COMPARISON OF PROCEDURES FOR ISOLATION OF MONOTERPENE HYDROCARBONS FROM FRESH NEEDLES OF *Picea abies* AND *Picea omorica*

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Extraction procedures (steam distillation, supercritical fluid extraction and solvent extraction) for isolation of monoterpene hydrocarbons from fresh needles of *Picea abies* and *Picea omorica* were optimised. The procedures were compared with the aim of minimizing consumption of needles and improving the extraction efficiency and repeatability. An influence of homogenisation procedures and storage conditions (liquid nitrogen, –18 and 4 °C) on the total content and composition of essential oils was studied. Cryogenic grinding (liquid nitrogen) combined with the extraction with cold hexane (extraction time 2 h) and subsequent GC-MS determination in freshly homogenised needles gives the best results (1.5–4 times better extraction efficiency, RSD < 10% for *P. abies* and < 25% for *P. omorica*). Limits of detections (3 S/N) for individual monoterpene hydrocarbons from units to tens of ng/g and recoveries 97.2–101.4% were found in fresh needles (calculated to fresh weight). While cooling to 4 °C is unacceptable, freezing at –18 °C for the period of 18 days in the dark gives also good results.

Key words: Plants; Isolation; Natural products; Monoterpenes; Extractions; Steam distillations; Supercritical fluid extraction; GC-MS; Gas chromatography; Mass spectrometry.

Several isolation and preconcentration procedures are routinely used for separation of monoterpene hydrocarbons (MTHs) from plant materials. The amount of isolated essential oil and its qualitative composition depends to a great extent on the experimental conditions during extraction and on the isolation procedure used. Steam destillation[1,2], simultaneous distillation and extraction (SDE)[3,4], solvent extraction[1–7], supercritical fluid extraction (SFE)[1,3] and solid phase micro-extraction (SPME)[8] are the most widely used procedures. Accelerated solvent extraction, a modern alternative to Soxhlet extraction[9], is used for extraction of less easily extractable organic compounds from solid samples with the same solvents commonly applied in Soxhlet extraction. The SPME (ref.[8]) is used for analysis of compounds oc-

curring in a headspace and it cannot be directly compared with other isolation procedures since it provides different type of information.

Each procedure presents specific problems when applied to real plant materials with complicated matrix. Possible degradation of thermolabile compounds and/or hydrolysis of soluble compounds are the main drawbacks of steam distillation and SDE procedures. Sabinene, present in trace quantities in the essential oil from *Picea*, is transformed to terpin-4-ol, α-terpinene, γ-terpinene and terpinolene[2], while the content of santhene[10] increases. A higher efficiency and almost quantitative extraction of essential oils from different matrices are the main advantages of the procedures. Unfortunately, the methods are not suitable for isolation of compounds not distilling with water steam. The SFE procedure using carbon dioxide has been shown a good alternative to the conventional extraction procedures. Application of toxic organic solvents and the extraction time are reduced in this case. On the other hand, matrix plays an important role, especially when plant materials are analysed[11].

The main aim of the present article is the optimisation and comparison of different extraction procedures for isolation of MTHs from fresh needles of *Picea abies* and *Picea omorica*, selection of the best procedure from the view point of extraction efficiency, repeatability and accuracy and possible reduction of sample consumption to prevent damage of control trees. The procedure should be applicable to the determination of concentration gradients of monoterpenes in different parts or even organs of a tree and should enable comparing composition of essential oils in individual branches or even in individual needles.

EXPERIMENTAL

Chemicals and Apparatus

Standard solutions of α-pinene, camphene, β-pinene, 3-carene, α-phellandrene, limonene and tetradecane were from Fluka (terpene standards for GC), organic solvents of HPLC grade (pentane, heptane, hexane, cyclohexane, ethanol, methanol, tetrachloromethane and isopropyl alcohol) were from Merck, liquid nitrogen was from AGA. Adstat and ANOVA computer programs were used for data treatment.

