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Supercritical fluid extraction as a tool for isolation of monoterpenes from coniferous needles and walnut-tree leaves

Research Article

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Abstract: Several monoterpenes, *i.e.*, (+)-α-pinene, (-)-camphene, sabinene, (-)-β-pinene, myrcene, R-(+)-limonene, (-)-bornylacetate, (-)-*trans*-caryophyllene and a-humulene were identified and determined by gas chromatography-mass spectrometry (GC-MS) in needles of Pinaceae (*Picea abies, P. omorika, P. pungens, P. Breweriana, Pinus nigra, P. mungo turra, P. black, P. sylvestris, Abies pinsapo, A. holophylla, A. Bronmuelleris, A. alba, Larix Kaempferi L. decidua*) and tree-leaves of Juglandaceae (*Juglans regia, J. nigra, J. sieboldiana var. Cordiformis*) families. Supercritical fluid extraction (SFE) was found to be very useful for their isolation at optimised conditions (needles/leaves: pressure 20/30 MPa, temperature 80/130°C, time of extraction 60/60 min, modifier chloroform/chloroform). Their seasonal distribution, evaluation of differences in concentrations and relative amounts in different trees and their varieties grown in different localities were evaluated.

Keywords: *Supercritical fluid extraction (SFE) • Gas chromatographic-mass spectrometric detection (GC-MS) • Terpenes • Juglandaceae • Pinaceae*

1. Introduction

Currently, sample treatment is the most tedious and time-consuming part of an analytical procedure. Several extraction techniques were tested as a possible substitution of „classical“ extraction techniques such as Soxhlet solvent extraction. Reduction of extraction time, elimination of toxic solvents and reduction of solvent consumption were the main objectives. Supercritical fluid extraction (SFE) is one of the most suitable methods for isolation of volatile organic compounds like terpenes from plant materials and other complicated natural matrices [1].

The extraordinary physico-chemical properties of supercritical fluids are the main reasons for increasing interest in SFE [2]. In comparison with a liquid, the diffusivity of a supercritical fluid is one order of magnitude higher whereas the viscosity is one order of magnitude lower. Thus the characteristics of mass transport in gaseous phase are combined with the very high solvation properties of liquids [1]. Carbon dioxide is one of the most commonly used supercritical fluids for SFE [3-5] due to its very low critical temperature (31°C) and pressure (7,38 MPa), zero toxicity, flame resistance and low reactivity. Its polarity and extraction strength is comparable with n-hexane whereas its extraction efficiency decreases with increasing polarity of analytes. The method is fast, instrumentally available and in comparison with “classical” solvent extractions it reduces consumption of expensive, toxic, harmful and even environmentally non-friendly organic solvents.

In addition to the Soxhlet solvent extraction, steam distillation [6], rapid steam distillation [7], extraction

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into n-hexane [8], Soxtec modified solvent extraction, accelerated solvent extraction at elevated pressure (ASE; or pressurised solvent extraction - PSE), sonication and microwave oven assisted extraction) can be applied for isolation of volatile organic substances from plant materials. A solid phase micro extraction (SPME) is very useful for isolation and preconcentration of the volatile substances from a gaseous phase and subsequent thermal desorption with a direct injection on a capillary GC column by a brief heat-up [9].

Gas chromatography with a flame-ionisation (FID) or mass spectrometric (MS) detection is the most commonly used technique for detection and quantification of the volatile substances. A high performance liquid chromatography with a UV-spectrophotometric [10-11], polarimetric, proton magnetic resonance [12] detection or detection using derivation spectrophotometry in UV range can also be applied for some groups of the substances.

Determination of volatile substances in plant materials was studied in several papers. The relationships of quantity and composition of terpenes were studied largely in coniferous trees and less often in foliage of other tree species to explain the chemotypes and diversity of terpenes in trees of different geographical origin [13] and effects of different stressors were studied. The influence of polluted urban air on composition of terpenes [14-15] and their role in atmospheric disorders [16] was also evaluated. Several papers were devoted to the comparison of different extraction techniques for isolation, purification and determination of essential oils from different plants [13-18].

Terpenes are highly volatile substances having typical coniferous aroma. They are most often extracted from different plant materials [19]. They are called isoprenoids due to the basic skeletal unit of isoprene that is magnified in the structure. Although the isoprene was not discovered in nature in native form, terpenoids and their oxygen derivatives (as polymeric substances) are widely distributed in different parts of plants *e.g.*, flowers, fruits, leaves, barks and roots. They are situated in resin ducts, papillas, glandular cells and intercellular spaces. They can be divided into several classes according to the number of carbon atoms, namely monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), triterpenes (C30), tetraterpenes (C40) and polyterpenes.

The aims of the present paper are an optimisation of an SFE extraction method for determination of terpenes from foliage of walnut-tree and coniferous needles, that would make possible fast and effective processing of more samples of plant materials for extensive monitoring studies, reduced time needed for extraction of studied volatile compounds, smaller quantity of harmful solvents used; and the production of final extracts which are directly analysed by GC-MS without necessity of clean-up.

2. Experimental Procedure

2.1. Chemicals and samples

Standards for gas chromatography: (+)-α-pinene, (-)-camphene, sabinene, (-)-β-pinene, myrcene, R-(+)-limonene, (-)-bornylacetate, (-)-*trans*-caryophyllene and α-humulene (all from Fluka, Buchs, Switzerland) were used for evaluation of extraction efficiency. n-Hexane (Fluka) was used for trapping of extracted substances. Carbon dioxide (2.0) was used for supercritical fluid extraction as an extraction agent. Helium (5.5; all from SIAD Czech, Braňany u Mostu, Czech Republic) was used for gas chromatography-mass spectrometry (GC-MS).

