

# Molecular resin composition of two taxodium taxa growing in different climate condition: Chromatographic and spectroscopic study

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## ABSTRACT

The molecular composition of resins from tree trunk, female cone, and needles (from its extract and a raw material) originating from two taxodiaceous varieties *Taxodium ascendens* growing in the Okefenokee Swamp located in the southeastern corner of Georgia (USA) and *Taxodium distichum* growing in the Průhonice Park located in the southeast of Prague (Czech Republic) were analyzed to determine chemical processes involved in the responses of plants to their environment. Despite expectation, any differences in terpenoids production in response to completely different geographic regions and climatic conditions were not found out using spectroscopic and chromatographic techniques. Obtained data confirmed the very high degree of terpenoids composition similarity between *T. ascendens* and *T. distichum*. Chromatographic spectra of female cone and needle extracts were dominated by monoterpenoids, while diterpenoids predominated in the resin from the tree trunk. Using derivatization with BF<sub>3</sub> and TMAH during pyrolysis, the same diterpenoids with various abietane structures were revealed in both varieties. In this study, spectral data of the original unseparated resin from female cone exudates were described for the first time, and an overview of aliphatic and aromatic structures, and the oxygen functional groups such as hydroxyl groups, carboxyl groups, esters and highly conjugated carbonyls was given. Studying variation in the amount and composition of terpenoids in needle extracts of *T. distichum* showed that some variability in terpenoids quality and quantity was related to the season and maturity of the needles.

**Keywords:** *Taxodium ascendens*, *Taxodium distichum*, terpenoids, ATR-FTIR, GC/MS, pyrolysis.

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## INTRODUCTION

Baldcypress, pondcypress, and montezuma cypress are three extant taxa recognized in the genus *Taxodium* (Britton, 1926). Baldcypress has a range along the Atlantic and Gulf Coast Plains of the United States, occurring mainly on rivers with alluvial floods and is particularly well adapted to wetland habitats. Pondcypress is restricted in distribution to Florida and the Gulf Coast east of Texas, occurring in black water rivers, ponds, and swamps, usually without alluvial flood deposits. Montezuma cypress is the southernmost taxa, occurring in Mexico and far southern Texas, generally tolerant of salt and alkaline soils, and less tolerant of

extended flooding. It typically grows next to water sources such as creeks, rivers, lakes, and ponds and performs better in deep loamy soils than in volcanic soils where firs, pines, and oaks are found. The three taxa are distinct in ecology, growing in different environments, but hybridise where they meet. It should be noted that the precise nomenclature for the genus *Taxodium* still remains a matter of some debate. *Taxodium rich.* is a small genus with one to three species. *Taxodium distichum* is widely accepted as the correct name for baldcypress. Disagreement arises with respect to the classification of the other two taxa. Pondcypress has

been treated as a distinct species, *T. ascendens* Brongn., and montezuma cypress is often treated as a distinct species, *T. mucronatum* Tenore (Arnold, 2002). But both are a botanical variety of *T. distichum*. According to many authors, there is no significant difference in reproductive morphology and anatomy, embryology, leaf flavonoid and bioflavonoid profiles, and DNA analysis (Tsumura et al., 1999) but even vegetative and ecological differences are insufficient to support a specific rank for them (Watson, 1983, 1985). Similarly, Lickey and Walker (2002) documented a very high degree of genetic similarity between *T. ascendens* and *T. distichum*, but among others, confirmed that these two taxa have a high degree of morphological plasticity exhibited in response to certain environmental factors. In 2007 Denny and Arnold published a lucid discussion of the historical nomenclature of the genus *Taxodium* and suggest that *T. distichum* is a single species with three botanical varieties: *T. distichum* (L. donovani) Rich. var. *distichum* (baldcypress), *T. distichum* var. *imbricarium* (Nutt.) Croom (pondcypress), and *T. distichum* var. *mexicanum* (Carriere Gordon) (montezuma cypress). Recently, Adams et al. (2012a) examined all three putative species using DNA sequences and also concluded that *Taxodium* is best treated as a monotypic genus with three varieties. Besides that, Cupressaceae sensu stricto (s.s.) was separated from Taxodiaceae by Pilger (1926) and the Taxodiaceae were at one time regarded as a distinct plant family comprising ten genera of coniferous trees. However, recent research has shown that the Taxodiaceae, with the single exception of *Sciadopitys*, should be merged into the family Cupressaceae. Based on the combination of morphological, anatomical and molecular data, both Cupressaceae and Taxodiaceae were incorporated in a single family Cupressaceae sensu lato (s.l.) (Gadek et al., 2000).

Cypress trees are valuable for restoring degraded coastal areas as well as for urban landscapes and other greening projects all over the world. *T. distichum* is cultivated for ornamental, economical, and pharmaceutical purposes. Essential oils from leaves and seeds are used for treatment of malaria, liver diseases, and the cone extract was used against *L. donovani* promastigotes *in vitro* (Naman et al., 2016). Sterols, fatty acids, tannins, polysaccharides, lignin derivatives, flavonol glycoside and biflavone are compounds that were isolated from *T. distichum* extracts and studied in detail (Logan and Thomas, 1985; Miller et al., 1993). In recent years, chromatographic techniques are often used to investigate taxodiaceous resin chemistry. Most chromatographic studies are focused on analyses of essential oils of *T. distichum*, obtained by steam distillation from fruits (El Tantawy et al., 1999; Kamkar and Bagher, 2002) and by hydrodistillation from cones, leaves, and branches (Flamini et al., 2000), leaves and fruits (Ogunwande et al., 2007). Other studies deal with the method of sample preparation. Dapić and Ristić

(2017) documented maceration of cones in absolute ethanol while Kusumoto et al. (2009) and Kusumoto et al. (2010) described the extraction of cones in hexane where several abietane-type diterpenoids have been isolated. Some others studies focused on geographical (Adams et al., 2012b), seasonal (Adams, 2012), and environmentally induced (Adams et al., 2014) variations in terpenoid composition. Not just the modern but also fossil taxodiaceous species have been studied and relations between them have been proven. Emphasis has especially been put on research of terpenoid compounds that are considered as valuable molecular markers for (paleo) systematics and phylogeny of conifers (Otto et al., 1997; Otto and Simoneit, 2001; Otto et al., 2003). Molecular markers were also used by Kunzmann et al. (2009) in investigations of fossil pollen and foliage of *Taxodium* from the upper Eocene to lower Miocene localities in central Europe in order to specify fossil remnants belonging to one species, *T. dubium* (Sternberg) Heer.

