CHEMIJA. 2006. Vol. 17. No. 4. P. 67–73

Characteristics of essential oil composition in the needles of young Scots pine (*Pinus sylvestris* L.) stands growing along an aerial ammonia gradient

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Content and composition of needle essential oils of young Scots pine stands (*Pinus sylvestris* L.) growing along an ammonia gradient (in the impact zone of a nitrogen fertilizer factory, JSC *Achema*, Jonava, Lithuania) have been studied. Eight young pine stands growing at a distance up to 22 km from the factory were chosen for sampling. Volatile components of the current-year and one-year-old needles were obtained by a simultaneous hydrodistillation-extraction of dried material and analysed by GC and GC/MS.

The yield of the essential oil varied from 0.25 to 0.47% and 0.25–0.49% in the current-year and one-year-old needles of pine, respectively. A slight increase of the oil yield was observed in the needles collected in pinewoods located at a distance up to 5 km from the factory. The chemical composition of the volatile oils of the dried needles collected at different distances was investigated. The most predominant fraction was found to be monoterpene hydrocarbons (58.4–72.4% and 45.0–60.8% in the current-year and one-year-old needles, respectively) with the major constituents α-pinene (19.8–35.0% and 18–28.0%) and δ-3-carene (22.7%–33.7% and 19.9–25.9%). Variability of the amounts of these major constituents in the needles of the stands growing at different distances from the factory were represented. Sesquiterpene hydrocarbons were the second dominating fraction in the oils and formed 14.5–25.7% and 18.5–33.1%, in the current-year and one-year-old needles, respectively. Under the effect of ammonia pollution, higher amounts of shorter chain terpenes and lower amounts of longer chain ones were produced, and this dependance was stronger expressed in the oils of the current-year needles.

Key words. *Pinus sylvestris*, *Pinaceae,* secondary metabolites, essential oil composition, monoterpenes, α-pinene, δ-3-carene, sesquiterpenes, diterpenes, industrial pollution, phytoindication

INTRODUCTION

The genus *Pinus* of the family *Pinaceae* (division *Pinophyta*) is comprised of more than 100 species and is spread worldwide. *Pinus sylvestris* L. (Scots pine) is the most widespread pine in the world. Scots pine's native land is Eurasia and it is adapted to different climatic conditions and grows on a wide variety of soil types.

Pinus sylvestris L. is an evergreen tree of 20-45 m height living up to 500 years. It is the most common tree species in Lithuania where pinewoods occupy about 38% of the total forest area.

Pine is a source of wood, it is also used to protect the erosion of soil and its buds, needles and bark are used in phytotherapy. Pine needles are rich in vitamin C, tannins, alkaloids and essential oils. Moreover, pines as all coniferous are very sensitive to air pollution, and the analysis of secondary metabolites allow to evaluate both the physiological state of a plant and the environmental conditions under which it is growing.

Scots pine is rich in monoterpene hydrocarbons such as α- and β-pinene, δ-3-carene, limonene, α- and γ-terpinene, (Z)-β-ocimene, myrcene, camphene, sabinene and terpinolene [1]. Other constituents include bornyl acetate, borneol, 1,8-cineole, citral, terpineol, α-cadinol, α-muurolol, (β)-caryophyllene, chamazulene and some acids [2]. The results of Scots pine essential oil composition research published in 70s and 80s years were reviewed by Lawrence [3]. Data on the essential oil composition variability of pines growing in different geographical locations and under various environmental

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conditions were presented in many studies [4–9], while the number of investigations of the volatile oils from pines growing in Lithuania is very limited.

Climatic and anthropogenic factors such as the extremes of temperature and rainfall, air and soil pollution have led to biochemical responses in the plants. Trees, especially coniferous, are very sensitive to unfavorable conditions. Changes in the amounts of secondary metabolites may be early indicators of an injury. The analysis of the secondary metabolites in *P. sylvestris* under stress factors such as mechanical damage, temperature decrease and pollution was carried out by Fuksman [10]. It has been shown that when affected by pollution and fungal infections, Scots pine begins to synthesise a higher amount of α-pinene and phenolic compounds.