Samples of coniferous needles of *Pinaceae* family (*Picea abies, P. omorika, P. pungens, P. Breweriana, Pinus nigra P. mungo turra, P. black, P. sylvestris, Abies pinsapo, A. holophylla, A. Bronmuelleris, A. alba, Larix Kaempferi, L. decidua*) and leaves of *Juglandaceae* family (*J. regia*, *J. nigra*, *J. sieboldiana var. Cordiformis*) were collected in Botanical gardens and/or were identified by dendrologists from Department of Dendrology of Mendel University. The samples were collected in Dewar tanks filled with a solid CO_2, transported to laboratory (maximum storage time 6 h) and immediately treated. The samples from Brno, Jevíčko, Velké Meziříčí and Bílá were homogenized (20 g ± 5 mg) using splintery grinder Yellow line A10 (IKA-Werke GmbH & Co., KG, Staufen, Germany) and approx. 0.5 g (± 5 mg) of homogenized sample was extracted using different extraction techniques, *i.e.*, supercritical fluid extraction, accelerated solvent extraction at elevated pressure, sonication, microwave assisted extraction, steam distillation (*c.* 10 g ± 5 mg), Soxhlet extraction (*c.* 2 g ± 5 mg) and solvent agitation (*c.* 2 g ± 5 mg).

2.2. Extraction procedures

2.2.1. Supercritical fluid extraction (SFE)

Supercritical fluid extractor SE-1 (SEKO-K, Brno, Czech Republic) was used as described previously. Homogenized samples (0.5 g ± 5 mg) in a stainless steel extraction cartridge (0.7 - 7 mL inner volume) were inserted into an extraction cell with two frits at its ends and hermetically sealed with an extraction seal and with a valve for overpressure control. Extraction medium (CO_2) containing extracted substances passed through the restrictor (a 30 μm *i.d.* fused silica capillary). Heating

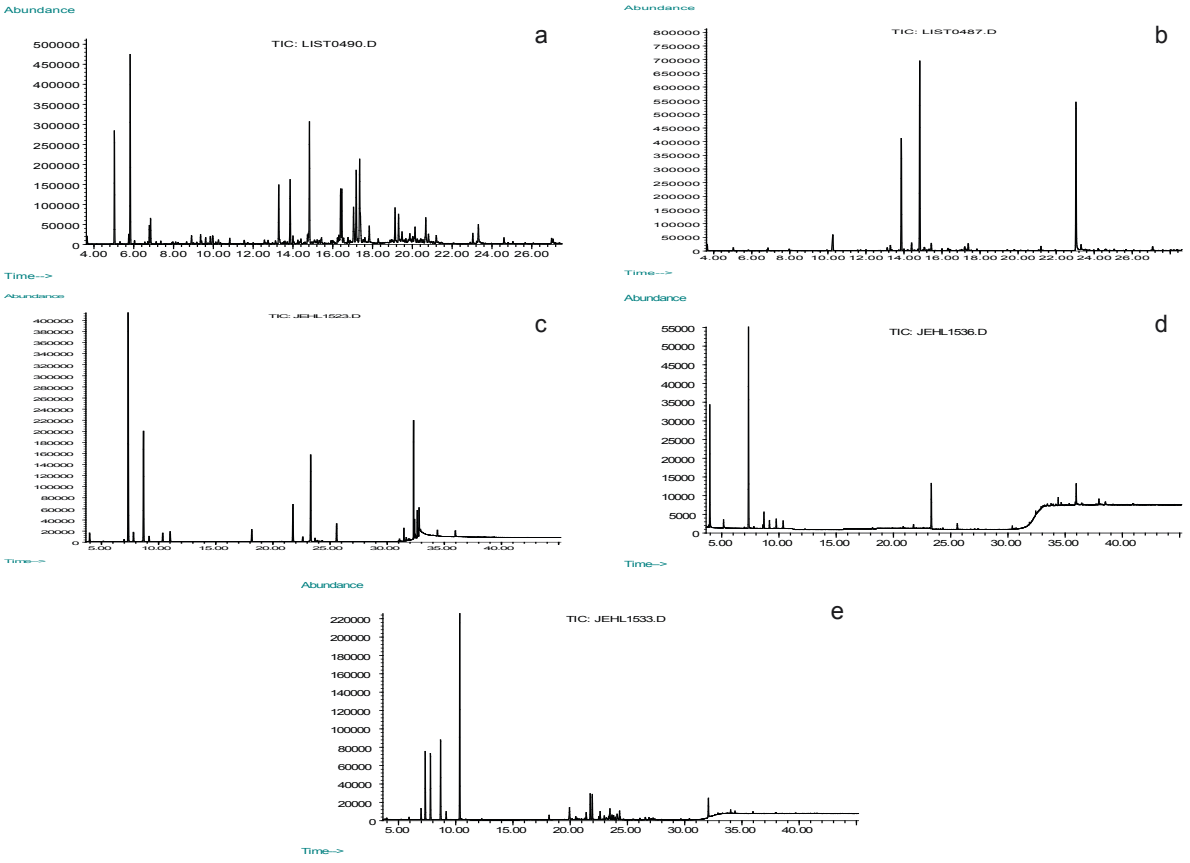

Figure 1. Chromatograms of extracts of J. regia L. (a), P. abies L. Karst. (b), Pinus nigra Arnold (c), Larix decidua Mill. (d), Abies alba Mill. (e).

of a restrictor was adjusted at 120°C to prevent restrictor blockage by a condensate. The volatile substances were trapped in n-hexane in a 30-mL glass vial at laboratory temperature. Heating and regulation of the trapping block were not used during analyses. Clean extract was injected directly into a GC-MS instrument.

2.2.2. Pressurized solvent extraction (PSE)

Pressurized solvent extraction (ASE) under elevated pressure was provided in a PSE-One instrument (Applied Separations Inc., Allentown, USA). Stainless steel cartridge (11 mL) was assembled with a metallic frit with a filter-paper disc placed on it. Further, 2 cm cotton plug was added, the cartridge was filled with a Spe-ed™ inert matrix (Applied Separations Inc., Allentown, USA), a filter-paper disc was placed on the column of sorbent and approximately 0.5 g of the homogenized biological sample was quantitatively transferred into the prepared cartridge. The sample was covered with a short column of the Spe-ed™ matrix and 2 cm cotton plug. The cartridge was finally sealed with a screw. Optimised conditions for PSE extraction using n-hexane as an extraction solvent were set at the temperature 120°C and 150 bar. Static extraction was carried out 3 times for 5 min. The extraction cartridge was filled with a solvent in each cycle, pressure was set on appreciate settings at high temperature and after period of the static extraction the final extract was collected into the glass vials. The volume of the final extract was approximately 20 mL. The extract was then directly injected into the GC-MS chromatographic system.