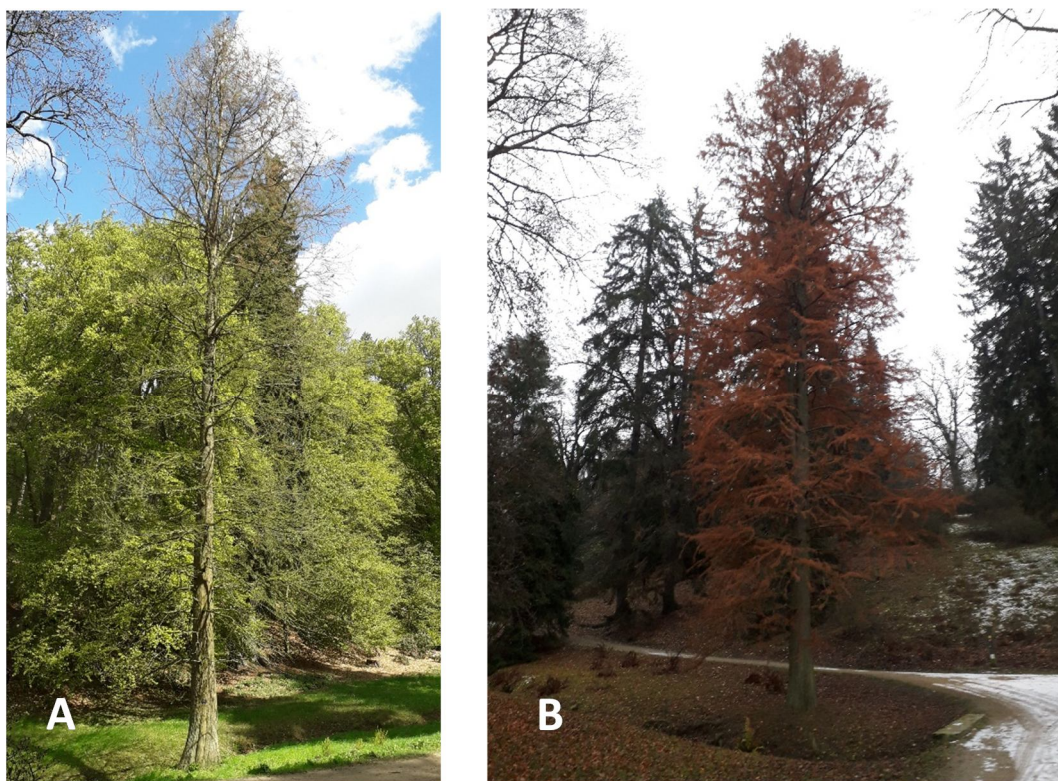
The aim of the present study is to contribute to our knowledge of taxodiaceous resins focusing on characterization of molecular composition of terpenoids. From previously published information about taxodiaceous resins, it is obvious that there are differences in its description and many questions are still unanswered. We studied the molecular composition of resins originating from two taxodiaceous varieties; *Taxodium ascendens* growing in the Okefenokee Swamp located in the southeastern corner of Georgia (USA) and *Taxodium distichum* growing in the Průhonice Park located in the southeast of Prague (Czech Republic). We were wondering if are there any differences between these two varieties growing in very different localities under different climate and natural conditions, especially those of humidity and temperature. There is also the question if are there significant differences among resins from tree trunk, female cone exudates, and needles, and also between analysis of samples from its extract or a raw material, and if so, what are they? The research on changes in the terpenoids variation in needles of *T. distichum* during the seasons was also included. To best answer these questions, several methods were used: Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR), gas chromatography/mass spectrometry (GC/MS), and pyrolysis methylation combined with gas chromatography/mass spectrometry (TMAH-Py-GC/MS) were conducted. Research on taxodiaceous resin from tree trunks by GC/MS and TMAH-Py-GC/MS are not described elsewhere. Measurements of female cone exudates by infrared spectra have also not yet been published; only spectral data describing isolated single substances, such as e.g. taxodone and taxodione are known (e.g. Kupchan et al., 1969). As is well-known, cypress trees have leaves that don't persist throughout the year; they appear in April and generally turn yellow and fall in October. Seasonal

variability in terpenoid compositions of *T. distichum* needles over a two-year period was studied using of head-space method (HS-GC/MS) for analysis of volatile compounds.

## MATERIALS AND METHODS

*T. distichum* (Figure 1, 2A) grow in the Průhonice Park located in the southeast of Prague, Czech Republic (49.9995944N, 14.5570811E). The natural park (area of 250 hectares) was founded in 1885 by Count Arnošt Emanuel Silva-Tarouca. He introduced exotic woody species and founded a dendrological collection of about 1,600 species of plants. Seeds of *T. distichum* were imported from the Montpellier botanical garden, France, and a

seed planting was held in 1923. The age of those trees is clearly 90 years. Resin from the tree trunk, female cones, and needles were sampled. Female cones were collected directly from the tree, and resin was removed from the trunk 1.5 meters above the ground, both in the summer (August) 2016. Needles were cut at a height of 2 to 3 meters above the ground on the southern side of the tree. Samplings of needles were carried out in the spring (April), summer (August), and autumn (November) in 2016 and 2017. *T. ascendens* (Figure 3, 2B) originated from the Okefenokee Swamp located in the southeastern corner of Georgia, USA (30.7101964N, 82.1719061W). The Okefenokee was formed over the past 6,500 years by the accumulation of peat in a shallow basin. The age of the studied tree is estimated as 50 years. Resin from the tree trunk and female cones were sampled in the summer (August) 2016. Collected samples were extracted or used in the raw state.



**Figure 1.** *Taxodium distichum* is growing in the Průhonice Park located in the southeast of Prague in the Czech Republic. A) A tree in the spring, B) in the autumn.

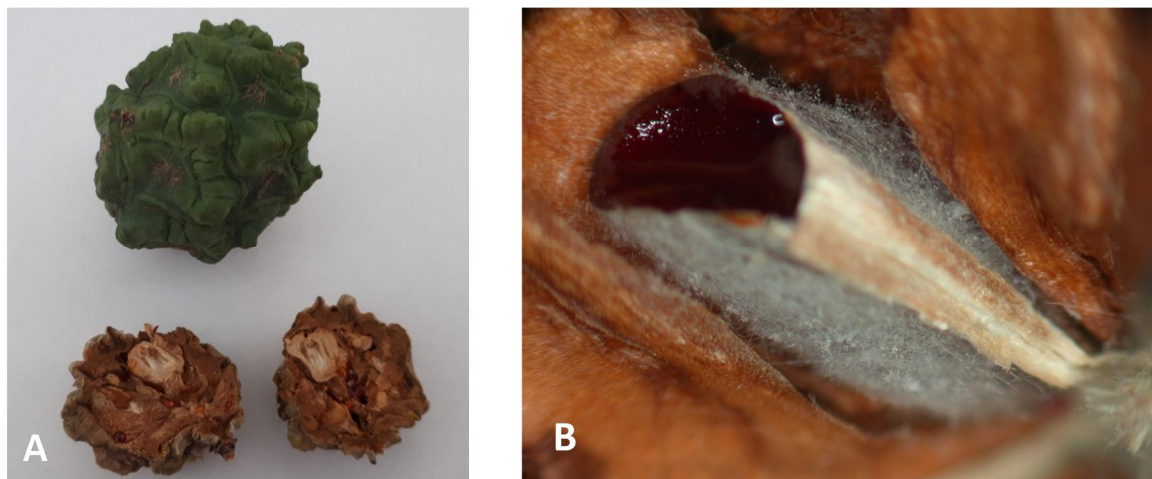
### ATR-FTIR analysis of female cone exudates

The exudates were carefully scraped from cone surfaces of *T. distichum* and *T. ascendens*, and measured using FTIR spectroscopy without any prior treatment. FTIR spectra of the exudate samples were collected on a Nicolet 6700 FTIR (Thermo Nicolet Instruments Co.) with a N<sub>2</sub> purging system. Spectra were acquired using a single reflection ATR (Attenuated Total Reflection) GladiATR accessory equipped with a single bounce diamond crystal (angle of incidence 45°). A total of 64 scans were averaged for each sample and the resolution was 4 cm<sup>-1</sup>. The spectra were rationed against a single-beam spectrum of the clean ATR crystal and converted into absorbance units using ATR correction. Data were collected in the range 4000 to 400 cm<sup>-1</sup>.

