Comparison of Terpene Composition in Engelmann Spruce (*Picea engelmannii*) Using Hydrodistillation, SPME and PLE

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Terpenes emitted by conifer trees are generally determined by analysing plant extracts or essential oils, prepared from foliage and cones using steam distillation. The application of these procedures limits experiments to cut plant materials. Recently headspace techniques have been adopted to examine terpene emission by living plants. This paper deals with the application of solid-phase micro-extraction (SPME) for the analysis of terpenes emitted by conifers foliage of Engelmann spruce (*Picea engelmannii*), including its seedlings. The compositions of SPME extracts obtained for destroyed and non-destroyed old and juvenile spruce needles were compared with the compositions of essential oils and pressurised liquid extraction (PLE) extracts corresponding to the same plant materials. No substantial differences have been found in the qualitative terpene composition estimated by analysing essential oil and PLE and SPME extracts from non-destroyed old and juvenile foliage. The disintegration of spruce needles results in the formation of a significant amount of myrcene in the case of the old conifer foliage and non-terpenoic compounds in the case of juvenile conifer foliage. This phenomenon can be attributed to enzymatic reactions occurring in the destroyed plant cells.

Key words: Terpenes, Engelmann Spruce, Solid-Phase Micro-Extraction

Introduction

Among the volatile substances emitted by plants, terpenes seem to be the most important. In nature they act as attractants for some pest insects and natural pesticides for others (Bernays and Chapman, 1994). Moreover, the presence of terpenes in the forest air appears to have good influence on human health (Miyazaki *et al.*, 1992), though some authors investigated them as air polluting substances (Geron *et al.*, 1994). Due to the significance of terpenes, their analysis is worth developing.

The compounds emitted by conifer trees are generally determined by analysing essential oils, prepared from foliage and cones using steam distillation, or extracts obtained from the same plant parts (Sjödin *et al.*, 1996). However, the application of these sample preparation procedures limits investigations to cut plant materials. Moreover, the composition of an essential oil or extract can be different from the composition of biogenic volatile organic compounds (BVOC) emitted by the plant (Tollsten and Bergstrom, 1988). To examine the emission by live plants, headspace techniques have recently been adopted (Dormont *et al.*, 1998). Yet, however, when classical headspace mode is used (*i.e.* the injection of an air sample taken from the space above the examined sample), very often there are difficulties with the detection of substances existing in small amounts in the headspace atmosphere. Then, the application of headspace analysis using the sample trapping procedure requires more complicated equipment. For these reasons, solid-phase micro-extraction (SPME) has been more and more employed in such cases.

SPME is a solvent-free extraction method in which analytes are directly absorbed into a fused-silica fibre coated with a polymer phase and then desorbed directly into a chromatographic injection port. This method was preliminary introduced by Arthur and Pawliszyn (1990) for the analysis of pollutants in water and air environmental samples. Since that time SPME has been significantly developed and recently frequently applied as a very

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helpful or even unique method in resolving various analytical problems (Pawliszyn, 1997). The analysis of volatile fractions of aromatic plants is one of the fields in which SPME will probably be commonly applied.

It is true that there are quite a lot of SPME applications for terpene analysis in various plant materials, but very few papers considering the employment of this technique for terpene analysis in conifers foliage (Schäfer *et al.*, 1995; Coleman *et al.*, 1997; Vereen *et al.*, 2000). Besides, the cited papers do not show whether there is any relation between the terpene composition estimated by means of the traditional and time consuming steam distillation and the composition estimated by the significantly cheaper, quicker and simpler SPME.

The main purpose of the study was to compare qualitatively the SPME-obtained composition of volatile compounds emitted from the conifer foliage with the composition of an essential oil obtained using the hydrodistillation method. It was decided to compare these data to the analogous ones obtained using pressurised liquid extraction (PLE), generally recognised as a very effective extraction method.