2.2.3. Steam distillation

Water steam distillation was performed in apparatus according to ČSN 58 0110 [5]. The milled plant material (*c.* 10 g ± 5 mg) was transferred quantitatively into the 1 l glass bottle and 400 mL of water was added. The mixture was distilled for 4 h. The obtained essential oil was diluted with n-hexane.

2.2.4. Soxhlet

Soxhlet-like extraction was provided in a fex-IKA apparatus (IKA – Werke GmbH & CO. KG, Staufen, Germany). The ground plant material (*c.* 2 g ± 5 mg) was transferred into an extraction bottle and 5 mL of n-hexane was added. The modified Soxhlet extraction was done at the temperature 160°C in two steps (30 min each).

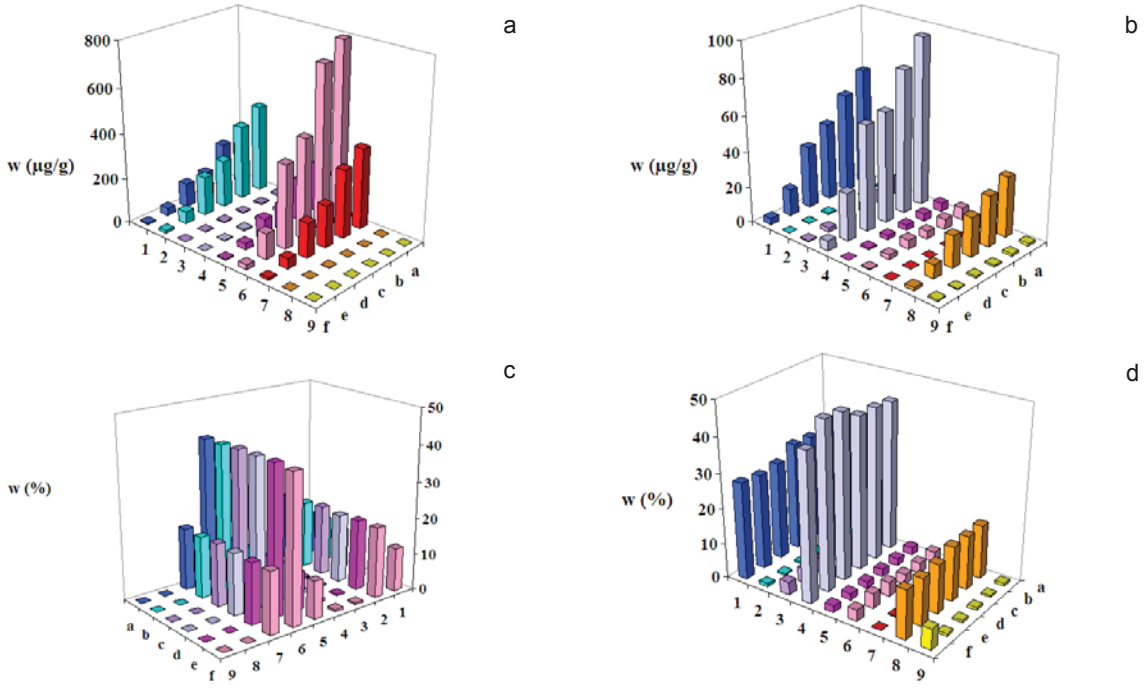

Figure 2. The effect of extraction methods on the amount (a, b) and representation (c, d) of monoterpenes in needles *P. pungens* and leaves *J. regia L.*

Individual terpenes: 1 - (+)-α-pinene, 2 - (-)-camphene, 3 – sabinene, 4 - (-)-β-pinene, 5 – myrcene, 6 - R-(+)-limonene, 7 - (-)-bornylacetate, 8 - (-)-*trans*-caryophyllene, 9 - α-humulene, extraction method: a – SFE, b – ASE, c – steam distilation, d – soxhlet, e – sonication, f - agitation

2.2.5. Sonication

Sonication was provided in an ultrasonic water bath K-5 (Kraintek, Podhajska, Slovakia). The milled plant material (*c.* 2 g ± 5 mg) was transferred into a 20-mL vial and extracted with 5 mL of n-hexane for 1 h at laboratory temperature.

2.2.6. Solvent agitation

Extraction with agitation of the solvent was provided on a T22 shaker (VD Lovena, Prague, Czech Republic). The milled plant material (*c.* 2 g ± 5 mg) was transferred into a 20 mL vial and extracted with 5 mL of n-hexane for 1 h at laboratory temperature.

2.3. Gas chromatography-mass spectrometry (GC-MS) procedure

A gas chromatograph HP-6890 with a HP-5973 mass spectrometric detector, equipped with an auto-sampler HP-6890 was used for determination of terpenes in extracts. The gas chromatograph was controlled with ChemStation software (version A.03.00). Separation of the compounds proceeded on a HP-5MS (5% Phenyl Methyl Siloxane, 30 m×0.25 mm, 0.25 µm film) chromatographic column (all from Agilent, Waldbronn, Germany) at a flow rate of helium 1 mL min^{-1}, split ratio 20:1, injector temperature 240°C, temperature of detector 250°C, using SIM mode at *m/z* 154-156 for (+)-α-pinene, (-)-camphene, (-)-β-pinene, myrcene, R-(+)-limonene and eucalyptol, at *m/z* 154-143 for (±)-linalool, at *m/z* 204-243 for (-)-bornylacetate, (-)-*trans*-caryophyllene and α-humulene and the temperature program: T_1 = 45°C, t_1 = 2 min, 8°C min^{-1} to T_2 = 300°C, t_2 = 15 min, for *c.* 47 min. Aliquots of 1 µL of the final extracts were dosed on the column. The final chromatograms are presented in Figs. 1a-e.