Changes of terpenes in the needles of white pine (*Pinus strobus*) growing in an urbanized environment were investigated [11]. Urban conditions with a significant environmental impact of traffic and industry and with subsequent changes in soil and climatic properties have caused some changes in the content and composition of the essential oils, mainly in the proportions of α- and β-pinene, myrcene, limonene, α-phellandrene, 1,8-cineole, terpineol, citral and carvone. The situation in the research of *Pinus sylvestris* L. in urban conditions along the transects was identical – the content of some monoterpenes such as carvone, bornyl acetate, β-pinene, terpineol increased, but the content of other monoterpenes such as citral, myrcene, camphene decreased in comparison with a relatively clear site [12]. Stress factors of the urban environment caused an increased production of terpenes, which was statistically significant as a protective mechanism against the environmental impact [11,12].

Previous studies have shown that UV-B radiation promotes terpene production in woody plants, suggesting that terpenes may also play a role in a response to ammonia pollution [13]. Nutrient availability on the secondary plant compounds in Scots pine was investigated in another study [14].

Since the 1980's researchers have widely examined the conditions of forests surrounding the largest stationary sources of atmospheric pollution in Lithuania. Several components of forest ecosystem were examined such as soil, air, trees and other organisms [15–20]. It was determined that the soil near an important pollution source in Lithuania, nitrogen fertilizer plant *Achema*, is acidic only at up to 4–8 km distance from the factory, with an increased Al^{3+} quantity [20]. Besides, an ammonia concentration gradient (26.0–3.5 $\mu g\ m^{-3}$) in the air was determined and the amount of this pollutant exceeded the critical concentration at the sites closest to the factory [19]. Characteristics of Scots pine growth in the environment polluted by the nitrogen fertilizer factory were discussed in another study [21], the results of long-term investigations of defoliation were represented. It was shown how the damaged trees had started to recover under a reduced pollution (emissions of nitrogen and sulphur oxides and the dust of mineral fertilizer). Since trees react very slowly to the environmental changes, it is often difficult to discern and to evaluate an invisible tree damage; sensitivity indicators for the latent tree changes should be found [22, 23]. The influence of industrial pollution in low levels (especially of ammonia) is not investigated.

The aim of this study is to characterize the changes of secondary metabolites (content and chemical composition of needle essential oils) of young Scots pines (*P. sylvestris*) growing in the impact zone of emissions from the nitrogen fertilizers factory.

MATERIALS AND METHODS

Young stands of Scots pine located at a distance of twenty two kilometers in the prevailing north-east wind direction from the nitrogen fertilizer factory (JSC *Achema*, Jonava district, Lithuania) were selected for sampling. A predominant soil type was arenosols. The needles of the current-year and one-year-old of young stands (up to 40 years of age) were collected from the middle part of the crowns of each tree in July, 2005. The plant material was dried at room temperature (20 °–25 °C) with ventilation.

The oils were isolated by a simultaneous hydrodistillation-extraction (a mixture of hexane and diethyl ether (1:1)) was used as an organic collecting solvent by a Clevenger-type apparatus. The isolation took 2 hours, and 15 g of the dried material was used for each experiment. The solutions of oil were dried over anhydrous sodium sulphate and stored in sealed vials in a refrigerator before analysis. The oil yield was conducted by hydrodistillation of 50 g of the dried needles, before distillation humidity of the dried material was measured using a Traceble® Term/Clock/Humidity Monitor (Fisher Scientific). Pale yellow oils were obtained in 0.2–0.6% and 0.2–0.3% of yield, respectively in the current-year and one-year-old needles on a dry mass basis.

GC analysis was carried out by an HP 5890(II) chromatograph equipped with an FID and a capillary column HP-FFAP (30 m × 0.25 mm, film thickness 0.25 mm). The GC oven temperature was set at 70 °C for 10 min and then programmed to 70 °–210 °C at a rate of 3 °C/min, and at 240 °C isothermal for 5 min, using He as a carrier gas (0.7 mL/min). The injector and detector temperatures were maintained at 200 °C and 250 °C, respectively.

The analyses by GC/MS were performed using a chromatograph HP 5890 interfaced to an HP 5971 mass spectrometer (ionization voltage 70 eV, m/z scan rage 35–350 Da, scan time – 0.6 s) and equipped with a capillary column CP-Sil 8 CB (50 m × 0.32 mm, film thickness 0.25 μm). The oven temperature was held at 60 °C for 2 min, then programmed to 60 °–160 °C at a rate of 5 °C/min, held for 1 min, then reprogrammed to 160 °–250 °C at a rate of 10 °C/min, and finally isothermal at 250 °C for 2 min, using He as a carrier gas (1.0 mL/min, split ratio 1:20). The injector and detector temperatures were 250 °C.

