O_{10}$	699.4108	MS^2: 333 (32), 349 (100), 363 (36), 365 (40); MS^3 of 349: 287 (47), 301 (56), 305 (100), 329 (31), 347 (75)

Measured $[M-H]^-$ *m/z* determined by ESI-QTOF-HR-MS was within 10 ppm of the monoisotopic mass.
[a]Relative abundancies lower than 30% are listed only in some cases.
hydr., hydrogenated; deRA, dehydrogenated RA; dideRA, didehydrogenated RA.

fungi (van Beek et al. 2007). To our knowledge, OH-RAs have not been characterized previously by ESI-MS.

High temperature medium-length column GC-MS analysis

Some semipreparative HPLC Frs. were analyzed by GC-MS equipped with a DB-1 HT 15-m column. Fujii et al. (1987) analyzed RA dimers by GC-MS with a 15-m GC column after methylation with diazomethane. This carcinogenic reagent is not used any more in our laboratory and thus some Frs. were methylated with TMAH. This was not successful for RA dimers, although the monomers were methylated. This is the reason why the Frs. were silylated.

Typical fragments in the mass spectra of silylated RAs are formed by the loss of 15 Da (CH_3), 118 Da (possibly two CH_3 + OTMS–H), and 133 Da (possibly HOTMS + C_3H_8). In the mass spectra of silylated OH- and oxo-RA monomers, loss of, e.g. a methyl group, the isopropyl group, 88 or 90 Da (possibly OTMS–H or HOTMS), 103 Da (possibly OTMS–H + CH_3), 118 Da, 133 Da, 208 Da (possibly two CH_3 and two OTMS groups) and 223 Da (possibly C_3H_7 and two HOTMS groups) can be observed. All these compounds have a detectable molecular ion.

In semipreparative HPLC Fr. 18, several compounds were tentatively identified as RA dimers (with both carboxyls in free form). For their mass spectra is typical the neutral molecule loss; the ion with the highest mass is probably the molecular ion. Three of the compounds showed a molecular ion at *m/z* 746, which could correspond to dimers of silylated deRA (300 + 72 + 302 + 72 Da). All compounds lost 116 Da, and two of them lost 188 Da, forming the base peak ion for one of them at *m/z* 630, and for another at *m/z* 558. Loss of 116 Da could correspond to CO_2TMS-H or (OTMS–H) + C_2H_4, whereas 188 Da could correspond to the 116 Da fragment + 72 Da [possibly C_5H_{12} or (TMS–H)]. Two of the compounds also lost 72 and 88 Da. Furthermore, one of the unidentified compounds could correspond to a dimer of deRA and RA, which has lost one of the isopropyl groups, and another to a dimer, which has lost both isopropyl groups. The molecular ions were *m/z* 702 [(300 + 72 + 302 + 72) – 44 Da] and *m/z* 658, respectively. These compounds, also, predominantly lost 116 Da, forming base peak ions at *m/z* 586 and at *m/z* 542, respectively. The molecular ion of the last discernible peak was *m/z* 760, which could correspond to an oxo-dimer of deRA and RA. The fragmentations were similar as for the other RA dimers. All the tentative RA dimers showed masses corresponding to silylated RA monomers, such as *m/z* 370 (silylated dideRA) or *m/z* 372 (silylated deRA), which confirms that they are RAs.

No discernible peaks eluted after the tentative oxo-diresin acid, although there was a rise of the baseline. This indicates that the OH-RA dimers were present in a too complex mixture in combination with low concentrations and/or low response for enabling characterization by GC-MS. The same could be observed by short column GC-FID analysis, as discussed above.

The detection of silylated compounds with masses around 700 Da, and with mass spectra indicating RA fragments, in the Frs. that other analytical methods indicated to contain RA dimers, further confirms that RA dimers are indeed present in the pine knot extract.