### GC/MS analysis of resin extracts

Samples were processed immediately after collection in the laboratory. Female cones (0.5 g) and needles (0.5 g) were cut with a clean scalpel into small pieces (1 mm) and extracted for 5 min with dichloromethane (DCM) in a sonicator. Resin from the tree trunk (0.1 g) was sonicated in DCM for 5 min as well. All solvent extracts were filtered through glass fiber filters and concentrated using a rotary evaporator to 1.5 ml of DCM. Half of the samples were subjected to derivatization with 14% boron trifluoride (BF<sub>3</sub>) in methanol. Hydrolysis and methylation were carried out at 90°C for 1 h. After that, 2 ml of water were added to the mixture, the vials were shaken manually for 30 s and the organic phase was separated by centrifugation at 2500xg for 2 min. The aqueous phase was





**Figure 2.** A) Female cones of *T. distichum*, B) female cone exudate of *T. ascendens*.



**Figure 3.** *Taxodium ascendens* is growing in Okefenokee Swamp located in the southeastern corner of Georgia in the USA. A) A tree in the summer; B) drops of resin on the tree trunk.

removed and the organic layer was then washed with water twice to remove excess reagents, prior to injection.

Chromatographic analysis was achieved using a Trace GC Ultra coupled to a DSQ II. – mass spectrometer (Thermo Scientific) on a fused silica capillary column TR-35MS (60 m × 0.25 mm i.d., 0.25 μm). The GC/MS operating conditions were as follows: temperature held at 40°C for 1 min, increased from 40 to 120°C at a rate of 15°C/min, to 200°C at a rate of 10°C/min, and to 300°C at a rate of 20°C/min with isothermal hold at 300°C for 10 min. Injections of 1 μl were carried out in splitless mode with the injector temperature at

220°C. The mass spectrometer was operated in the electron impact mode at 70 eV and scanned from 40 to 650 Da. Individual compounds were identified by comparison of mass spectra from the National Institute of Standards and Technology mass spectral library (NIST) and literature.

#### TMAH-Py-GC/MS analysis of raw resin

Analytical flash pyrolysis was performed at 610°C (Curie-Point) for

20 s, using a CDS Pyroprobe 5150 (CDS Analytical, LLC, USA) directly connected to the injection port of a GC/MS instrument described above. Both the interface and the transfer lines were heated to 300°C. The analysis was carried out with a temperature program from 40°C, increasing from 40 to 120°C at a heating rate of 15°C/min, to 200°C at a rate of 10°C/min, and then to 300°C at a rate of 20°C/min with isothermal hold at 300°C for 10 min. The mass spectrometer was operated in the electron impact mode at 70 eV and scanned from 40 to 650 Da. The area of each individual peak was divided by the total area of the integrated total ion chromatogram (TIC) and expressed as the relative abundance of the total area, in percent. Pyrolysis was combined with methylation using a 25% (w/w) solution of tetramethylammonium hydroxide (TMAH) (Sigma-Aldrich). All of the indicated pyrolysis products were identified by comparison of mass spectra from NIST.

### HS-GC/MS analysis of volatile compounds from needles of *Taxodium distichum*

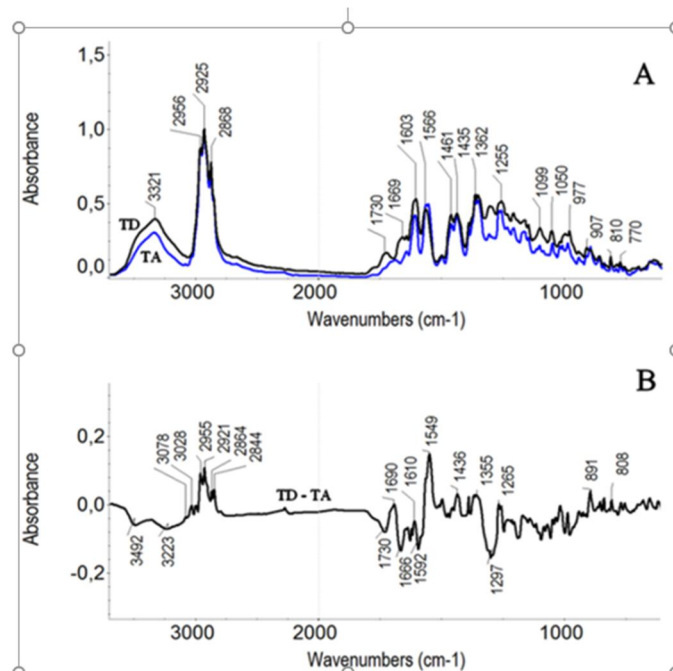
Volatile compounds were analysed using head-space gas extraction. Needles (1 g) were cut into small pieces (1 mm) and immediately crimped into head-space vials. The agitator temperature was 100°C for 10 min. HS-GC/MS analysis was achieved on a fused silica capillary column DB-5 (30 m × 0.25 mm i.d., 0.25 μm). The operating conditions were as follows: temperature held at 40°C for 1 min, increased to 120°C at a rate of 8°C/min, to 250°C at a rate of 15°C/min with an isothermal hold at 250°C for 1 min. The mass spectrometer was operated in the electron impact mode at 70 eV and scanned from 29 to 450 Da. The sample (2 ml) was injected in splitless mode with the injector temperature at 250°C. Data were acquired and processed with the Xcalibur software (ThermoElectron). Individual compounds were identified by comparison with mass spectra from library data (NIST) and literature.

All reagents were of pesticide grade. DCM was purchased from the P-Lab company (Czech Republic), and the derivatization reagents were obtained from Sigma-Aldrich (Czech Republic).

## RESULTS AND DISCUSSION

### Analysis of resin from female cone exudates of *Taxodium ascendens* and *Taxodium distichum*

Infrared spectra of *T. ascendens* and *T. distichum* cone exudates had similar features to other coniferous resin spectra. The most intensive bands at 2925 and 2956 cm<sup>-1</sup> were assigned to antisymmetric stretching vibrations of methylene and methyl groups (Figure 4, Table 1). The band at 2868 cm<sup>-1</sup> was ascribed to symmetric stretching vibrations of the methyl group. Other bands of the aliphatic C-H bonds were found in the 1500 to 1300 cm<sup>-1</sup> region for the deformation vibrations (Table 1). The low intensive stretching band at 3080 cm<sup>-1</sup> was ascribed to aromatic C-H bonds. The spectral regions 3800 to 3100 cm<sup>-1</sup> (the range of O-H stretching vibrations) and 1800 - 1500 cm<sup>-1</sup> (the range of C=O and aromatic C=C stretching vibration, and O-H deformation modes) of the studied samples were band fitted by a mixed Gauss-Lorentz function. Five bands with different hydrogen bonding were found. The band at 3321 cm<sup>-1</sup> had the highest intensity with strong hydrogen bonding. The band



**Figure 4.** Infrared spectra of cone exudates: ATR spectra of *T. distichum* (TD) and *T. ascendens* (TA) (A). Differences in spectra of *T. distichum* and *T. ascendens* (B). The bands are listed and interpreted in Table 1.

positions and their intensities corresponded to positions and the number of OH groups in phenolic molecules. The rich collection of spectral features in the range 1800 to 1500 cm<sup>-1</sup> contained bands whose number and intensities are consistent with the types of carbonyl groups (e.g. quinones and flavones) and their interactions with OH groups: bands at 1669, 1657, 1642, 1623, 1612, 1603, 1566, 1550 cm<sup>-1</sup> were detected.