The percentage composition of the oils was computed from GC peak areas without the correction factors. Qualitative analysis was based on a comparison of retention times, indexes and mass spectra with the corresponding data in the literature [24] and computer mass spectra libraries (Wiley and NBS 54K).

RESULTS AND DISCUSSION

The amount of the needle essential oil yield (% v/w) on a dry mass basis of pine stands growing at different distances from the nitrogen fertilizer factory was investigated. At the growing localities closest (at 0.5 km) to the plant, the amounts of the oils were: 0.35–0.47% and 0.38–0.4% in the west direction, 0.33–0.38% and 0.41–0.42% in the north direction, and 0.25–0.35% and 0.30–0.39% in the prevailing north-east wind direction in the current-year and one-year-old needles, respectively. The changes in the amount of the oils of the needles collected from young pine stands located along 22 km transect in the prevailing north-east wind direction are represented in the Fig. 1. A slight increase of the oil yield was observed in the needles collected from pinewoods located at a distance up to 5 km from the factory, and this pattern was stronger in one-year-old ones. A response to pollution in the volatile oil content was not strong, although the sites selected for the study had the mean monthly concentration of ammonia 3.9 μg m^{-3} in the air at a distance of 22 km from the pollution source and 4.9 times higher amount with an extreme value over 19.1 μg m^{-3} at the stand at 0.5 km from the plant in the north-east direction.

The chemical variation in the oil composition of Scots pine needles collected from 17 localities in Lithuania was investigated by Venskutonis et al [25]. α-Pinene (18.5–33.0%) and δ-3-carene (9.1–24.6%) were found

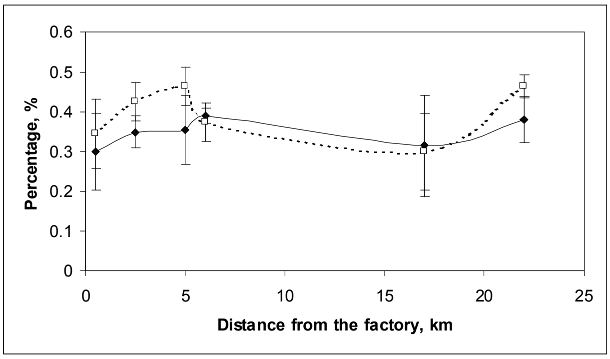

Fig.1. Variation of the essential oil yield from the needles of young Scots pine (Pinus *sylvestris* L.) stands growing at different distances from the nitrogen fertilizer factory *Achema* Oil quantity was represented as an average mean (n = 4, confidence interval 95% and significance level α = 0.05) —◆— current-year needles, ---□--- one-year-old needles

to be major constituents in almost all the investigated oils with only one exception when germacrene-D-4-ol was predominant in one of the samples (13.2%). As the main sesquiterpenoides, β-caryophyllene (2.6–4.9%), germacrene D (1.4–6.5%), bicyclogermacrene (2.1–6.2%), δ-cadinene (4.7–11.6%) and γ-cadinene (3.0–5.5%), germacrene D-4-ol (2.6–13.2%), cubenol (2.0–5.1%) and a-cadinol (1.9–7.7%) were determined in the study. The content of the oils was about 1% in many samples. It is difficult to compare the oil yield data of our results with that from the above study where the age neither of the trees nor of the needles was indicated.

The chemical composition of the oils investigated in this study is given in Table, the relative quantities of the constituents are represented as intervals of the average means of four repetitions. Also, volatile oils of pine stands growing in different directions (west, north and north-east) to the sites closest (at 0.5 km) to the fertilizers plant were analysed. As the main fraction of the oils was found to be monoterpene hydrocarbons comprising 58.4–72.4% and 45.0–60.8% of the total oil from the current-year and one-year-old needles (collected at distances up to 22 km in the direction of north-east prevailing wind) respectively. It is in agreement with published results of pine species in the literature [1–3, 6, 7, 9, 25–27]. Among fifteen monoterpenes, α-pinene (19.8–35.0% and 18–28.0%) and δ-3-carene (22.7%–33.7% and 19.9–25.9%) were the main constituents in the oils and relative percentage of these compounds was found higher in the oils of the current year needles. A relative amount of these predominant monoterpene hydrocarbons was higher in the oils comparing with the data obtained from the needle oils collected at unpolluted localities [25]. According to the literature, higher total volatile terpene concentrations were reported in the leaves of plants growing in an environment of low nitrogen and phosphorus. A higher content of terpenes in the current-year needles in comparison with the one-year-old needles was established in the oils from pine grown under urban stress [12]. Relative concentrations of predominant constituents of the essential oils obtained from the needles of pines growing at different distances from the factory are presented in Figs. 2 and 3. An increase in the amount of α-pinene and δ-3-carene was observed in the stands at a distance up to 6 km from the pollution source. All the investigated volatile oils could be attributed to the α-pinene and δ-3-carene chemotype.