RA dimers have not been characterized previously in natural samples. However, AbA dimers (diAbAs) have been prepared by acid catalysis (Sinclair et al. 1970, 1971; Fujii et al. 1987). Also isopimaric and levopimaric acid dimers were prepared (Sinclair et al. 1970). Different diAbAs formed were numerous (about 40); the three major components were shown to be heptacyclic dimers linked at carbon atoms 7-7′ and 14-14′ or 7′-14 and 14′-7, whereas three

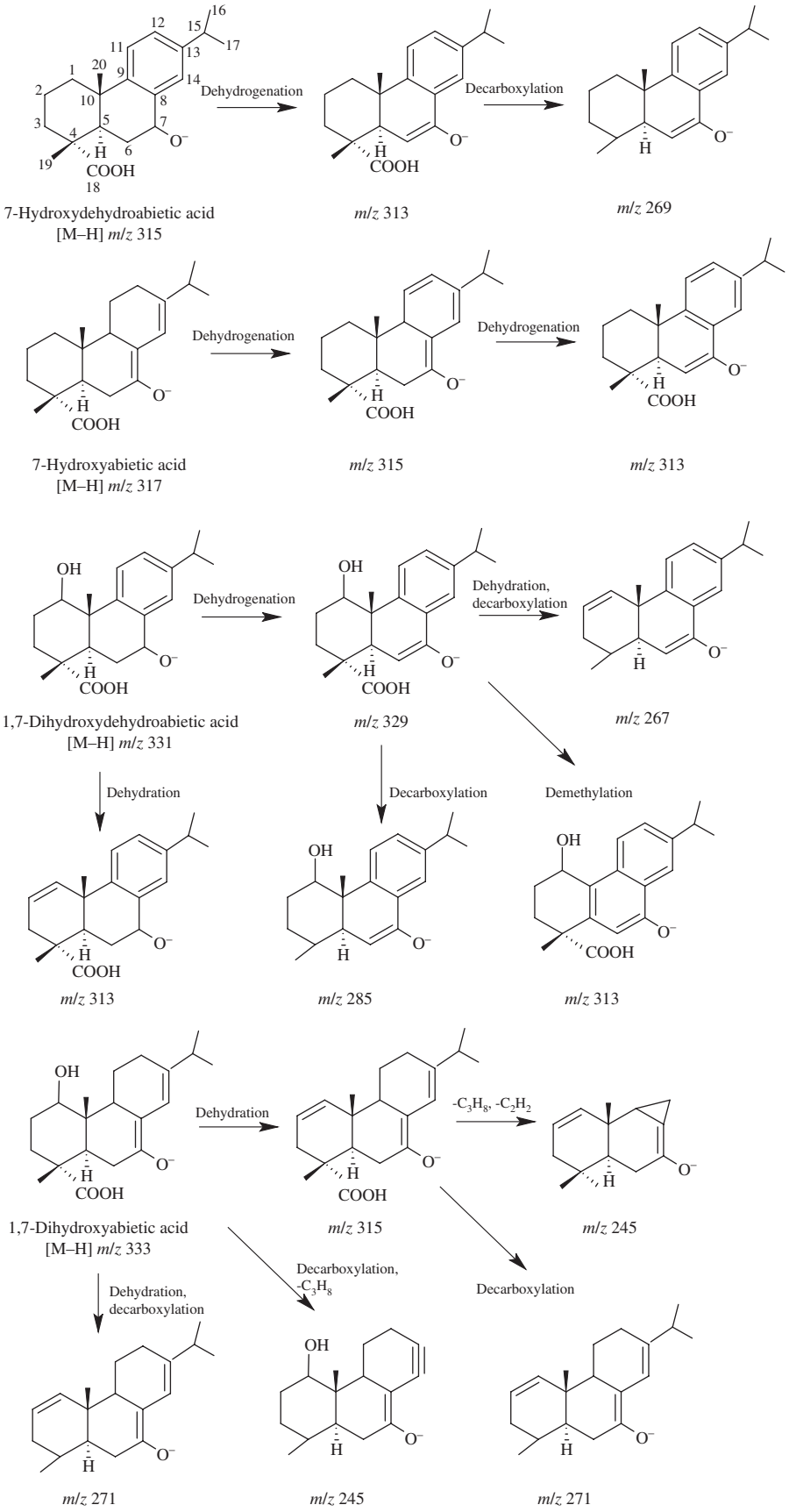

Figure 7: Suggested pathways for MS fragmentation of hydroxylated RA by ESI-IT-MS.