An HP-6890 gas chromatograph equipped with a mass spectrometric detector HP-5673 and an HP-INNOWax column (30 m × 0.25 mm × 0.5 μm film of poly(ethylene glycol), Hewlett–Packard) was used for determination of monoterpene hydrocarbons[11]. The flow rate of helium was 1.7 ml/min and the injector temperature was 250 °C. The temperature program: 60 °C (0 min)//ramp 5 °C/min//100 °C//ramp 30 °C/min//240 °C (15 min). Mass selective detector HP-5673 was used for the MS determination. The detector was tuned in the selected ion monitoring (SIM) mode, and the most intensive three ions in each molecular

ion cluster were monitored. Spectra were recorded at 91, 93, 121 *m/z* and from the 5th minute also at 68 *m/z* to improve sensitivity for limonene.

Calibration curves (6–12 concentrations of Fluka standards, triplicate injections) were strictly linear in content range from units of ng/g up to hundreds of μg/g with regression coefficients $r > 0.9999$. Limits of detection [(3 S/N (signal to noise ratio)] for individual monoterpene hydrocarbons α-pinene 58.64 ng/g, camphene 73.65 ng/g, β-pinene 22.42 ng/g, 3-carene 3.34 ng/g, α-phellandrene 11.3 ng/g and limonene 9.34 ng/g and recoveries 97.2–101.4% were determined for samples of fresh needles (calculated to fresh weight).

Samples

P. abies: MTHs were determined in samples collected from a control tree situated in the southern part of the Drahanská uplands (area of Sobìšice, northern surroundings of Brno, altitude 420 m, mild warm and dry climate, biotic granodiorit parent rocks, eutric Cambisol soil). A tree from natural seeding is situated close to the fringe of a mixed wood on the northern side. No remarkable negative changes were noted. Needles are soft, with no evident colour changes. The first whorl is situated 150 cm above the ground.

P. omorica: A control tree is situated in a city park in the northern part of Brno, altitude 420 m, mild warm and dry climate, tertiary marl parent rocks, garden mixed soil). The control tree is situated in the middle of a small group of *P. omorica* trees of the same habitat. No remarkable negative changes were noted. Needles are soft, strong and dark green. The first whorl is situated 100 cm above the ground.

The needles (1–9 years old) were collected from different whorls of the central part of the control trees and mixed samples were prepared for each tree. The samples were stored in a cool box (0 °C) after collecting, immediately transported into laboratory and further stored at –18 °C before analyses if necessary.

Homogenisation of Plants Materials

The needles (1–2 g of the mixed sample) were homogenised by cryogenic grinding to soft powder (*ca* 50 μm particle size) using a Vibrom 2 S (Jebavý Ltd., Czech Republic) homogeniser. Its internal grinding space, all grinding parts and needles were cooled with 300 ml of liquid nitrogen and needles were homogenised for 2 min after evaporation of most nitrogen (*ca* 90%). The needle powder (0.3–1 g) was weighed into 20 ml vials and 2–3 g of cold hexane was added. Vials were hermetically closed and samples were shaken for 2 h. The clear extracts were transferred quantitatively into 2 ml vials and analysed by GC-MS. Extracts were stored at –18 °C when necessary. Needles cut to small pieces of different size (0.5–1, 1–3 and 3–5 mm) or whole needles without any pretreatment were also treated in the same way.

Extraction Methods

Steam distillation: Standard apparatus for determination of essential oils according to the Czech Standard ČSN 58 0110 (ISO-ČSN 6571) was used. A homogenised sample (1 g ± 0.1 mg of powdered needles) was distilled with 400 ml of deionised water for 4 h. Essential oils were collected in the hexane layer (*ca* 2 ml) and the solution was analysed by GC-MS with tetradecane as an internal standard.

Supercritical fluid extraction: An apparatus SE-1 (SEKO-K, Brno) was used for SFE extraction (0.5 g ± 0.1 mg of homogenised needles) at 7.5 MPa, 45 °C and a 60-min extraction period with carbon dioxide as an extraction fluid (no modifier was used). Essential oils were collected in hexane and the final solution was analysed by GC-MS with tetradecane as an internal standard.

Solvent extraction: Homogenised samples (0.3–0.5 g ± 0.1 mg of homogenised needles) were transferred into vials and 3–4 ml of organic solvents (pentane, hexane, cyclohexane or tetrachloromethane) was added. The vials were hermetically closed and shaken for 0.5, 1, 2, 4 or 6 h with a laboratory shaker. Clear extracts were analysed by GC-MS with tetradecane as an internal standard without any additional treatment.