As results from the literature cited above, the SPME-obtained composition of volatile compounds emitted by plants may depend on their age and the type of sample pre-treatment. For these reasons the second aim of the investigations was to determine:

if there are differences in the the SPME-obtained composition of volatile species emitted by old and juvenile conifers foliage;

if the SPME-obtained composition can be dependent on the way of conifer sample pre-treatment (dependent on sample destruction), and

if there are differences in SPME-obtained composition of volatile species emitted by conifers foliage of seedlings both live and plucked off.

The Engelmann spruce (*Picea engelmannii*) from the Botanical Garden in Lublin was used for the investigation as both the tree and the seedlings grown from its seeds were easily available.

Experimental

Plant materials

Old and juvenile foliages and seedlings of Engelmann spruce (*Picea engelmannii*) were used for investigation. The foliages were collected in May from a 37-year-old tree growing in the Botanical Garden of Maria Curie Sklodowska University in Lublin. The seedlings (2 years old) were grown from the seeds of the same tree.

Sample preparation procedures

Hydrodistillation

Essential oils from old and juvenile foliage of the Engelmann spruce tree were obtained by hydrodistillation with the addition of xylene using the Dering apparatus. In both cases the same amounts of the plant material were used (30 g). Due to the small amount of seedlings available this material was not hydrodistilled.

Pressurised liquid extraction

PLE was performed with a Dionex ASE200 instrument (Dionex Corp., Sunnyvale, CA, USA). The exactly weighed portions of the plant material (1 g) were mixed with neutral glass and placed into a 22-ml stainless steel extraction cell. The application of a neutral glass, playing the role of a dispersion agent, is recommended to reduce the volume of the solvent used for the extraction (ASE200 Operator's Manual). The content of the cell was extracted under the following conditions: extracting solvent, hexane; extraction temperature, 100 °C; extraction pressure, 60 bar; static extraction time, 10 min.

The volume of the collected extract was 25 ml. Each sample was extracted three times under the same conditions. Between runs the system was washed with the extraction solvent.

Seedlings were not subjected to the PLE procedure because of too small amounts of the material.

Headspace SPME procedure

In order to answer the questions stated in Introduction, two types of material were investigated in the headspace SPME experiments:

- a freshly plucked off old and juvenile foliage of Engelmann spruce, and
- Engelmann spruce seedlings grown from the same tree's seeds.

The old and juvenile foliage was used either immediately after cutting or destroyed before performing further measurements.

As SPME fibre, a 75 μm Carboxen-PDMS fibre (Supelco, Bellefonte, PA, USA) placed into a SPME holder for manual sampling was applied in

the investigations. The fibre was conditioned according to the supplier's prescription. The SPME extraction procedure was as follows: the SPME fibre was introduced for 30 min into the thermostated vial (25 °C) containing 0.3 g of the foliage and closed tightly with a rubber seal. Then the fibre was immediately transferred into a GC injector port and the 2-minute thermo-desorption process was carried out.

The same procedure was repeated for the seedling foliage, but only 0.1 g of the material was used for the SPME extraction.

As mentioned above, a part of the experiments was performed with destroyed materials. For this purpose separate portions of foliage were folded in aluminium foil and immersed in liquid nitrogen for 30 min. After this period the frozen material was quickly homogenised and immediately transferred into a thermostated vial for 20 min before SPME extraction. Further steps of the procedure were the same as described above.

The SPME extracts from non-destroyed and destroyed conifer foliage are hereafter called SPME-1 and SPME-2, respectively.

In experiments with live Engelmann spruce seedlings, the seedlings were placed inside a glass bell with a port with a rubber seal in its upper part for introducing SPME fibre.

GC/MS measurements

SPME, PLE extracts and essential oil samples were analysed by the GC/MS system ITS-40 (Finnigan MAT, San Jose, CA, USA). The chromatograph injector was equipped with Merlin Microseal Septum (Supelco, USA). A DB-5MS fused-silica capillary column (30 m × 0.25 mm, 0.25 μm) (J&W Scientific, USA) was used. Helium (grade 5.0) was used as a carrier gas. A split-splitless injector was operated in the splitless mode for all chromatographic runs. The injector's temperature was 280 °C. The following temperature programmes were applied: 2 min at 40 °C and then a linear temperature increase up to 280 °C at the rate 4 °C/min for essential oils analysis and 8 °C/min for SPME extracts analysis. The mass spectrometer was operated in EI mode at 70 eV; manifold temperature was 220 °C. The mass spectra were measured in the range 35–300 amu.