Unlike other coniferous resins, members of Pinaceae, Cupressaceae or Araucariaceae (Tappert et al., 2011), the spectra of both cone exudates studied presents the characteristic vibrational modes (~1600, ~1550 cm<sup>-1</sup>, and ~3220 cm<sup>-1</sup>) indicating the presence of plant phenolic compounds containing one or more hydroxyl groups bonded to the aromatic rings in their molecules. Hydroxyflavones (e.g. quercetin and its glycosides), lignans, diterpenoid abietane phenols (e.g. ferruginol, totarol), and hydroxylated quinones (e.g. taxoquinone) together with other compounds form a complex mixture (Otto et al., 2003; Zhang et al., 2009; Naman, 2015) and significantly influence the infrared spectra due to the presence of the hydrogen bond system. For example, one of the most biologically active polyhydroxy-substituted derivatives of flavanol is quercetin (3,5,7,3',4'-pentahydroxyflavone) which displays hydrogen bonding, leading to the formation of either 6- or 5-membered intramolecular rings that are essential for conformational stability. In the infrared spectrum of quercetin, two bands of H-bonded O-H groups at 3368 and 3290 cm<sup>-1</sup>

**Table 1.** Assignment of ATR-FTIR vibrational bands for taxodium exudates.

Band position (cm <sup>-1</sup> )	Assignment
3604, 3508, 3431, 3321, 3195	$\nu(\text{O-H})$ strong H-bonded
3080	$\nu(\text{CH})_{\text{ar}}$
2956	$\nu^{\text{as}}(\text{CH}_3)$
2925	$\nu^{\text{as}}(\text{CH}_2)$
2868	$\nu^{\text{s}}(\text{CH}_3)$
1730, 1689	$\nu(\text{C=O})$ in COOH
1669	$\nu(\text{C=O}) + \nu(\text{C=C}) + \delta(\text{O-H})$
1657	$\nu(\text{C=O}) + \nu(\text{C=C}) + \delta(\text{O-H})$
1642	$\nu(\text{C=O}) + \nu(\text{C=C}) + \delta(\text{O-H})$
1623	$\nu(\text{C=O}) + \nu(\text{C=C}) + \delta(\text{O-H})$
1612	$\nu(\text{C=O}) + \nu(\text{C=C}) + \delta(\text{O-H})$
1603	$\nu(\text{C=O}) + \nu(\text{C=C}) + \delta(\text{O-H})$
1566	$\nu(\text{C=O}) + \nu(\text{C=C}) + \delta(\text{O-H})$
1550	$\nu(\text{C=O}) + \nu(\text{C=C}) + \delta(\text{O-H})$
1497	$\nu(\text{C=O}) + \nu(\text{C=C}) + \delta(\text{O-H})$
1461	$\delta(\text{CH}_2/\text{CH}_3) + \nu(\text{C=C}) + \delta(\text{O-H})$
1435	$\delta(\text{CH}_2/\text{CH}_3) + \nu(\text{C=C}) + \delta(\text{O-H})$
1388	$\delta(\text{CH}_3)$ gem
1362	$\delta(\text{CH}_3)$ gem
1350	$\nu(\text{C=C}) + \delta(\text{O-H}) + \delta(\text{CH}_3) + \delta(\text{CH}_2)$
1304	$\delta(\text{O-H}) + \nu(\text{C-O}) + \delta(\text{C-C-H})$
1255	$\nu(\text{C-O}) + \delta(\text{C-C-H})$
1206	$\nu(\text{C-O}) + \delta(\text{C-C-H}), \nu(\text{C-C})$
1160	$\nu(\text{C-O}) + \nu(\text{C-C})$
1145	$\nu(\text{C-O}) + \delta(\text{O-H})$
1099	$\nu(\text{C-O}) + \nu(\text{C-C}) + \delta(\text{O-H})$
1050	$\nu(\text{C-O}) + \nu(\text{C-C})$ ring
1017	$\nu(\text{C-O}) + \nu(\text{C-C})$ ring
995	$\rho(\text{CH}_2/\text{CH}_3), \gamma(\text{O-H})$ in COOH
977	$\rho(\text{CH}_2/\text{CH}_3), \gamma(\text{O-H})$ in COOH
940	$\rho(\text{CH}_2/\text{CH}_3), \gamma(\text{O-H})$ in COOH
907	$\rho(\text{CH}_2/\text{CH}_3), \gamma(\text{O-H})$ in COOH
891	$\rho(\text{CH}_2), \delta(\text{CH}_3), \gamma(\text{C-H})_{\text{ar}}$
855	$\rho(\text{CH}_2), \delta(\text{CH}_3), \gamma(\text{C-H})_{\text{ar}}$
810	$\rho(\text{CH}_2), \delta(\text{CH}_3), \gamma(\text{C-H})_{\text{ar}}$ (
786	$\rho(\text{CH}_2), \delta(\text{CH}_3), \gamma(\text{C-H})_{\text{ar}}$ (
770	$\rho(\text{CH}_2), \delta(\text{CH}_3), \gamma(\text{C-H})_{\text{ar}}$ (
754	$\rho(\text{CH}_2), \delta(\text{CH}_3), \gamma(\text{C-H})_{\text{ar}}$ (
720	$\rho(\text{CH}_2)$
695	$\nu(\text{C-C})$
677	$\nu(\text{C-C})$
650	
633	
617	
598	
586	
574	$\delta(\text{C-C-O})$
551	$\delta(\text{C-O-C}) + \delta(\text{C-C-O})$
520	$\delta(\text{C-O-C}) + \delta(\text{C-C-O})$
497	$\delta(\text{C-C-O})$

Source: Socrates (1994), Schulz and Baranska (2007), Tappert et al. (2011), Machado et al. (2013) and Naman (2015).

appeared, together with prominent vibrational bands at 1670, 1662, 1615, 1559, 1520, 1512, 1503  $\text{cm}^{-1}$ , representing complex vibrations of  $\nu(\text{C}=\text{O})$ ,  $\nu(\text{C}=\text{C})$  and  $\delta(\text{O}-\text{H})$  (Machado et al., 2013). Also, the polyphenolic compound norlignan, isolated from the branches and leaves of *T. ascendens* (Zhang et al., 2009) appeared in the infrared spectral bands at 3421  $\text{cm}^{-1}$  (O-H) and 1614  $\text{cm}^{-1}$  ( $\text{C}=\text{C}_{\text{ar}}$ ). Similar positions of bands as the aromatic phenolic compounds described above were also present for the diterpenoid abietane phenols and hydroxylated quinones. The typical example is ferruginol, which had spectral bands at 3264  $\text{cm}^{-1}$  (OH), 1620, 1582, 1511  $\text{cm}^{-1}$ , and taxochinon that showed bands at 3572, 3340, 1672, 1654, 1618 and 1597  $\text{cm}^{-1}$  (Naman, 2015). Due to the presence of a high intensity band at 1603  $\text{cm}^{-1}$  in the spectra, which can be attributed mainly to the stretching vibrations of the carbonyl groups, it is possible to consider the presence of compounds with strong hydrogen bonding as shifting other bands to lower wavenumbers; e.g. strongly H-bonded 3-hydroxyflavone has similar intensive band positions at 1607 and 1563  $\text{cm}^{-1}$ .