A set of seven standard calibration solutions in a concentration range 0.05–1.5 µg g^{-1} was prepared for quantification of terpenes in coniferous needles and in leaves of a walnut-tree. Calibration curves were constructed as a dependence of peak areas vs. concentrations of standard solutions. They were strictly linear (r = 1.000 for (+)-α-pinene, (-)-camphene, (-)-β-pinene, r = 0.999 for myrcene, R-(+)-limonene, eucalyptol, (-)-bornylacetate, (-)-*trans*-caryophyllene and α-humulene and r = 0.998 for (±)-linalool) in the whole concentration range. Each determination was repeated 5-times and the results are given as average values.

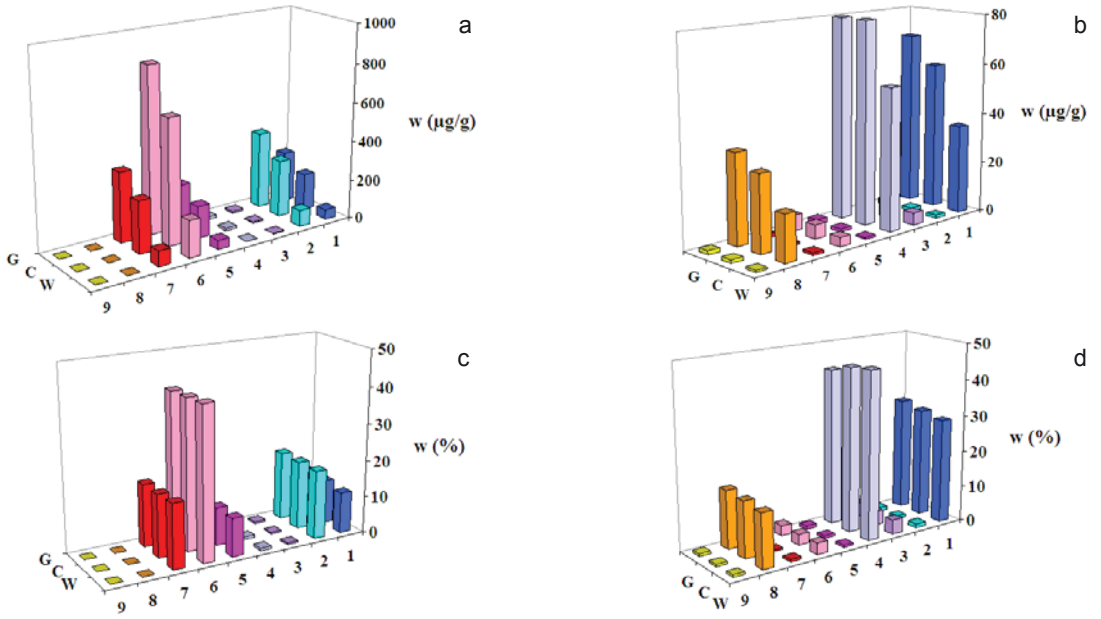

Figure 3. The effect of sample preparation method on the amount (a, b) and representation (c, d) of monoterpenes in needles *P. pungens* and leaves *J. regia L.*

Individual terpenes: 1 - (+)-α-pinene, 2 - (-)-camphene, 3 – sabinene, 4 - (-)-β-pinene, 5 – myrcene, 6 - R-(+)-limonene, 7 - (-)-bornylacetate, 8 - (-)-*trans*-caryophyllene, 9 - α-humulene, sample preparation: W – whole, C – cut, G - milled

3. Results and Discussion

3.1. Influence of an extraction procedure on the total amount and relative distribution of terpenes

Homogenized samples of *P. pungens* and *J. regia,* were used for comparison of six extraction techniques (supercritical fluid extraction, accelerated solvent extraction at elevated pressure, sonication, steam distillation, Soxhlet extraction and solvent agitation) for extraction of terpenes from coniferous needles and foliage of walnut-tree.

The highest amounts of terpenes (see Figs. 2 for comparison) were extracted from both *P. pungens* needles and *J. regia* leaves using supercritical fluid extraction (2.11 mg g^{-1}, 0.22 mg g^{-1}), accelerated solvent extraction at elevated pressure (1.79 mg g^{-1}, 0.19 mg g^{-1}), steam distillation (1.09 mg g^{-1}, 0,14 mg g^{-1}), Soxhlet extraction (0.91 mg g^{-1}, 0.13 µg g^{-1}), sonication (0.28 mg g^{-1}, 0.06 mg g^{-1}) and finally solvent agitation (0.05 mg g^{-1}, 0.01 mg g^{-1}).

Relative amounts of individual terpenes insignificantly changed for different extraction methods. The relative amounts of (-)-camphene in *P. pungens* needles were insignificantly higher for sonication, steam distillation and solvent agitation compared to the other methods of extraction. The elevated relative amounts of (-)-β-pinene were determined using Soxhlet extraction and sonication. The relative amounts of (-)-β-pinene and R-(+)-limonene in *J. regia* were higher for Soxhlet extraction and sonication. Higher relative amounts of α-humulene were obtained using solvent agitation. A negligible effect of extraction technique was observed for the rest of the terpenes.

3.2. Influence of sample treatment on total amount and relative distribution of terpenes

The effect of sample pre-treatment on a content of substances of interest in extracts was studied for *P. pungens* needles and *J. regia* foliage. The whole (untreated), cut and milled needles and leaves were used for extraction. The highest efficiency of the extraction was achieved for milled samples (2.10 mg g^{-1}, 0.23 mg g^{-1}), further for cut samples (1.56 mg g^{-1}, 0.20 mg g^{-1}) and in the end while using whole samples (0.45 mg g^{-1}, 0.12 mg g^{-1}) as can be seen in Fig. 3.