Qualitative analysis was carried out comparing the obtained MS spectra with spectra from the libraries [NIST library, terpene library (Finnigan MAT), and own library]. The obtained results were additionally confirmed using own and published temperature retention indexes.

Statistical analysis

The data are presented as mean value ± SD. Statistical analysis was performed by means of Student's t-test for non-dependent samples.

Results and Discussion

Table I lists the retention indices and the peak area percentages of the compounds found in essential oils obtained from juvenile and old foliage of *Picea engelmannii.* As results from the presented data, the compositions of both oils differ to a large extent. In the essential oil obtained from juvenile foliage α- and β-pinene dominate; their contents are 23.3% and 29.7%, respectively. Also present in the oil in great amounts were: Δ^3-carene (9.4%), myrcene (5.5%), limonene (5.4%), β-phellandrene (3.3%) and santene (1.0%). In the old foliage essential oil α-pinene and β-pinene exist in much smaller amounts (2.3% and 1.2%, respectively), with myrcene and camphor dominating (12.2% and 14.9%, respectively). Moreover, contrary to the juvenile foliage essential oil, the old foliage essential oil contains significantly greater amounts of such compounds as borneole (5.2%), camphene hydrate (5.0%), piperitone (4.6%), and fenchone (3.0%). There is only 0.2% of Δ^3-carene, while its content is very big (9.4%) in the essential oil from juvenile foliage. It can be seen that the essential oil from the old foliage is more differentiated than that from juvenile foliage.

As stated in Introduction, the main aim of the paper was to compare the composition of essential oils obtained by hydrodistillation and by the SPME method from conifer foliage. The analysis of the SPME extract reveals a generally similar composition of terpenes to that presented in Table I for essential oils. However, the quantitative relations between the main components are a little different. It is difficult to estimate the relations between the concentrations for all compounds, which would enable a more detailed comparison of both methods, as the amounts of some compounds extracted by the SPME fibre are too small to give a distinct signal on the total ion chromatogram. A few reasons may be responsible for obtaining a too small sample in the SPME method:

Table I. The percentage concentration of compounds found in essential oils obtained from juvenile and old foliage of Engelmann spruce (*Picea engelmannii*).