The comparison of the infrared spectra of *T. ascendens* and *T. distichum* reveals that they were similar with respect to functional groups, and only differed in the intensities of their bands. The main differences in the structures of both samples could be clearly detected by subtracting the infrared spectra (Figure 4B). The differences detected were a higher content of aliphatic C-H bonds (2965, 2921, 2864, 2841  $\text{cm}^{-1}$ ), aromatic C-H (3078 and 3026  $\text{cm}^{-1}$ ) and carboxyl groups (1690  $\text{cm}^{-1}$ ) for *T. distichum* cone exudate and a higher content of hydroxyl groups (negative bands at 3492 and 3223  $\text{cm}^{-1}$ ) and esters at 1730  $\text{cm}^{-1}$  in *T. ascendens* cone exudates (Figure 2A). Also, the positions of bands characteristic of hydroxyflavones, hydroxyquinones and lignans (1610, 1690, 1666, and 1592  $\text{cm}^{-1}$ ) showed structural differences in the two specimens of taxodium cone exudates.

Chromatographic spectra of female cone exudates in dichloromethane extracts of both varieties revealed higher relative contents of monoterpenoids over diterpenoids (Table 2, Figure 5), and  $\alpha$ -pinene was the major compound (Figure 5B, D). The monoterpenoids myrcene and  $\beta$ -pinene were not detected in extracts of *T. distichum*. This is interesting because for *T. distichum*, a tree growing in Italy (Flamini et al., 2000) Egypt (El Tantawy et al., 1999), and Nigeria (Ogunwande et al., 2007) the presence of myrcene and  $\beta$ -pinene in female cone extracts was proven. On the contrary, in female cone extracts of a tree growing in China, neither myrcene nor  $\beta$ -pinene were detected and  $\alpha$ -pinene as a typical compound was also not proven (Zhou et al., 2012). Moreover, compared to other studies in female cone extracts of both taxa, a higher proportion of diterpenoids was found. As stated by Flamini et al. (2000) terpenoids in his study were represented almost exclusively by monoterpenoids with  $\alpha$ -pinene and limonene being

predominant, with the exception of small quantities of sesquiterpenoids. El Tantawy et al. (1999) also showed predominantly  $\alpha$ -pinene with a less intense contribution of other monoterpenoid compounds and no diterpenoids in cone extracts. Similarly, Ogunwande et al. (2007) reported a higher content of  $\alpha$ -pinene among the monoterpenoids in cone extracts but diterpenoids were not detected. In all these studies, the terpenoid fraction was isolated by hydrodistillation. Only in the study of Kusumoto et al. (2010) were twelve diterpenoids demonstrated in samples of cones that were extracted in hexane. These results indicate a significant dependence on the extraction method that was used. Solvent extraction seems to be more effective for isolating terpenoids, especially those of higher molecular weight.

Results of GC/MS and ATR-FTIR analyses agree with the presence of plant phenolic compounds containing one or more hydroxyl groups bonded to aromatic rings, and particularly with the presence of diterpenoid abietane phenols such as totarol and sugiol (Figure 5, Table 2). The only difference between these two taxa was in band intensities.

Derivatization procedure of female cone exudate extracts resulted in the formation of methyl esters of fatty acids in resins (Table 3). The chromatographic profile of both taxa showed a very similar and recognizable pattern of methylated compounds with characteristic abietane structures of diterpenoids.

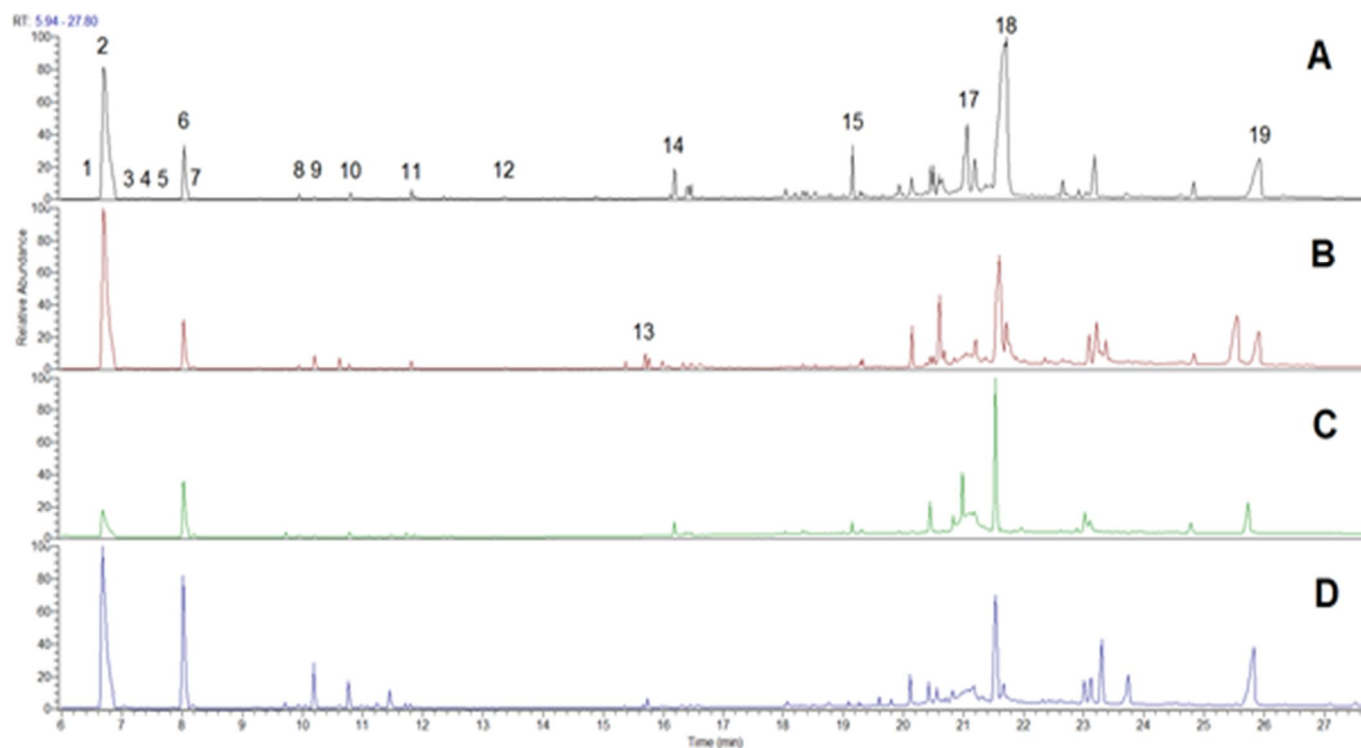
Pyrolysis-GC/MS can help to characterize materials including non-volatile or complex materials. To improve GC/MS detection of polar compounds, derivatisation with tetramethylammonium hydroxide (TMAH) was applied. The principal identified products (Table 4) were mainly various substituted diterpenoids with methoxy- group, belonging to the abietane family. According to Tapert et al. (2011) resins can be subdivided into two distinct resin types that reflect compositional differences in their terpenoid constituents; the first type of resin so-called pinaceous resin is produced by members of Pinaceae and consists mainly of diterpenoids that are based on abietane/pimarane skeletal structures. The second type so-called cupressaceous resin is associated with members of the Cupressaceae, Sciadopityaceae, Araucariaceae, and Podocarpaceae and consists mainly of diterpenoids that are based on labdanoid structures. Likewise, Chiavari et al. (2002) documented a large amount of abietane compounds in resins of Pinaceae, while mainly labdane compounds were detected in resins of Cupressaceae.