Sesquiterpene hydrocarbons were the second dominated fraction in the oils and formed 14.5–25.7% and 18.5–35.1% in the current-year and one-year-old needles, respectively. The quantity of sesquiterpenoids was higher in the oils of the one-year-old needles compared to the current-year needles. A relative amount of sesquiterpenes was slightly lower as compared to the data from a previous study where samples were collected at unpolluted locations of the country [25].

Diterpenoids (manoyl oxide, abietadiene, abieta-8(14),1 3(15)-diene and a tentatively identified compound

Table. **Variation of the essential oil composition (%, average means, n = 4) of the needles (current-year and one-year-old) of young *Pinus sylvestris* growing in the impact zone of nitrogen fertilizers factory "Achema"**

Compound	RI	Interval (c)	Interval (c + 1)
Tricyclene+α-Thujene	929	0.3–1.4	t – 0.8
α-Pinene	**939**	**19.8–35.0**	**18.0–28.0**
Camphene	953	0.8–2.2	1.5–4.7
Sabinene+β-Pinene	980	0.2–1.4	1.3–2.7
Myrcene	991	0.8–1.1	0.9–3.3
δ-3-Carene	**1002**	**22.7–33.7**	**19.9–25.9**
p-Cymene	1026	0.2–0.5	t – 0.3
Limonene+β-Phellandrene	1031	0.7–1.4	0.9–2.7
(Z)-β-Ocimene	1037	t – 0.5	0–0.3
(E)-β-Ocimene	1050	0.4–2.0	0.2–1.5
γ-Terpinene	1060	0.3–0.9	0.5–1.3
Terpinolene	1089	0.6–2.9	0.5–2.7
n-Undecane	1100	t – 0.2	0–0.3
Borneol	1169	0.4–1.1	0.3–1.2
p-Mentha-1,5-dien-8-ol	1170	t – 0.2	0.3–1.2
Terpinen-4-ol	1177	0.6–2.4	0.6–1.2
m-Cymen-8-ol	1180	0–0.3	0–0.2
p-Cymen-8-ol	1183	t – 0.4	0–0.4
α-Terpineol	1189	0.3–1.2	0.6–3.3
Decanone-2	1192	0–0.3	0–0.2
Thymol, methyl ether	1235	0–0.7	0–0.6
Bornyl acetate	1289	1.0–3.4	0.5–2.4
Undecanone-2	1294	0–0.8	0.1–0.8
(2E, 4E)-Decadienal	1317	0–0.3	0.0
δ-Elemene	1338	0.4–2.0	t – 1.4
Terpinyl acetate	1349	t – 1.4	0.4–1.2
α-Cubebene	1351	t – 0.5	t – 0.5
α-Copaene	1377	0.2–0.6	0.1–0.6
β-Bourbonene	1386	0.1–0.5	t – 0.4
β-Cubebene	1388	0.2–0.4	t
β-Elemene	1391	0.7–2.3	0.7–1.8
(E)-Caryophyllene	1419	2.6–7.0	2.7–6.5
β-Copaene	1432	0.1–0.8	t – 0.4
β-Gurjunene	1432	t – 0.5	t
Aromadendrene	1441	0.1–1.2	0.2–0.8
trans-Muurola-3,5-diene	1454	t – 0.5	0.1–0.3
α-Humulene	1455	0.8–1.8	0.6–1.4
cis-Muurola-4(14),5-diene	1467	0.1–0.4	t – 0.3
γ-Muurolene	1480	0.8–7.3	0.8–1.9
Germacrene D	1485	0.8–6.9	0.6–7.0
β-Selinene	1490	0.5–1.4	t
trans-Muurola-4(14),5-diene	1495	0–0.4	0–0.5
Bicyclogermacrene	1500	0.2–2.0	0.5–3.6
α-Muurolene	1500	1.5–5.0	0.6–4.1
γ-Cadinene	1514	1.2–9.3	1.7–11.1
δ-Cadinene	1523	2.9–9.0	1.3–10.6
trans-Cadina-1(2),4-diene	1535	0.1–0.7	0.1–0.4
α-Cadinene	1539	0.2–0.5	0.2–0.4
α-Calacorene	1546	t – 0.4	0–0.2
β-Calacorene	1566	0–0.2	0–0.1
Hexenyl benzoate-3Z	1567	0–0.2	0–0.2
Germacrene-D-4-ol	1576	0.5–5.2	2.1–6.7
Spathulenol	1578	0.5–1.5	0.7–2.3