No	Compound	RI[a]	Content (%) in	
			juvenile foliage	old foliage
1	Santene	887	1.05	0.2
2	Tricyclene	919	0.2	0.3
3	α-Thujene	924	Tr[b]	Tr
4	α-Pinene	931	23.3	2.3
5	Camphene	946	1.7	3.5
6	Benzaldehyde	959	Tr	5.6
7	Sabinene	971	1.0	0.1
8	β-Pinene	975	29.7	1.2
9	Myrcene	989	5.5	12.2
10	α-Phellandrene	1004	0.2	0.1
11	Δ^3-Carene	1007	9.4	0.2
12	α-Terpinene	1015	0.2	0.1
13	*p*-Cymene	1023	0.1	0.1
14	Limonene	1028	5.4	1.6
15	β-Phellandrene	1029	3.3	0.6
16	1,8-Cineole	1031	Tr	2.0
17	γ-Terpinene	1057	0.3	0.1
18	Tolualdehyde	1068	Tr	0.2
19	Terpinolene	1084	1.5	0.5
20	Fenchone	1088	0.3	3.0
21	Dehydro-*p*-cymene	1089	0.3	0.1
22	Methyl benzoate	1094	Tr	0.2
23	Linalool	1100	0.6	6.5
24	*n*-Nonanal	1106	0.2	0.1
25	1,3,8-*p*-Menthatriene	1112	0.2	0.3
26	Endo-fenchol	1119	Tr	0.8
27	*cis*-*p*-Menth-2-en-1-ol	1125	Tr	0.5
28	*trans*-*p*-Menth-2-en-1-ol	1144	Tr	0.3
29	Camphor	1147	1.0	14.9
30	Camphene hydrate	1155	0.3	5.0
31	Isoborneol	1164	Tr	0.2
32	Borneol	1173	0.3	5.2
33	Pinocamphone isomer	1177	0.2	Tr
34	4-Terpineol	1182	0.3	0.4
35	Methyl salicylate	1193	Tr	0.2
36	α-Terpineol	1197	0.3	3.2
37	Estragole	1200	Tr	0.4
38	Verbenone	1209	Tr	0.4
39	*n*-Decanal	1209	0.2	Tr
40	*trans*-Piperitol	1211	Tr	0.2
41	*trans*-Carveole	1221	Tr	0.1
42	Citronellol	1231	Tr	2.8
43	Thymol methyl ether	1232	0.2	Tr
44	Neral	1241	Tr	0.1
45	Nerol	1254	Tr	0.4
46	Piperitone	1259	Tr	4.6
47	Geranial	1271	Tr	0.1
48	*trans*-Cinnamaldehyde	1275	Tr	1.7
49	Bornyl acetate	1287	1.1	3.0
50	Citronellyl acetate	1357	Tr	0.5
51	Geranyl acetate	1383	0.1	0.9
52	β-Elemene	1394	Tr	0.2
53	Longifoene	1414	0.4	Tr
54	β-Caryophyllene	1425	0.5	Tr
55	α-Ionone	1427	0.1	0.1
56	*(Z)-trans*-α-Bergamotene	1439	0.2	Tr
57	*cis*-β-Farnesene	1460	0.2	Tr
58	Germacrene D	1470	0.3	0.3
59	β-Ionone	1485	Tr	0.3
60	α-Farnesene	1512	0.9	0.1

Table I (continued).

No	Compound	RI[a]	Content (%) in	
			juvenile foliage	old foliage
61	β-Bisabolene	1515	0.3	0.1
62	β-Himachalene	1516	0.5	Tr
63	*cis*-γ-Bisabolene	1517	0.5	Tr
64	γ-Cadinene	1521	Tr	0.4
65	δ-Cadinene	1526	0.3	1.7
66	*trans*-γ-Bisabolene	1535	1.0	Tr
67	Unknown	1548	0.3	0.1
68	*trans*-Nerolidol	1570	Tr	Tr
69	*cis*-3-Hexenyl benzoate	1580	0.4	0.3
70	4-β-Hydroxygermacra-1(10),5-diene	1585	1.1	2.2
71	τ-Cadinol	1652	0.2	0.7
72	τ-Muurolol	1654	0.1	1.2
73	δ-Cadinol	1656	Tr	0.4
74	α-Cadinol	1666	0.8	3.3
75	α-Bisabolol	1698	1.5	Tr

[a] Temperature program retention index.
[b] Tr: Trace amount (< 0.1%).

– too small capacity of the SPME fibre;
– too small terpene amount absorbed due to a short absorption time;
– too small terpene amount desorbed resulting from a too short desorption time or a too low desorption temperature, and
– too low vapour pressure (concentration) of the compounds emitted by the plant material.