However, our results of TMAH-Py-GC/MS analysis proved that terpenoids with abietane structures were present in both taxa of *Taxodium* (Figure 6). It is evident that these abietane compounds were not extracted into the solvent since they were not recorded in the GC/MS chromatograms. However, using pyrolysis to analyse crude resin material, these abietane structures were proven. This result confirms that for demonstrating



**Table 2.** List of chemical compounds identified in extracts of resin from the tree trunk and female cones of *T. ascendens* and *T. distichum*. Numbers of compounds correspond to those given in the chromatograms in Figure 5.

Compound	MW	<i>T. ascendens</i>		<i>T. distichum</i>	
		Trunk 2016	Cone 2016	Trunk 2016	Cone 2016
		%Area	%Area	%Area	%Area
1 Tricyclene	136	0.05	0.07	0.05	0.11
2 $\alpha$ -pinene	136	29.39	48.26	16.06	39.17
3 Camphene	136	0.07	0.23	0.05	0.42
4 $\beta$ -myrcene	136	0.10	0.12	-	-
5 $\beta$ -pinene	136	0.05	0.07	-	-
6 D-limonene	136	5.42	7.51	19.24	17.46
7 o-cymene	134	0.09	0.29	0.48	0.50
8 p-mentha-2,8-dienol	152	0.03	0.07	1.22	0.54
9 Camphenol, 6-	152	0.32	0.42	0.22	0.43
10 2(10)-pinen-3-ol	152	0.24	1.74	0.65	4.05
11 2(10)-pinen-3-one	152	0.46	0.57	1.23	2.45
12 Bornyl acetate	196	0.50	0.69	0.25	0.35
13 Caryophyllene oxide	220	-	0.94	-	0.72
14 Agarospirol	222	208	-	2.56	-
15 Labda-8(20)-14-dien-13-ol	290	2.51	-	1.64	-
16 Dehydroabietane	270	-	3.15	-	1.90
17 Pimaral	286	1.44	0.71	7.80	1.66
18 Totarol	286	46.85	26.08	36.75	15.35
19 Sugiol	300	10.41	9.07	11.79	14.89



**Figure 5.** Total ion chromatograms (TIC) of extracts of resin from the tree trunk and female cones of *T. ascendens*: extract of resin from tree trunk (A) and female cone extract (B), and *T. distichum*: extract of resin from tree trunk (C), female cone extract (D). For list of compounds see Table 2.



**Table 3.** List of compounds identified in extracts of resin from female cone exudates and the tree trunk of *T. distichum* and *T. ascendens* after derivatization with BF<sub>3</sub>.

Compound	MW	<i>T. distichum</i>		<i>T. ascendens</i>	
		Cone 2016		Cone 2016	Trunk 2016
		%Area		%Area	%Area
1 4-(2-Methoxypropan-2-yl)-1-methylcyclohex-1-ene	168	18.91	23.62	21.05	
2 1-Ethyl-4,4-dimethyl-cyclohex-2-2n-1-ol	154	0.59	0.79	-	
3 Octanal, 7-methoxy-3,7-dimethyl-	186	12.96	16.31	13.99	
4 Dammar-22-en-3-ol, 20,24-epoxyl-24-methyl-, acetate, (3 $\beta$ , 24S)-	444	-	0.13	-	
5 Hexadecanoic acid, methyl ester	270	1.44	0.92	-	
6 7-isopropyl-1,1,4a-trimethyl-1,2,3,4,4a,9,10,10a-octahydrophenanthrene	270	-	0.76	-	
7 Imidazole, 2-phenylazoamino-1-methyl-	201	-	0.08	-	
8 S-indacene-1,7-dione, 2,3,5,6-trrahydro-3,3,4,5,5,8-hexamethyl-	270	-	0.38	8.87	
9 2,3-Dehydroferruginol, trimethylsilyl ester	356	10.77	9.76	-	
10 Podocarpa-8,11,13-triene-7 $\beta$ ,13-diol, 14-isopropyl-	302	32.80	19.64	20.04	
11 Ferruginol	286	7.93	6.70	17.59	
12 3-(3',4'-Dimethoxyphenyl)-6-ethoxy-4-methylcoumarin	340	5.97	10.94	-	
13 9(1H)-Phenanthrenone, 2,3,4,4a,10,10a-hexahydro-6-hydroxy-	300	5.09	4.19	18.46	
14 Di(pentamethylphenyl)ketone	322	1.39	2.41	-	
15 Podocarpa-1,8,11,13-tetraen-3-one, 14-isopropyl-1,13-dimethoxy-	342	2.16	3.37	-	

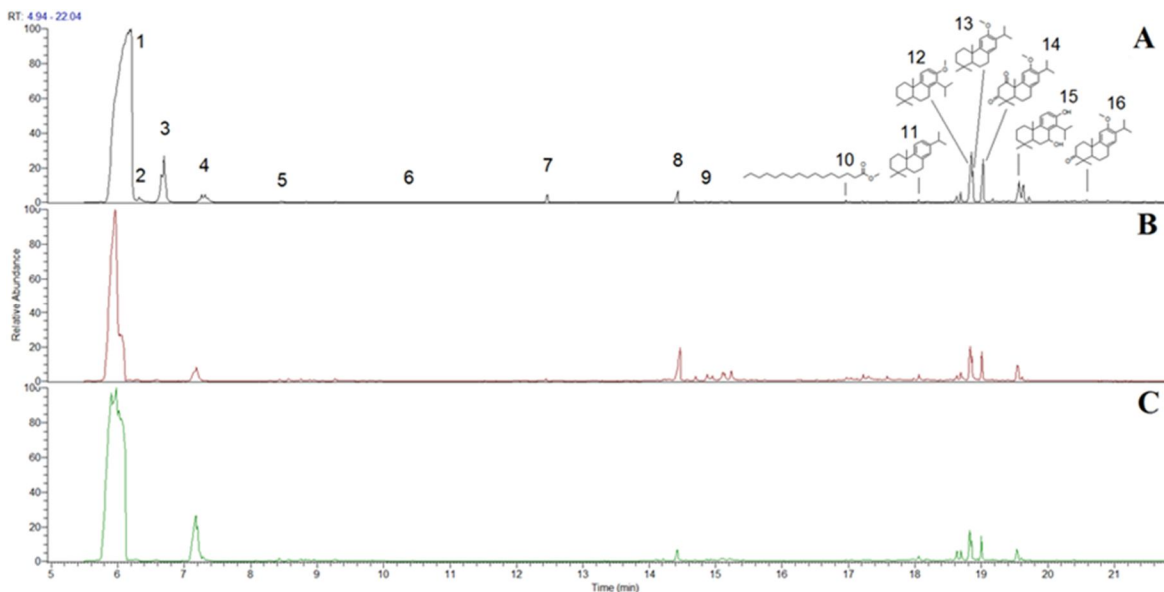
**Table 4.** List of compounds identified by TMAH-Py-GC/MS analysis of female cone resin of *T. distichum*, and female cone resin and resin from the tree trunk of *T. ascendens*. Numbers of compounds correspond to those given in the chromatograms in Figure 6.