The dependence of relative distribution of particular terpenes on treatment procedure was not significant. It is possible to see only the very mild increase of the concentration of (+)-α-pinene and myrcene and a mild decrease of the R-limonene, in accordance to sample preparation procedures, from whole to the milled leaves.

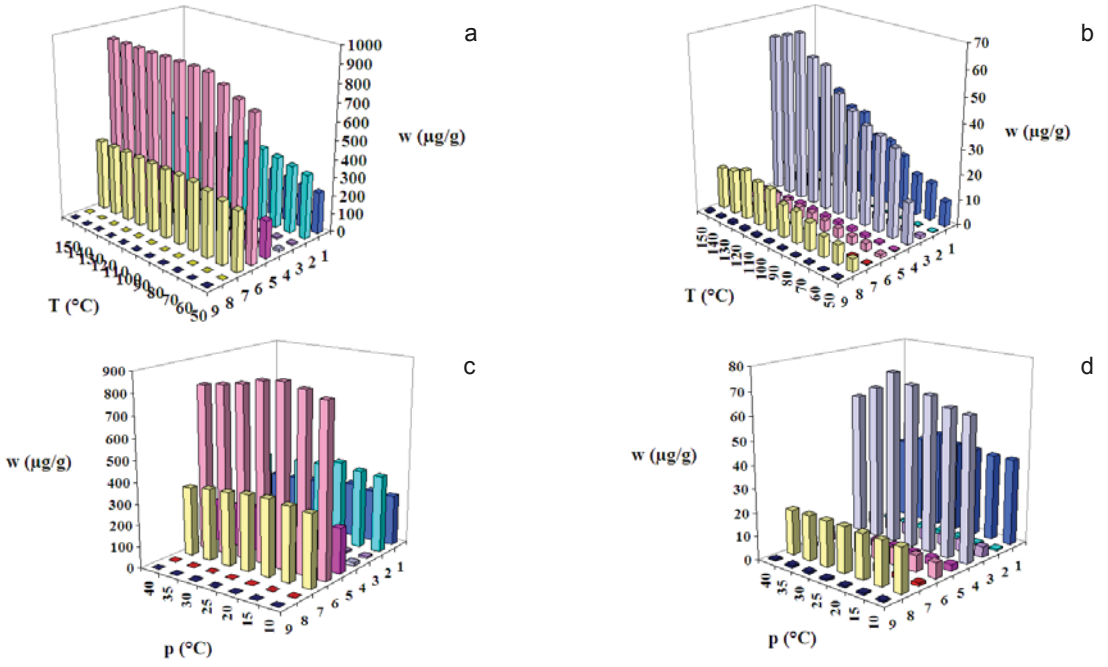

Figure 4. Optimization of temperature (a, b) and pressure (c, d) in needles *P. pungens* and leaves *J. regia L.*

Individual terpenes: 1 - (+)-α-pinene, 2 - (-)-camphene, 3 – sabinene, 4 - (-)-β-pinene, 5 – myrcene, 6 - R-(+)-limonene, 7 - (-)-bornylacetate, 8 - (-)-*trans*-caryophyllene, 9 - α-humulene,

3.3. Optimisation of extraction procedures

3.3.1. Optimisation of supercritical fluid extraction (SFE) procedure

The homogenous samples of needles of *P. pungens and* foliage of *J. regia* were used for optimisation of extraction temperature and pressure of the SFE procedure.

A pressure of 20 MPa and a temperature of 80°C were found to be the best for the extraction of terpenes from needles and 30 MPa and 130°C were optimum for extraction from leaves, respectively (see Fig. 4). Therefore, these conditions were selected for subsequent experiments. High molecular mass substances were extracted predominantly at the elevated temperatures. The substances complicated not only the extraction itself but also the subsequent GC-MS analyses. The relative distribution of particular terpenes was not affected. A mild increase of (+)-α-pinene, myrcene and α-humulene and a mild decrease of the R-(+)-limonene were observed at higher temperature. The relative distribution did not change with the increase of the pressure.

Further, a study of optimal number of cycles for static extraction and selection of a suitable modifier was done (see Fig. 5) at the selected experimental conditions (temperature of 80/130°C and pressure of 20/30 MPa for needles/leaves, respectively). Nearly 99.2% of the total amount of the volatile compounds was obtained after the 60 min cycle as can be seen from Fig. 5. Chloroform was selected as the most efficient modifier in both cases.

The relative distribution of particular terpenes was not affected by the duration of extraction procedures for both types of samples. A mild increase of concentration of (+)-α-pinene, camphene, (-)-bornylacetate and a mild decrease of the R-(+)-limonene for extracts from needles with time was observed. A mild increase of concentration of (+)-α-pinene and a very mild decrease of the (-)-β-pinene was observed for leaves. The relative distribution did not change with increase in pressure.

3.4. Influence of tree variety on total amount and relative distribution of terpenes

Mixed samples of the foliage of walnut-tree and that of coniferous needles were collected for determination of dependencies of quantity and distribution of terpenes on the kind of tree. The optimised SFE was used for extraction of milled samples. Fourteen kinds of trees of the *Pinaceae* family (*Picea abies, P. omorika, P. pungens, P. Breweriana, Pinus nigra, P. mungo turra, P. black, P. sylvestris, Abies pinsapo, A. holophylla, A. Bronmuelleris, A. alba, Larix Kaempferi, L. decidua*) and three from the *Juglandaceae* family (*J. regia*, *J. nigra*, *J. sieboldiana var. Cordiformis*) from city of Brno were selected. Several mixed samples were collected from each tree (one year old needles were collected only). The samples were homogenized and the terpenes were extracted with SFE and determined by GC-MS (see Fig. 6).