Additional experiments, in which SPME fibres of different capacity were used and various absorption and desorption times were applied, showed that the first three possibilities can be excluded. These additional experiments did not exclude that some substances could be present deeply inside the plant cell (Tulloch, 1976). If yes, their concentration in the air above the sample is too low. In order to confirm this suggestion it was decided to perform SPME experiments also with destroyed plant material, facilitating the emission of volatile substances from conifer foliage (SPME marked as SPME-2). To avoid the loss of volatile compounds, the investigated materials were destroyed after freezing in liquid nitrogen. As appears from performed experiments, the destruction of the material causes the release of greater amounts of volatile compounds. In this case the estimation of the concentration relations among a greater number of compounds is possible. Fig. 1 illustrates the difference in the total amount of terpenes extracted from non-destroyed (SPME-1) and destroyed (SPME-2) old and juvenile Engelmann spruce needles. The presented data relate the total amount of terpenes extracted from the investigated foliages to the total amount of terpenes extracted from old destroyed foliage (the total amount of terpenes extracted from old destroyed foliage was assumed to be 100%). As appears from these data, in the case of old foliage, SPME-2 gives more than a 10-fold increase in the amount of extracted terpenes than SPME-1. The presented phenomenon is probably connected with the presence of a tight wax layer, which covers old needles and obstructs the emission of terpenes (Lewinsohn *et al.*, 1991). (In the state of nature, the increase of terpene emission is caused by the damage of foliage, *e.g.* by beetles.) There is no such big difference between the SPME-1 and

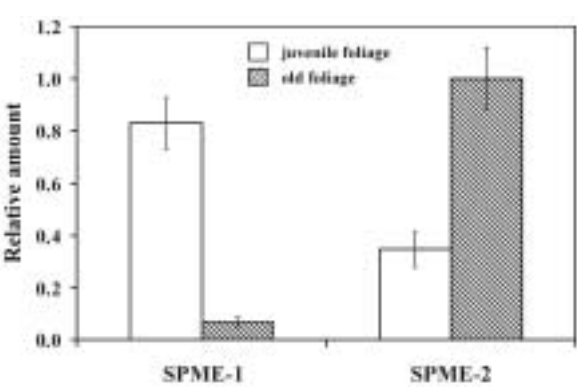

Fig. 1. Graphs representing the relation between total terpene amounts estimated for old (bars with slope lines) and juvenile (white bars) Engelmann spruce conifer foliage by analysing SPME extracts from whole (SPME-1) and destructed (SPME-2) needles (the total amount of terpenes extracted from old destroyed foliage was assumed to be 100%).

SPME-2 data in the case of juvenile foliage with thin wax protection. Moreover, contrary to old foliage, the destruction of juvenile foliage results in the emission decrease of the total amount of terpenes, which is difficult to account for. The enzymatic reactions occurring after the disintegration of juvenile foliage cells may be responsible for the observed decrease. Another possibility is that the juvenile needles contain a greater amount of extremely volatile substances which, despite of the low destruction temperature of the plant material, are lost before SPME. The presence of two non-terpenoic compounds in extracts from the destroyed juvenile foliage (SPME-2) partly supports the first explanation. These compounds are either released from the disintegrated plant cells or are formed in the homogenate as the effect of reactions occurring in the plant material after its disintegration (Mayer *et al.*, 2003; Lichtenthaler *et al.*, 1997). These compounds do not exist in SPME-1 and PLE extracts, nor in the essential oil of the same foliage.

The terpene compositions of the essential oils and of SPME and PLE extracts, with respect to the most representative terpenes, are compared in Fig. 2 (juvenile foliage) and Fig. 3 (old foliage). The content of the selected terpenes was normalised to α-pinene, whose amounts in essential oils and both types of extracts (SPME and PLE) were assumed to equal 1. Such assumption allows for an easy comparison of different character samples, such as these used in the experiments.

Considering the results for juvenile foliage (Fig. 2) it can be concluded that the terpene composition of the PLE extract is similar to that of the essential oil, with a slightly smaller content of some less volatile terpenes. The qualitative compositions of the SPME-1 and SPME-2 extracts are also similar to the compositions of the corresponding essential oils but, contrary to PLE extracts, they are characterised by a bigger content of less volatile terpenes than the essential oil.

In the case of the old foliage (Fig. 3) there are no substantial differences in the quantitative terpene composition between the sample preparation procedures used, except for SPME-2. In the headspace of the old destroyed foliage, a high content of myrcene is observed. The ratio of myrcene to α-pinene content estimated for the old foliage from the analysis of the essential oil and PLE or SPME-1 extracts is contained in the range 1:1 – 5:1, while from SPME-2 it equals 37:1. Such striking discrepancy cannot be explained by the presence of a large myrcene amount in the deep parts of the old needles or by the presence of a thick wax shelter because the application of steam distillation, recognised as a very exhaustive sample preparation method for terpene analysis, should give a myrcene to α-pinene ratio similar to the SPME-2 value. It may be cautiously assumed that the ratio change should rather be attributed to enzymatic reactions occurring after the disintegration of foliage tissues.