Caryophyllene oxide	1580	0.6–1.7	0.4–4.4
Gleenol	1587	0–0.4	t – 2.5
β-Oplopenone	1608	0–0.5	0–0.5
1,10-di-epi-Cubenol	1619	0.4–1.1	0.3–2.5
1-epi-Cubenol	1629	0.7–2.0	0.8–4.2
epi-α-Cadinol+	1640		
epi-a-Muurolol+a-Muurolol	1642	4.3–6.7	3.5–8.7
α-Cadinol	1654	4.9–7.8	2.5–9.6
Eudesma-4(15),7-dien-1-β-ol	1688	t – 0.6	0–0.5
Manoyl oxide	1988	0–0.4	t – 0.4
Abietadiene	2088	0–0.3	t – 0.6
Unknown	2105	0.3–4.0	0.3–6.8
Abieta-8(14),13(15)-diene	2154	0–0.4	0–0.2
Total		94.4–98.7	95.9–98.4
Monoterpene hydrocarbons		58.4–72.4	45.0–60.8
Oxygenated monoterpenes		3.2–5.9	3.0–6.7
Sesquiterpene hydrocarbons		14.5–25.7	18.5–33.1
Oxygenated sesquiterpenes		12.0–14.4	7.8–18.1
Diterpenoids		0.4–4.1	0.3–7.0

RI – retention index on nonpolar column CP- Sil 8 CB
current-year needles indicaed by letter c and one-year-old ones – by (c + 1)
t – traces (<0.05%)
unknown: MW = 257, m/z: 191(100), 43(75), 81(67), 69(64), 55(64), 96(64), 109(43), 137(18), 123(30), 177(30), 149(12), 204(6)

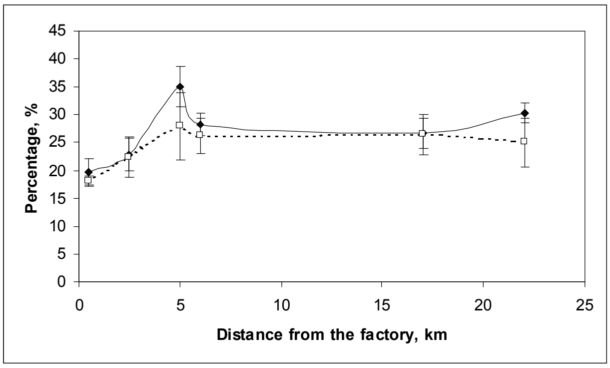

Fig. 2. Variation of α-pinene concentration in the needle essential oils of young Scots pine *(Pinus sylvestris* L.) stands growing at different distances from the nitrogen fertilizer factory "Achema".
Relative percentage was represented as an average mean (n = 4, confidence interval 95% and signifi-cance level α = 0.05)
—◆— current-year needles, ---□--- one-year-old needles

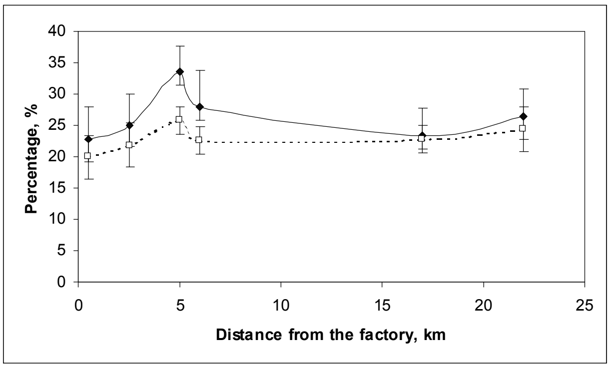

Fig. 3. Variation of δ-3-carene concentration in the needle essential oils of young Scots pine *(Pinus sylvestris* L.) stands growing at different distances from the nitrogen fertilizer factory "Achema".
Relative percentage was represented as an average mean (n = 4, confidence interval 95% and significance level α = 0.05)
—◆— current-year needles, ---□--- one-year-old needles

attributed to this class) comprised 0.4–4.1% and 0.3–7.0% of the oils of needles of different age, relative amount of diterpene fraction was higher in the one-year-old needles.