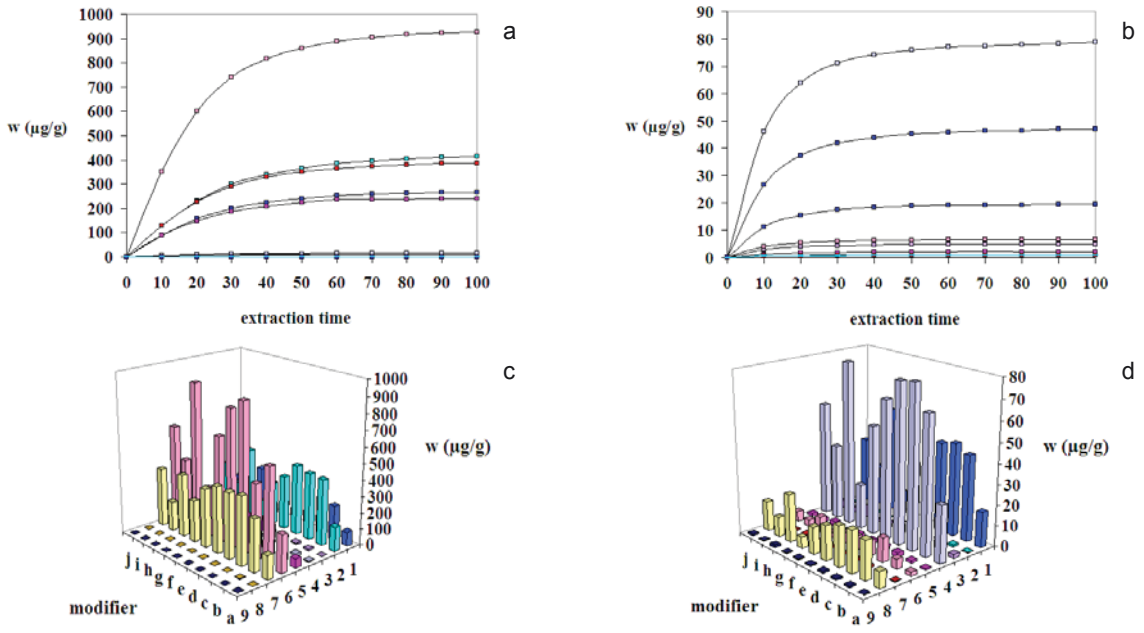

Figure 5. Optimization of extraction time (a, b) and modifier (c, d) in needles *P. pungens* and leaves *J. regia L.*

Individual terpenes: 1 - (+)-α-pinene, 2 - (-)-camphene, 3 – sabinene, 4 - (-)-β-pinene, 5 – myrcene, 6 - R-(+)-limonene, 7 - (-)-bornylacetate, 8 - (-)-*trans*-caryophyllene, 9 - α-humulene,
modifier: a – without modifier, b – methanol, c – ethanol, d – acetone, e – acetonitrile, f – hexane, g – dichlormethane, h – chloroform, i – toluene, j - water

The highest quantity of the studied terpenes in *Pinaceae* (see Fig. 7) was found for samples of *A. holophylla* (7.09 mg g^{-1}), *P. abies.* (5.59 mg g^{-1}), *A. alba.* (4.69 mg g^{-1}), *A. Bronmuelleris* (4.33 mg g^{-1}), *Pinus nigra* (3.22 mg g^{-1}), *Pinus mungo turra* (2.56 mg g^{-1}), *P. omorika* (2.40 mg g^{-1}), *P. pungens* (2.11 mg g^{-1}), *Pinus black* (1.76 mg g^{-1}), *Abies pinsapo* (1.65 mg g^{-1}), *Pinus sylvestris* (0.96 mg g^{-1}), *P. Breweriana* (0.73 mg g^{-1}), *L. Kaempferi* (0.66 mg g^{-1}), *L. decidua* 0.37 mg g^{-1}) and in *Juglandaceae* family for *J. sieboldiana var. Cordiformis* (0.33 mg g^{-1}), *J. regia* (0.28 mg g^{-1}) and the lowest amount for *J. nigra* (0.18 mg g^{-1}). Also the relative distribution of the particular compounds considerably varied with the kind of tree.

3.5. Influence of location on total amount and relative distribution of terpenes

The foliage of walnut-tree royal (*J. regia*) and needles from *P. abies* from four different locations (Brno, Jevíčko, Velké Meziříčí and Bílá) were collected for determination of dependencies of quantity and distribution of terpenes on locations in the same period. Several mixed samples were collected from each location. The samples were milled, extracted by supercritical fluid extraction (SFE) and analysed by GC-MS. As can be seen from Fig. 7, the highest quantity of the terpenes was found in the needles from Velké Meziříčí (2.78 mg g^{-1}) and Bílá (2.78 mg g^{-1}), followed by locality Brno (2.71 mg g^{-1}), Jevíčko (2.64 mg g^{-1}) and in leaves from the location Velké Meziříčí (0.30 mg g^{-1}), Brno (0.28 mg g^{-1}), Jevíčko (0.28 mg g^{-1}), Bílá (0.25 mg g^{-1}).

The relative distribution of particular terpenes was nearly unchanged for individual locations and both materials with exception of mildly increasing quantity of (-)-bornylacetate in needles from the locations Jevíčko and Bílá and foliage from the location Bílá, (-)-*trans*-caryophyllene in needles from the location Bílá and myrcene in foliage from the location Bílá.

3.6. Influence of vegetation stage on total amount and relative distribution of terpenes

Several mixed samples of coniferous needles of *P. abies* (one and two years old) and foliage of *J. regia* were collected from April to September from selected trees for determination of dependencies of quantity and distribution of terpenes on vegetative stages in location of Bílá. The samples were homogenized, the terpenes were extracted with SFE and analysed by GC-MS (see Fig. 8).