RESULTS AND DISCUSSION

Essential oils are concentrated in needles in resin ducts. Monoterpene hydrocarbons, important constituents of essential oils, are emitted very slowly through the wax layer under normal conditions and they are trace constituents of the surrounding micro-atmosphere. MTHs easily evaporate or polymerize (natural protection of trees) after damage of the thin surface layer. The careful sample manipulation seems to be the most important for prevention of MTHs losses and for their accurate determination.

Relative amounts of MTHs were virtually constant for all the homogenisation procedures but the yields were much higher for cryogenically ground needles (Table I) for *P. abies* and *P. omorica*. Figure 1 shows a comparison of results for α-pinene and different extraction procedures. The same trends were also observed for other monoterpenes. The best repeatability was obtained for cutting the needles to small pieces of uniform size, the extraction efficiency decreased with their size. Reproducible cutting was time-

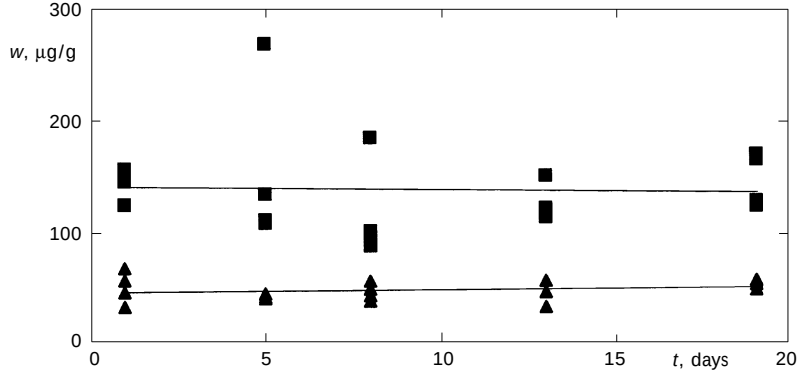

FIG. 1
Content of α-pinene (μg/g of fresh weight) in ground needles stored at –18 °C as a function of storage time; *P. omonica* (■), *P. abies* (▲)

TABLE I

Content (μg/g of fresh weight), relative contenta of monoterpene hydrocarbons (%, in parentheses) and relative standard deviation for different separation methods and procedures for homogenisation of fresh needles

Method	α-Pinene	Camphene	β-Pinene	3-Carene	α-Phellandrene	Limonene
			Solvent extraction			
Grinding[b]	44.4(23.4)/4.91	84.1(44.3)/4.96	6.95(3.67)/7.20	0.07(0.04)/24.45	0.21(0.11)/9.04	53.9(28.4)/9.83
Cutting[b]	20.0(25.4)/31.61	36.4(46.2)/30.74	2.81(3.57)/30.40	0.04(0.05)/79.03	0.11(0.14)/10.96	19.4(24.6)/35.41
Grinding[c]	777.8(34.3)/17.42	841.6(37.1)/18.00	49.4(2.18)/17.13	4.96(0.22)/24.42	23.3(1.02)/16.10	572.3(25.2)/18.68
Cutting[c]	498.8(33.9)/1.04	536.3(36.5)/1.24	31.1(2.12)/2.29	3.74(0.25)/7.04	15.6(1.06)/1.94	385.4(26.2)/1.81
			SFE extraction			
Grinding[b]	44.3(24.6)/3.17	78.7(43.7)3.08	6.81(3.78)/5.82	0.07(0.04)/6.38	0.19(0.11)/11.18	50.2(27.8)/10.87
Cutting[b]	19.0(26.4)/40.6	32.4(45.0)/40.99	2.75(3.82)/30.61	[d]	[d]	17.7(24.7)/39.13
Whole[b]	2.45(21.8)/6.6	3.73(33.1)/9.16	0.55(4.92)/29.61	[d]	[d]	4.53(40.2)/14.52
Grinding[c]	444.2(33.5)/7.99	484.7(36.5)/8.48	28.2(2.13)/8.95	3.02(0.23)/26.43	13.7(1.03)/9.08	352.5(26.6)/9.7
Cutting[c]	246.2(34.0)/17.97	263.6(36.4)/17.38	14.8(2.04)/20.48	1.89(0.26)/22.95	7.50(1.04)/16.57	189.3(26.2)/16.36
Whole[b]	54.9(33.6)/23.20	56.4(34.5)/23.53	3.28(2.00)/21.11	0.43(0.26)/28.15	1.91(1.17)/23.13	46.4(28.4)/23.95
			Steam destillation			
Grinding[b]	14.0(19.6)/4.59	31.9(44.7)/0.03	2.53(3.5)/14.44	0.02(0.02)/6.95	0.10(0.14)/9.61	22.8(32.0)/6.96
Grinding[c]	77.2(27.3)/27.76	91.8(32.4)/24.52	7.92(2.8)/28.13	1.08(0.4)/29.75	4.09(1.4)/24.26	101.2(35.7)/21.79