In the case of old non-destroyed foliage, the amounts of terpenes extracted by the SPME fibre were very small, due to the wax shelter, and decreased with decreasing terpene volatility.

The composition of the terpene fractions emitted by conifer foliage of 2-year-old seedlings of *Picea engelmannii* grown from seeds of the investigated tree was examined using the SPME extracts. Three types of materials were used in the experiments: whole live seedlings, freshly plucked off

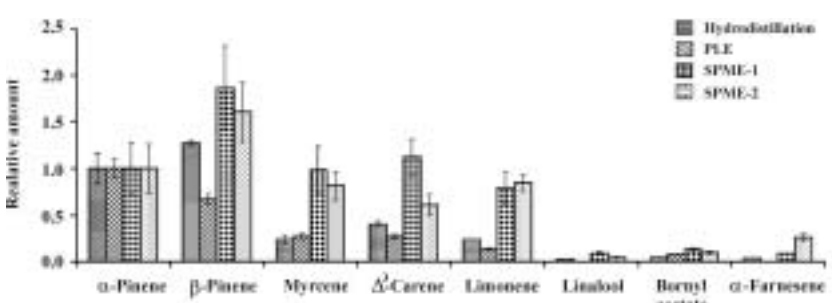

Fig. 2. Graphs representing the relation between the most abundant terpenes estimated for juvenile Engelmann spruce conifer foliage by analysing essential oil, PLE extracts, SPME-1 extracts and SPME-2 extracts (the content of terpenes was normalised to α-pinene, whose amount was assumed to equal 1).

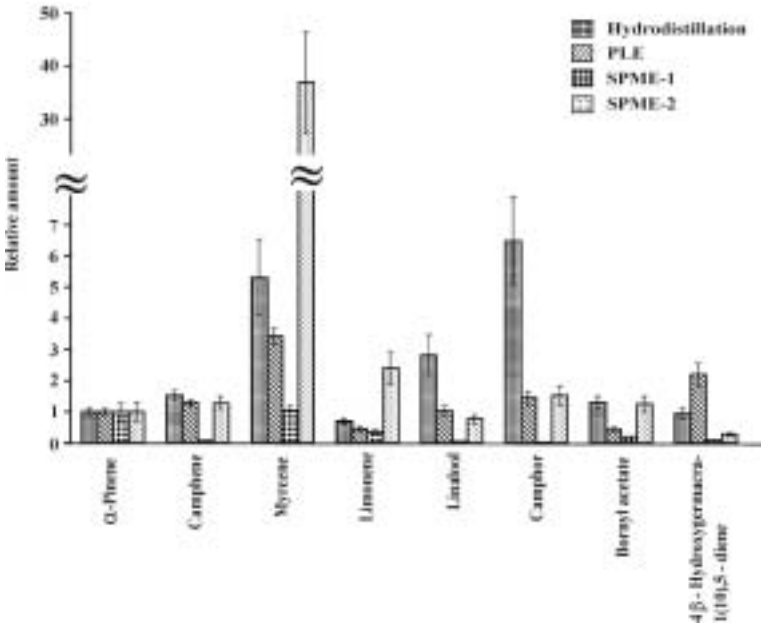

Fig. 3. Graphs representing the relation between the most abundant terpenes estimated for old Engelmann spruce conifer foliage by analysing essential oil, PLE extracts, SPME-1 extracts and SPME-2 extracts (the content of terpenes was normalised to α-pinene, whose amount was assumed to equal 1).

needles, and destroyed needles. The results of these investigations can be summarised as follows. The qualitative composition of terpenes emitted by seedlings is similar to that estimated for the spruce needles plucked off a fully grown tree. Quantitatively, this composition resembles the one emitted by the mixture of juvenile and old conifer foliage. It is not surprising if one takes into account the presence of juvenile needles in the conifer foliage of 2-year-old seedlings.