The total amount of the identified constituents (including the tentatively identified compound) comprised 94.4–98.7% and 95.9–98.4% of total oil in the current-year and one-year-old needles, respectively.

CONCLUSIONS

Changes in the needle essential oil yield were not considerable, just a slight increase of the oil yield was observed in the needles collected from pinewoods located at a distance up to 5 km from the factory, and this tendency was stronger in the one-year-old needles; despite the fact that the gradient of ammonia pollution is significant in the sites selected for the study. Also, a higher content of the dominated monoterpenes, α-pinene and δ-3-carene, was observed at the same localities. Under the effect of industrial pollution, higher amounts of shorter chain terpenes and lower amounts of longer chain terpens are produced, and this dependance was stronger expressed in the oils of the current-year needles.

Our study showed that the current-year needles of young pines were more informative than one-year-old ones for the indication of the effect of ammonia pollution. An increase or a decrease in the amount of the essential oil yield and the quantity of predominant constituents may occur under the influence of pollution. Our data are in agreement with earlier obtained facts that changes in the content of terpenes in the needles of pine have shown that a group of the secondary metabolites is an important biochemical marker of the environmental impact on woody plants.

ACKNOWLEDGEMENTS

This work was supported by the Lithuanian State Science and Studies Foundation, through Project FIBISTRESS ("Sumedėjusių augalų fiziologinės ir biocheminės reakcijos į gamtinių ir antropogeninių veiksnių stresą"), the grant No. C-05033/05.

Also, J. Šližytė and A. Judžentienė are grateful to Science Council of Lithuania and Department of Science and Higher Education (Ministry of Education and Science) for financial support (No. V17-13/BP-123). A part of this research was carried out during the project "Studentų mokslinė praktika".

Received 23 October 2006
Accepted 27 October 2006

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PAPRASTOSIOS PUŠIES (*PINUS SYLVESTRIS* L.) JAUNUOLYNŲ, AUGANČIŲ AMONIAKU UŽTERŠTOSE VIETOSE, SPYGLIŲ ETERINIŲ ALIEJŲ POKYČIAI

Santrauka

Tirta paprastosios pušies pirmamečių ir antramečių spyglių, surinktų azotinių trąšų gamyklos „Achema" (Jonava) įtakos zonoje augančiuose jaunuolynuose, eterinių aliejų cheminė sudėtis. Spyglių mėginiai buvo surinkti 0,5–22 km atstumu nuo gamyklos. Hidrodistiliacijos būdu gauti eteriniai aliejai buvo analizuojami dujų chromatografijos/masių spektrometrijos metodu. Eterinio aliejaus išgava kito nuo 0,25 iki 0,47% pirmamečiuose ir nuo 0,25 iki 0,49% antramečiuose spygliuose. Monoterpeniniai angliavandeniliai vyravo visuose tirtuose aliejuose ir pirmamečiuose ir antramečiuose spygliuose sudarė atitinkamai 58,4–72,4% ir 45,0–60,8%. Seskviterpeniniai angliavandeniliai sudarė 14,5–25,7% aliejaus pirmamečiuose ir 18,5–33,1% antramečiuose spygliuose.

Eterinio aliejaus kiekis šiek tiek padidėjo spygliuose, surinktuose 5 km atstumu į šiaurės rytus nuo gamyklos augančiuose jaunuolynuose. Vyraujančių eterinių aliejų komponentų – monoterpenų α-pineno ir δ-3-kareno – koncentracijos taip pat padidėjo spygliuose, surinktuose vietovėse, esančiose 5 km atstumu nuo taršos šaltinio.

Pramoninės taršos sąlygomis pušys gamina daugiau terpeninių angliavandenilių, turinčių trumpesnę anglies atomų grandinę ir mažesnį kiekį lakiųjų terpeninių junginių su ilgesne atomų grandine. Ši tendencija labiau pasireiškia pirmamečiuose spygliuose.