The total amount of terpenes dramatically varied with vegetation season. The amount of terpenes in the needle samples of *P. abies* (May – September with exception of the youngest ones) decreased with the increasing vegetation period and the relative distribution varied markedly. The highest amount of

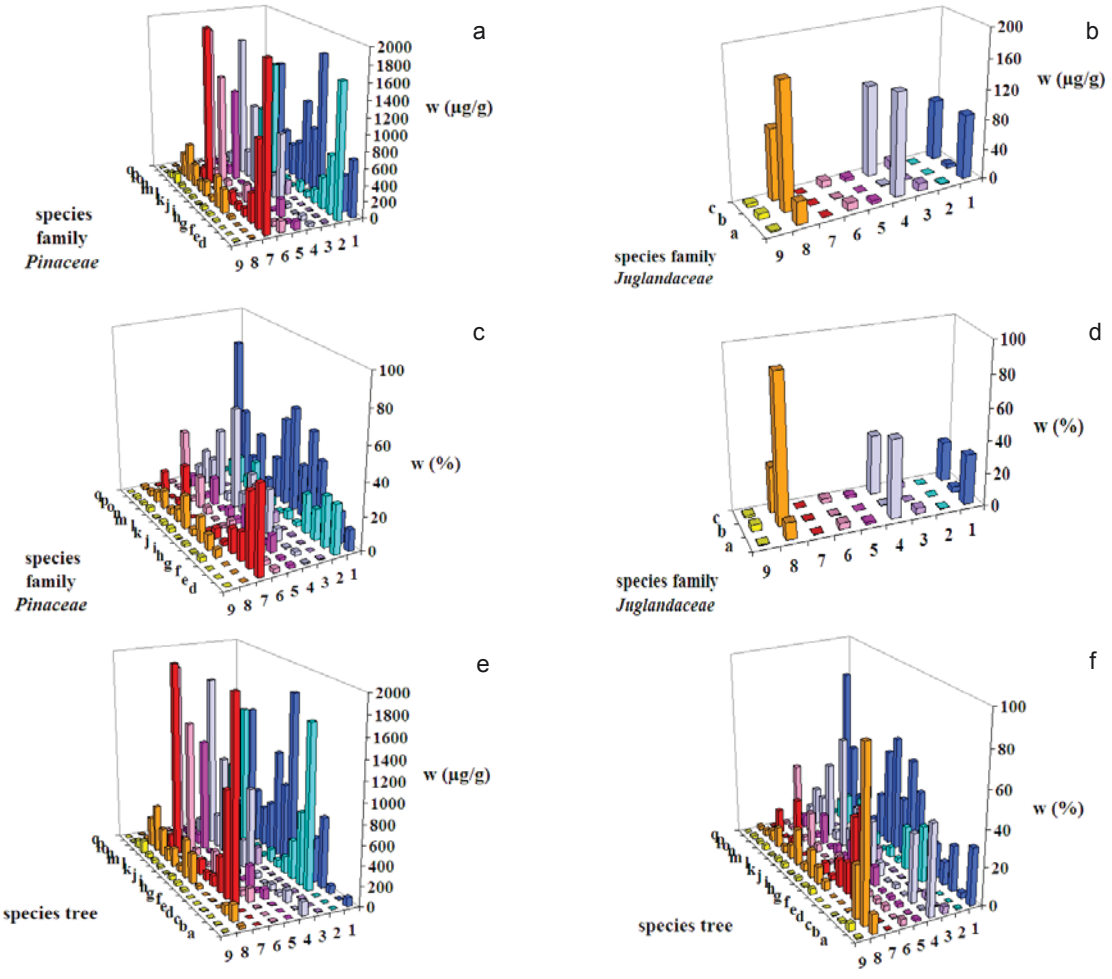

Figure 6. The effect of species tree on the amount (a, b, e) and representation (c, d, f) of terpenes in tree family Pinaceae and Juglandaceae, Individual terpenes: 1 - (+)-α-pinene, 2 - (-)-camphene, 3 – sabinene, 4 - (-)-β-pinene, 5 – myrcene, 6 - R-(+)-limonene, 7 - (-)-bornylacetate, 8 - (-)-*trans*-caryophyllene, 9 - α-humulene, species family *Pinaceae* and *Juglandaceae*: a - *Juglans regia*, b - *J. nigra* c - *J. sieboldiana var. Cordiformis*, e - *Picea abies*, f - *P. pungens*, g - *P. Breweriana*, h - *Pinus nigra*, i - *P. mungo turra*, j - *P. black*, k - *P. sylvestris*, l - *Abies pinsapo*, m - *A. holophylla*, n - *A. Bronmuelleris*, o - *A. alba*, p - *Larix Kaempferi*, q - *L. decidua*

terpenes of interest was found in samples collected in July (1.76 mg g^{-1}). The relative distribution of terpenes insignificantly changed in the one-year-old needles. Dramatic decrease of terpenes in *J. regia* foliage was observed from April to September with exception of July with the more dramatic decrease (up to 0.20 mg g^{-1}). The relative distribution of terpenes significantly changed for (+)-α-pinene, (-)-β-pinene and (-)-*trans*-caryophyllene.

4. Conclusions

The SFE technique was found to be suitable for extraction of volatile substances (*i.e.*, terpenes) from solid plant materials (needles, foliage *etc.*). The optimised SFE procedure (pressure, temperature, modifier *etc.*) markedly reduces time of extraction of volatile substances of interest from homogenized samples of coniferous needles and foliage in comparison with "classical" extraction methods. SFE also effectively reduces the consumption of harmful organic solvents (only small amounts of modifiers are used). The final extracts do not need any further clean-up steps and they can be directly injected into a GC-MS system. Due to the short extraction times the method is also very suitable for analyses of a huge number of samples in screening studies. The proposed SFE procedure (the selected experimental conditions - time 60 min, chloroform as a modifier, temperature of 80/130°C, pressure of 20/30 MPa for needles/leaves, respectively) was applied for determination of terpenes in needles of *Pinaceae* family and foliage of *Juglandaceae* family. The dependence of the total content and the relative distribution of particular terpenes on kind of tree, location, vegetation season were also studied.