SPME terpene compositions almost the same as for the whole seedlings can be found analysing the needles plucked off seedlings, yet with a slightly increased concentration of myrcene in SPME extracts. A distinctly higher myrcene level is observed in SPME extracts corresponding with the plucked off and destroyed needles of the seedlings. It partly confirms the supposition that myrcene is produced during the destruction of foliage tissues. In this case the myrcene level is not as high as for the old conifer foliage but the conifer foliage plucked off the examined seedlings contained both juvenile and 2-year-old needles.

Arthur C. L. and Pawliszyn J. (1990), Solid phase microextraction with thermal desorption using fused silica optical fibers. Anal. Chem. **62**, 2145–2148.

ASE 2000 (1995), Accelerated Solvent Extractor Operator's Manual, Document No. 031149, Revision 01. Dionex, Sunnyvale, CA, Sect. 3–5.

Bernays E. A. and Chapman R. F. (1994), Host-plant Selection by Phytophagous Insects. Chapman & Hall, London.

Coleman III W. M. and Lawrence B. M. (1997), A comparison of selected analytical approaches to the analysis of an essential oil. Flavour Fragr. J. **12**, 1–8.

Dormont L., Roques A., and Malosse C. (1998), Cone and foliage volatiles emitted by *Pinus cembra* and some related conifer species. Phytochemistry **49**, 1269–1277.

Geron C. D., Guenther A. B., and Pierce T. E. (1994), An improved model for estimating emissions of volatile organic compounds from forests in the eastern United States. J. Geophys. Res. **99(D6)**, 12733–12791.

Lewinsohn E., Gijzen M., Savage T. J., and Croteau R. (1991), Defense mechanism of conifers: relationship of monoterpene cyclase activity to anatomical specialisation and oleoresin monoterpene content. Plant Physiol. **96**, 38–43.

Lichtenthaler H. K., Schwender J., Disch A., and Rohmer M. (1997), Biosynthesis of isoprenoids in higher plant chloroplasts proceeds via a mevalonate-independent pathway. FEBS Lett. **400**, 271–274.

Mayer F., Takeoka G., Buttery R., Nam Y., Naim M., Bezman Y., and Rabinowitch H. (2003), Aroma of fresh field tomatoes. Freshness and Shelf Life of Foods (ACS Symposium Series) (Cadwallader K. and Weenen H., ed.) **836**, 144–161.

Miyazaki Y., Motohashi Y., and Kobayashi Y. (1992), Changes in mood by inhalation of essential oils in humans. I. Effects of essential oils on papillary light reflex, performance, sensory evaluation and profile of mood states. Mokuzai Gakkaishi **38**, 903–908.

Pawliszyn J. (1997), Solid Phase Microextraction: Theory and Practice. Wiley-VCH, New York.

Schäfer B., Hennig P., and Engewald W. (1995), Analysis of monoterpenes from conifer needles using solid phase micro extraction. HRC – J. High Res. Chromat. **18**, 587–592.

Sjödin K., Persson M., BorgKarlson A. K., and Norin T. (1996), Enantiomeric compositions of monoterpene hydrocarbons in different tissues of four individuals of *Pinus sylvestris*. Phytochemistry **41**, 439–445.

Tollsten L. and Bergstrom G. (1988), Headscape volatiles of whole plant and macerated plant parts of *Brassica* and *Sinapis*. Phytochemistry **27**, 4013–4018.

Tulloch A. P. (1976), In: Chemistry and Biochemistry of Natural Waxes (Kolattukudy P. E., ed.). Elsevier, Amsterdam, p. 235.

Vereen D. A., McCall J. P., and Butcher D. J. (2000), Solid phase microextraction for the determination of volatile organics in the foliage of Fraser fir (*Abies fraseri*). Microchem. J. **65**, 269–276.