Compound	MW	<i>T. distichum</i>		<i>T. ascendens</i>	
		Cone 2016		Cone 2016	Trunk 2016
		%Area		%Area	%Area
1 $\alpha$ -Pinene	136	84.60	82.07	87.78	
2 Camphene	136	0.11	0.23	0.22	
3 $\beta$ -Myrcene	136	5.35	0.37	0.17	
4 D-Limonene	136	1.46	3.45	7.05	
5 Bicyclo[2.2.1] heptane, 2-methoxy-1,7,7-trimethyl-	168	0.09	0.23	0.20	
6 Bornyl acetate	196	0.03	-	-	
7 Caryophyllene	204	0.43	0.19	-	
8 Caryophyllene oxide	220	0.68	4.76	0.67	
9 Agarospirol	222	0.03	0.47	0.09	
10 Palmitic acid, methylester	270	0.09	0.11	0.03	
11 Dehydroabietane	270	0.10	0.49	0.22	
12 14-Isopropyl-13-methoxypodocarpa-6,8,11,13-tetraene	298	2.78	2.72	1.30	
13 Podocarpa-8,11,13-trien, 13-isopropyl-12-methoxy-	300	0.72	1.06	0.58	
14 Podocarpa-8,11,13-triene-1,3-dione, 13-isopropyl-12-methoxy-	328	2.27	2.08	0.94	
15 14-isopropylpodcarpa-8,11,13-triene-7,13-diol	302	1.16	1.77	0.76	
16 Podocarpa-8,11,13-trien-3-one, 13-isopropyl-12-methoxy-	314	0.09	-	-	

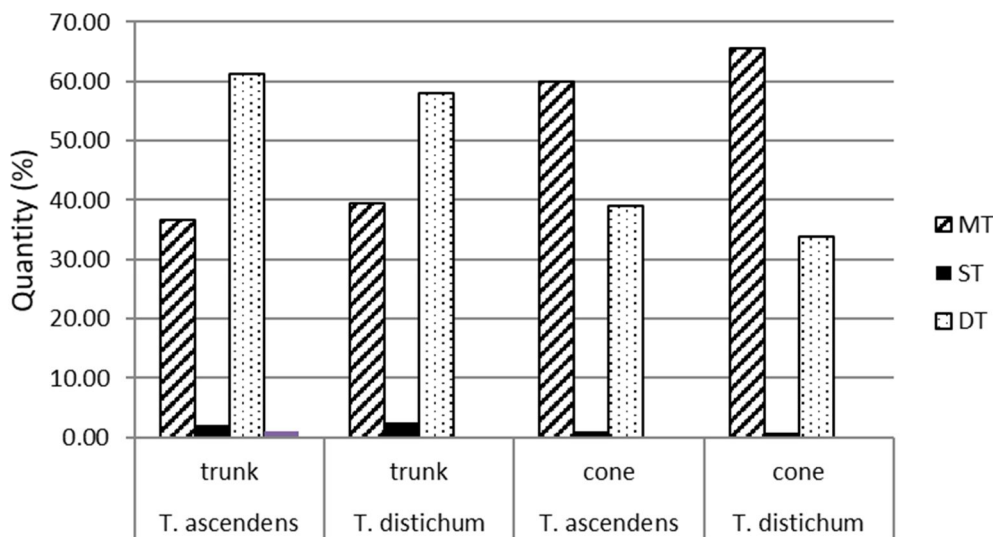
terpenoids of higher molecular weight, in our case abietane structures, analysis of the raw samples of resin is more appropriate. In addition, TMAH-Py-GC/MS of *T. distichum* resin from cone exudates also resulted in the detection of the monoterpene  $\beta$ -myrcene and the diterpenoid dehydroabietane which were not seen using GC/MS analysis of resin extracts.

#### Analysis of resin from the tree trunks of *Taxodium ascendens* and *Taxodium distichum*

The extracts exhibited higher amounts of diterpenoids than observed in female cone exudates (Table 2, Figure 7). Totarol and sugiol formed a substantial proportion of diterpenoids with small amounts of pimaral and



**Figure 6.** Total ion chromatograms (TIC) of TMAH-Py-GC/MS analysis of the resin from female cone exudates of *T. distichum* (A), female cone exudates of *T. ascendens* (B) and resin of the tree trunk of *T. ascendens* (C). For list of compounds see Table 4.



**Figure 7.** Comparison of the content of mono- (MT), sesqui- (ST), and diterpenoids (DT) in extracts of resin from the tree trunk and female cones of *T. ascendens* and *T. distichum*.

dehydroabietane (Figure 5A, C). Diterpenoids of the abietane-type were associated with antitermitic function (Kusumoto et al., 2009). Diterpenoids totarol and sugiol were also detected as the major compounds in the fossil species *T. dubium* (Otto et al., 1997). Extracts also contained a higher amount of sesquiterpenoids than extracts of female cones of both taxa (Table 5). Small differences in the quantity of terpenoids between these two taxa were observed. The major compound in extracts of resin from *T. distichum* was totarol (36.75%), followed

by limonene (19.24%) and  $\alpha$ -pinene (16.06%), while resins of *T. ascendens* also contained higher levels of totarol (46.85%) and  $\alpha$ -pinene (29.39%), but limonene reached only 5.42%. Extracts also differed in the presence of  $\beta$ -myrcene and  $\beta$ -pinene in the resin of *T. ascendens* (although in trace amounts). Only the resin from the tree trunk of *T. ascendens* was available in sufficient amounts for derivatization followed by GC/MS analysis. In the list of compounds (Table 3), fewer compounds identified in resins from the tree trunk are

**Table 5.** List of compounds identified in needle extracts of *T. distichum*. Samples were collected in the spring (April), summer (August), and autumn (November) of 2016 and 2017.