[a] In % of total MTHs; [b] *P. abies*; [c] *P. omorica*; [c] close to LOD.

consuming and it was difficult to obtain pieces of precisely defined size even in the case of a single operator. Unacceptable low extraction efficiency was obtained for whole needles.

Homogenisation by cryogenic grinding gave the best results when internal grinding space, all grinding parts and needles were cooled with 300 ml of liquid nitrogen and needles were homogenized for 2 min after evaporation of most nitrogen (*ca*. 90%). When a smaller volume of nitrogen was used, the grinding parts were insufficiently cooled and serious loss of MTHs was observed. The same problems appeared due to the increasing temperature of grinding parts when the grinding time was longer then 2 min. A shorter time led to insufficient homogenisation. The best results were obtained when approximately 1 g of one year old fresh needles was grinded. Smaller (one half) amounts of older, better developed and harder, three years old needles could be used for homogenisation. Cryogenic grinding under liquid nitrogen is the most suitable method for sample homogenisation of biological material (needles). Cutting of needles gives a lower extraction efficiency compared with the ground material. The lowest yields were obtained when whole needles were used for extraction.

A defined volume of cold organic solvent (3–4 ml) should be immediately added to the homogenised powder to prevent loss of MTHs and also water condensation on the powder surface. Of the tested organic solvents, hexane and cyclohexane were found the best for solvent extraction at room temperature in all cases. Serious peak tailing of α-pinene appeared when heptane was used as an extractant. Very low reproducibility was obtained for GC-MS analysis of pentane extracts. On the basis of the results, hexane was selected as the best solvent due to its lower boiling point and better separation of its peak from the peaks of other substances in GC-MS analysis. Hexane extracts were clear, without the traces of waxes. They were directly used for GC analysis. No remarkable decrease of lifetime of the GC column was observed after 800 injections. Extraction time of 2 h was selected as the optimum; a longer extraction (up to 6 h) did not increase the extraction yield of MTHs.

Co-extraction of natural pigments appeared when solvent extraction and SFE procedure were used in agreement with the literature data[12]. Low extraction efficiency and co-extraction of natural pigments complicate the GC-MS analysis mainly when polar solvents, such as methanol and ethanol were used for extraction of MTHs. For example, the extraction yield of α-pinene for methanol was 65.7 μg/g compared with 248 μg/g for hexane.

Chromatography on a 5 cm column of non-activated aluminium oxide with hexane as a mobile phase should be used to separate MTHs from

co-extracted natural pigments and other substances. The extract was evaporated to 1 ml under stream of pure nitrogen and the resulting solution was introduced onto the column. The retained substances were eluted from three well-separated zones with hexane three fractions being collected. The first fraction contained nonpolar MTHs and sesquiterpenes with the exception of bornyl acetate. Carotenoids, bornyl acetate and several other unidentified substances (probably degradation products of carotenoids) were present in the second (yellowish) fraction, while the green natural pigments were strongly adsorbed at the top of the column. The preconcentration and purification steps led to approximately 60% losses of the initial amounts of MTHs in the original extract but they were useful for determination of substances with higher boiling points. Both steps are not recommended for sample pre-treatment when MTHs of lower boiling point should be determined.