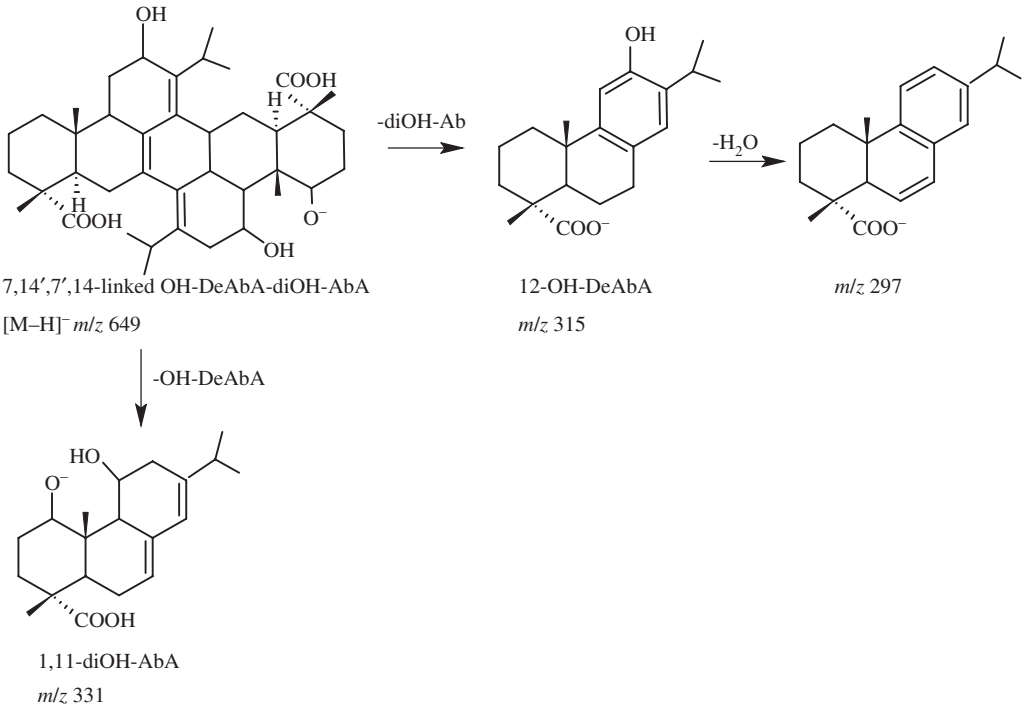

Figure 8: Example of the structure of a hydroxylated RA dimer and suggested pathways for its MS fragmentation by ESI-IT-MS.

structures were linked by a single C-C bond (7-7′ or 7-14′) and had one lactone ring each (Fujii et al. 1987). Gigante et al. (1986) prepared a 7-7′-linked AbA dimer without lactone ring. RA dimers are used commercially in adhesives.

Conclusions

The HMM Fr. of a Scots pine KnW-EtOH extract was a very complex mixture, which seemed to contain mainly RA and FA oligomers, but also oligomers of sterols and stilbenes. The dominating RA moiety is DeAbA and the MM maxima of the oligomeric Frs. ranged from around 500 to 2200 Da. The oligomers contained both free and esterified carboxyl groups, and they were more stable under alkaline than acidic conditions. However, the largest part of the oligomers was stable under both acid and alkaline conditions, which indicates that they were linked by stable C-C bonds. Most of the HMM material was present in a mixture with low-MM compounds, and the separation was challenging. After separation by GPC, flash and silica column chromatography, and semipreparative HPLC, the Frs. still consisted of complex mixtures of FA and RA monomers and oligomers, making identification of individual compounds by NMR impossible. Thus intermonomeric linkages remain unknown. However, by high temperature GC-MS and by ESI-MS, a large number of RA dimers were tentatively identified.

Acknowledgments: This work is part of the activities at the Johan Gadolin Process Chemistry Centre, a Centre of Excellence financed by Åbo Akademi University.

Author contributions: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.
Research funding: None declared.
Employment or leadership: None declared.
Honorarium: None declared.

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