Compound	MW	Apr 2016	Aug 2016	Nov 2016	Apr 2017	Aug 2017	Nov 2017
		%Area	%Area	%Area	%Area	%Area	%Area
1 Tricyclene	136	0.31	0.28	0.14	0.22	-	0.30
2 $\alpha$ -pinene	136	71.47	54.63	4.00	20.03	39.94	29.21
3 Camphene	136	0.99	1.16	0.10	0.63	2.56	2.34
4 $\beta$ -phellandrene	136	-	0.66	-	-	-	-
5 $\beta$ -pinene	136	-	1.75	-	-	0.94	-
6 D-limonene	136	8.52	7.55	1.18	-	6.83	-
7 p-mentha-2,8-dienol	152	0.19	0.63	0.03	1.57	1.44	2.72
8 2(10)-pinen-3-ol	152	0.38	1.77	0.07	28.16	3.18	11.33
9 2(10)-pinen-3-one	150	0.36	2.11	0.04	22.29	2.53	12.03
10 Bornyl acetate	196	0.03	0.06	0.02	2.67	-	-
11 Caryophyllene	204	11.61	2.49	0.89	-	-	-
12 4(15),5-murolene	204	0.40	0.92	-	-	-	-
13 Caryophyllene oxide	220	-	-	1.64	-	8.89	-
14 Longipinocarvone	218	0.86	4.42	-	-	-	-
15 Dehydroabietane	270	-	2.31	-	4.34	2.24	1.58
16 Sugiol	300	-	-	83.62	2.44	-	-
17 Totarol	286	4.90	19.26	8.26	17.65	31.46	40.50

evident compared with female cone exudates. Conversely, TMAH-Py-GC/MS analyses (Table 4, Figure 6) showed very uniform results of chemical composition in tree trunk and female cone exudate resins. Unfortunately, there are no literature reports on the chemical composition of extant *Taxodium* resins removed from tree trunks to compare with our results.

#### Analysis of resin from needles of *Taxodium distichum*

GC/MS analyses showed substantial differences in the terpenoid compositions of needle extracts, and extracts of resin from the tree trunk and female cone exudates of *T. distichum*. Monoterpenoids were the predominant fraction in needle extracts, with sesquiterpenoids presented in a trace amount. In contrast, with extracts of resin from tree trunk and female cone exudates, monoterpenoids  $\beta$ -phellandrene, 2(10)-pinene, and p-mentha-2,8-dienol, and sesquiterpenoid longipinocarvone were detected. On the contrary, the monoterpenoid o-cymene, the sesquiterpenoid agarospirol, and diterpenoids labda-8(20)-14-dien-13-ol and pimaral were not proven. There were also differences in composition during the year. Monoterpenoids with the major constituent  $\alpha$ -pinene were the main fraction in extracts, except for needles collected in November 2016, where the major compound diterpenoid sugiol (83.62%) was detected (Table 5). Bornyl acetate, as an oxygenated monoterpenoid, was present in trace amounts in the year

2016, however higher amounts (2.53%) were found in the spring of 2017. Sesquiterpenoids 2(10)-pinen-3-ol and 2(10)-pinen-3-one were proven in all extracts of both years, however, in April 2017 they achieved their highest amount relative to other compounds. The diterpenoid totarol was detected in all extracts of needles in both years, but during 2017 its quantity increased.

Variations were also observed between years. Significant decreases in  $\alpha$ -pinene, limonene, and caryophyllene were observed in the summer of 2016 (54.63, 7.55 and 2.49%, respectively) and then even more in the autumn of 2016 (4, 1.18 and 0.89, respectively) (Table 5). Similarly, Adams (2012) and Adams et al. (2014), in studies on seasonal variations of terpenoids of *T. distichum* growing in Texas (USA), found that the amount of certain terpenoids increased in young needles from April to May, then rapidly declined, and became constant until October, when the needles yellowed and gradually fell. Variability in the amounts of terpenoids was attributed to maturation of the needles.

Headspace analysis was used to examine volatile compounds in the needles (Table 6). No significant quantitative variations in terpenoids were observed. Compared to needle extract analyses, almost unchanging and reconcilable amount of  $\alpha$ -pinene, camphene, and limonene in both years, 2016 and 2017, were detected. While the amounts of  $\alpha$ -pinene were very high other compounds were detected in trace amounts, showing no quantitative changes, neither during season nor during the years. Some mono-, sesqui-, and diterpenoids were only detected using HS-GC/MS.

**Table 6.** List of volatile compounds identified in needles of *T. distichum* by HS-GC/MS. Samples were collected in the spring (April), summer (August), and autumn (November) of 2016 and 2017.

Compound	MW	Apr 2016	Aug 2016	Nov 2016	Apr 2017	Aug 2017	Nov 2017
		%Area	%Area	%Area	%Area	%Area	%Area
1 Tricyclene	136	0.26	0.26	0.27	0.20	-	-
2 $\alpha$ -pinene	136	79.59	73.58	69.26	75.26	81.34	76.91
3 Camphene	136	0.98	2.99	0.88	2.00	1.48	0.60
4 $\beta$ -pinene	136	0.21	-	-	1.11	1.66	1.15
5 $\beta$ -pinene (stereoisomer)	136	3.27	1.73	1.28	0.25	-	-
6 o-cymene	134	-	1.03	3.69	-	-	-
7 D-limonene	136	11.49	12.37	23.01	19.33	15.51	19.79
8 $\tau$ -terpinen	136	-	0.22	-	0.13	-	-
9 p-mentha-1,4(8)-diene	136	0.37	0.38	0.02	0.23	-	-
10 Alcanfor	152	0.18	-	-	-	-	0.17
11 p-menth-1-en-4-ol	154	0.10	-	-	0.09	-	-
12 p-menth-1-en-8-ol	154	0.10	1.60	-	0.04	-	0.39
13 Bornyl acetate	196	-	1.51	0.03	-	-	-
14 Caryophyllene	204	2.54	3.37	1.44	1.37	-	0.58
15 Germanacrene D	204	0.69	-	-	-	-	-
16 4(15),5-muurolene	204	0.14	0.24	-	-	-	-
17 Cadina-1(10),4-diene	204	0.09	0.71	-	-	-	-
18 Caryophyllene oxide	220	-	-	0.12	-	-	0.42

## CONCLUSION

Using ATR-FTIR and GC/MS techniques with derivatization using  $\text{BF}_3$  and TMAH during pyrolyzing, new facts on taxodiaceous resins were revealed.

The infrared spectra of female cone exudates showed the presence of intensive bands at  $\sim 1600$  and  $\sim 1550 \text{ cm}^{-1}$ , which indicate the presence of phenolic substances such as hydroxyflavoditerpenoid, hydroxyquinones or lignans, and that is what distinguishes taxodiaceous resins from another conifer's resins. Subtracting the *T. ascendens* and *T. distichum* spectra, the structural differences were found only in intensities of aliphatic and aromatic structures, OH, COOH, and COOR groups and phenolic compounds.

Chromatographic spectra of resin extracts gained from trees that are growing in different environments surprisingly showed very similar terpenoids composition; with diterpenoid totarol dominating in resins from the tree trunk, while monoterpenoid  $\alpha$ -pinene dominated in resins from cone exudates and needles.

The use of derivatizations has proved to be very effective: by derivatization with  $\text{BF}_3$  the presence of many other abietane structures of diterpenoids in resin extracts was demonstrated, and the principal identified products of TMAH-pyrolysis of raw material were various substituted diterpenoids with methoxy- groups belonging to the abietane structural class.

Although *T. ascendens* and *T. distichum* were designated as varieties, and in addition these two trees grow in different geographic regions no differences in the

production of terpenoids have not been revealed.

We showed that the extraction in dichloromethane was more effective for the extraction of diterpenoids than extraction in hexane or hydrodistillation.

Variations in terpenoid quantity in needle extracts of *T. distichum* were demonstrated and shown to be related to individual seasons and maturity of the needles. However, using HS-GC/MS, cross-season variabilities in volatile terpenoids were not observed.

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