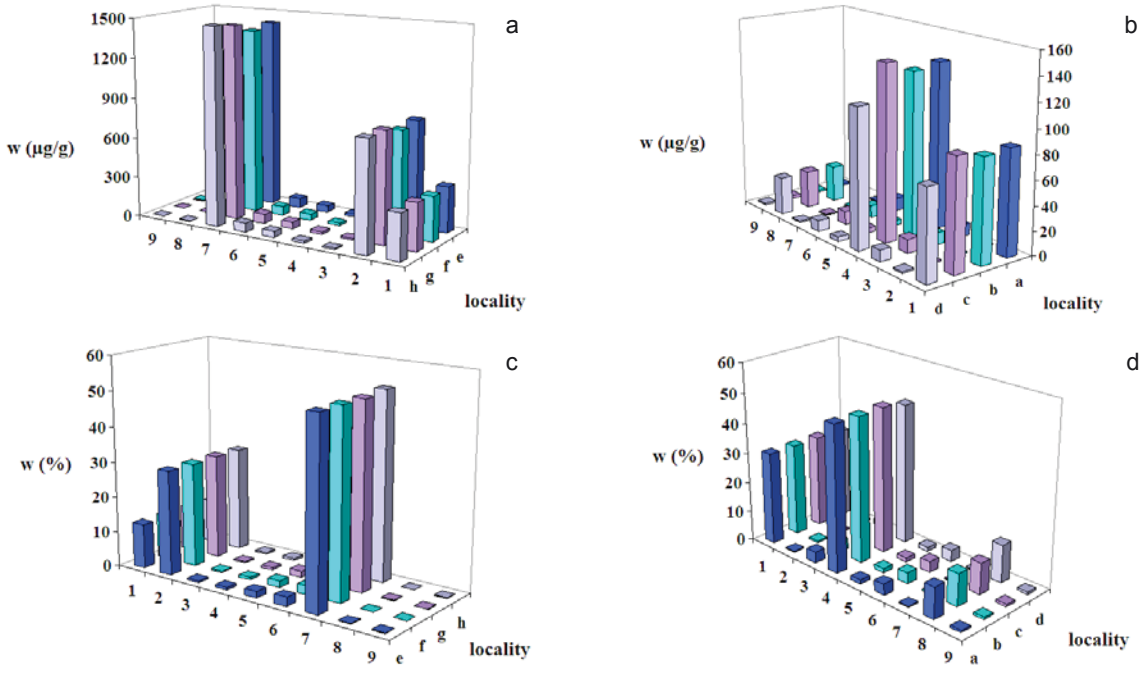

Figure 7. The effect of locality on the amount (a,b) an representation (c,d) of terpenes in *P. abies L. Karst*. and *J. regia L.*

Individual terpenes: 1 - (+)-α-pinene, 2 - (-)-camphene, 3 – sabinene, 4 - (-)-β-pinene, 5 – myrcene, 6 - R-(+)-limonene, 7 - (-)-bornylacetate, 8 - (-)-*trans*-caryophyllene, 9 - α-humulene,
locality: a, e – Brno, b, f – Jevíčko, c, g – V. Meziříčí, d, h – Bílá

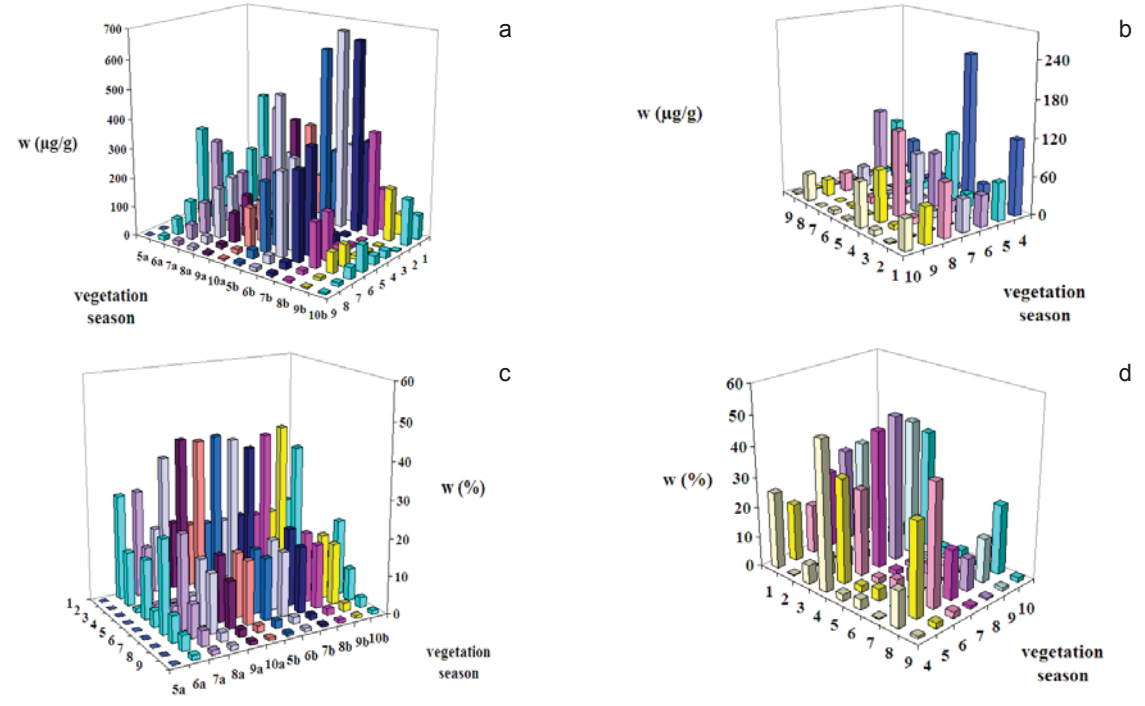

Figure 8. The effect of vegetation season on the amount (a,b) and representation (c,d) of terpenes in *P. abies L. Karst*. and *J. regia L.*

Individual terpenes: 1 - (+)-α-pinene, 2 - (-)-camphene, 3 – sabinene, 4 - (-)-β-pinene, 5 – myrcene, 6 - R-(+)-limonene, 7 - (-)-bornylacetate, 8 - (-)-*trans*-caryophyllene, 9 - α-humulene,
vegetation season: April – October, a - this-year needles, b - previous year needles

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