The extraction efficiency was only 60% for the SFE procedure at 45 °C, 7.5 MPa and the 60-min extraction time. Co-extraction of natural pigments appeared when higher pressure and/or temperature were applied. Thus mild extraction conditions, a higher separation selectivity and lower extraction efficiency were preferred in our case to prevent co-extraction of natural pigments and/or of substances with higher boiling points. Their presence in extracts prolonged the time for GC-MS analysis. A comparison of the homogenisation procedures for both species (*P. abies* and *P. omorica*) is given in Table I. Concentrations of MTHs differ in the same way as for solvent ex-

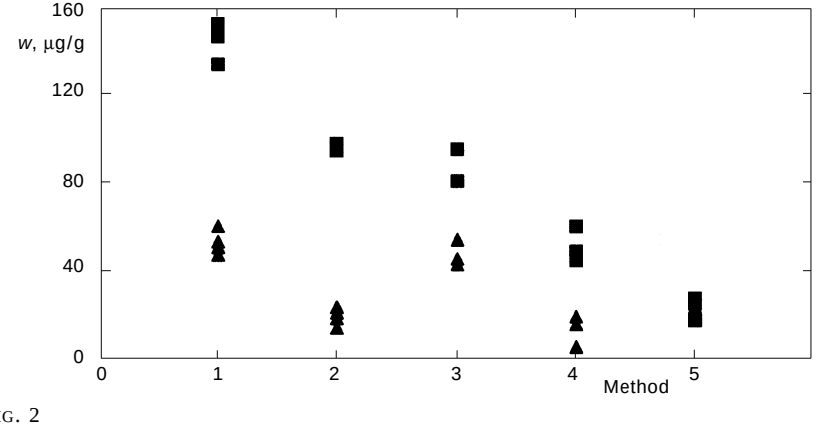

FIG. 2

Influence of different extraction procedures on the content of α-pinene (μg/g of fresh weight) and its repeatability in needles of *P. abies* (▲) and *P. omorica* (■). 1 hexane extraction, grinding; 2 hexane extraction, cutting; 3 SFE, grinding; 4 SFE, cutting; 5 steam distillation, grinding

traction with cold hexane. Cryogenic grinding gives the highest extraction yields and the highest repeatability (relative standard deviation RSD < 10%). RSDs for 3-carene were higher (<25%) for *Picea omorica*.

According the literature data[1,2], steam distillation is a suitable extraction procedure for determination of total amount and composition of essential oils. The relative amounts were constant in all our experiments (see results for *P. abies* and *P. omorica* in Table I). The procedure is time-consuming (2–4 h, 8 h is also recommended[1,2]), the reproducibility is low (RSD 50%) and the extraction efficiency is 85–90%. Steam distillation separates selectively mono- and sesquiterpenes from natural pigments (yellowish extracts were reported[12] when applied to savory, peppermint and dragohead), no additional pre-treatment steps being needed before GC-MS determination. The main drawback of the procedure is the necessity to exactly control the level of hexane in the cooling tube and the problems associated with the quantitative transfer of MTHs in hexane solution into volumetric flasks. The latter problem is well solved in SDE (ref.[3]).

Two ways ANOVA analysis indicated that the tree species and extraction procedure had a significant ($p < 0.050$) effect on the total MTH content. The best results were obtained for simple solvent extraction with cold hexane in both species *P. abies* and *P. omorica*. The method is preferable for the determination of MTHs. The SFE method produces similar results for ground (RSD < 12%) and cut (RSD < 30–40%) needles of *P. abies*. Lower contents of MTHs were obtained for *P. omorica*. The lowest contents of MTHs and worse repeatabilities (RSDs 24–30%) were obtained for steam distillation. A higher selectivity is the main advantage of the method although a longer time is needed (2–4 h) for complete extraction.

The sample storage under liquid nitrogen in containers seems to be the best alternative for long-term storage since no significant changes in the total content of MTHs and in percentages of individual MTHs were observed. Concentrations of four major oxygenated terpenes and myrcene increased when the samples were stored at –18 °C for a very long time while the concentrations of other MTHs decreased. Regular analysis of samples during storage of needles ground in a freezer at –18 °C for 18 days in a glass bottle confirmed that the concentrations of all MTHs and also ratios of the MTHs were constant (see Fig. 2 for α-pinene in *P. abies* and *P. omorica*). Short-term storage at 4 °C in a refrigerator was not acceptable since concentrations of MTHs decreased by 15% daily.

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