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ENANTIOMERIC COMPOSITIONS OF MONOTERPENE HYDROCARBONS IN DIFFERENT TISSUES OF FOUR INDIVIDUALS OF *PINUS SYLVESTRIS*

KRISTINA SJÖDIN,* MONIKA PERSSON, ANNA-KARIN BORG-KARLSON and TORBJÖRN NORIN

Department of Chemistry, Organic Chemistry, Royal Institute of Technology, S-100 44 Stockholm, Sweden

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Key Word Index—*Pinus sylvestris*; Pinaceae; monoterpene hydrocarbons; camphene; 3-carene; limonene; β-phellandrene; α-pinene; β-pinene; sabinene; enantiomers; chiral analysis.

Abstract—The relative amounts of volatiles, mainly monoterpene hydrocarbons, were determined in eight different tissues of each of four individuals of *Pinus sylvestris*. The four trees represented widely different monoterpene compositions. Two-dimensional gas chromatography, using columns with stationary phases containing functionalized cyclodextrins, allowed the determination of the enantiomeric compositions of seven major chiral monoterpene hydrocarbons. Large differences in the relative amounts of the monoterpene hydrocarbons as well as in their enantiomeric compositions were found both within and between individuals. The extremely large relative amounts of (+)-3-carene or (−)-limonene found in the samples of 1-year-old phloem and bark of branches and in the shoots of some of the trees were not found in the xylem of the trunk or root or in the needles of the same trees. Only the (+)-enantiomer of 3-carene was detected. The (−)-enantiomers of β-pinene, sabinene and β-phellandrene dominated over the corresponding (+)-enantiomers in all samples.

INTRODUCTION

A prerequisite for studies of insect–conifer relationships is a knowledge of the contents and variations of attractants within and among the trees. Monoterpene hydrocarbons play an important role in the complex interactions between forest insects and their conifer hosts. The primary attraction of several Scolytidae and Curculionidae to monoterpene hydrocarbons is well known [1, 2]. From a biosynthetic point of view, the two enantiomers of a chiral monoterpene must be considered as two separate constituents [3] and they often exhibit differences in biological activity. The importance of the chirality of these constituents for the primary attraction is, however, far less well known. The attraction of *Tomicus piniperda* (L.) to α-pinene [4] and the reduction of the attraction of *Hylobius abietis* (L.) to different baits by limonene [5] have been reported to be independent of the enantiomeric compositions of these monoterpenes. The opposite has been reported for the attractiveness of α-pinene to *Dendroctonus valens* (Leconte) [6, 7]. However, the results reported in the last two investigations are somewhat contradictory. The importance of the enantiomeric composition of α-pinene for the production of active pheromone components has, however, been established [8]. These types of investigations have been hampered by the difficulties not only in determining enantiomeric compositions but also in obtaining test compounds of high enantiomeric purity. The gas chromatography columns now commercially available, which are based on derivatives of cyclodextrin [9], provide a tool for the chiral analysis which makes it possible to overcome at least the first difficulty.

The prevailing coniferous species of economic importance in Scandinavia are Scots pine, *Pinus sylvestris* L., and Norway spruce, *Picea abies* (L.) Karst. The results of investigations on the enantiomeric compositions of monoterpene hydrocarbons in *P. abies* have recently been presented [10–12]. Large variations in the relative amounts of the constituents as well as in the enantiomeric compositions of most of the chiral monoterpenes, between and within trees, have been revealed.

In contrast, the knowledge of the enantiomeric compositions of monoterpene hydrocarbons in different tissues of *P. sylvestris* is very scarce. A few results of analyses of needle oils have been presented by König [13] and by König *et al.* [14]. Hiltunen *et al.* [15] determined the enantiomeric ratios and the relative amounts of chiral monoterpene hydrocarbons in pine needle oils. Some information on the enantiomeric compositions of the chiral monoterpene hydrocarbons in the xylem has been presented in a recent investigation on topcut trees [16].

In this investigation the results of analyses of samples from eight different tissues of four specimens of *P. sylvestris* are presented. As suggested by Hiltunen *et al.* [17],

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*Author to whom correspondence should be addressed.

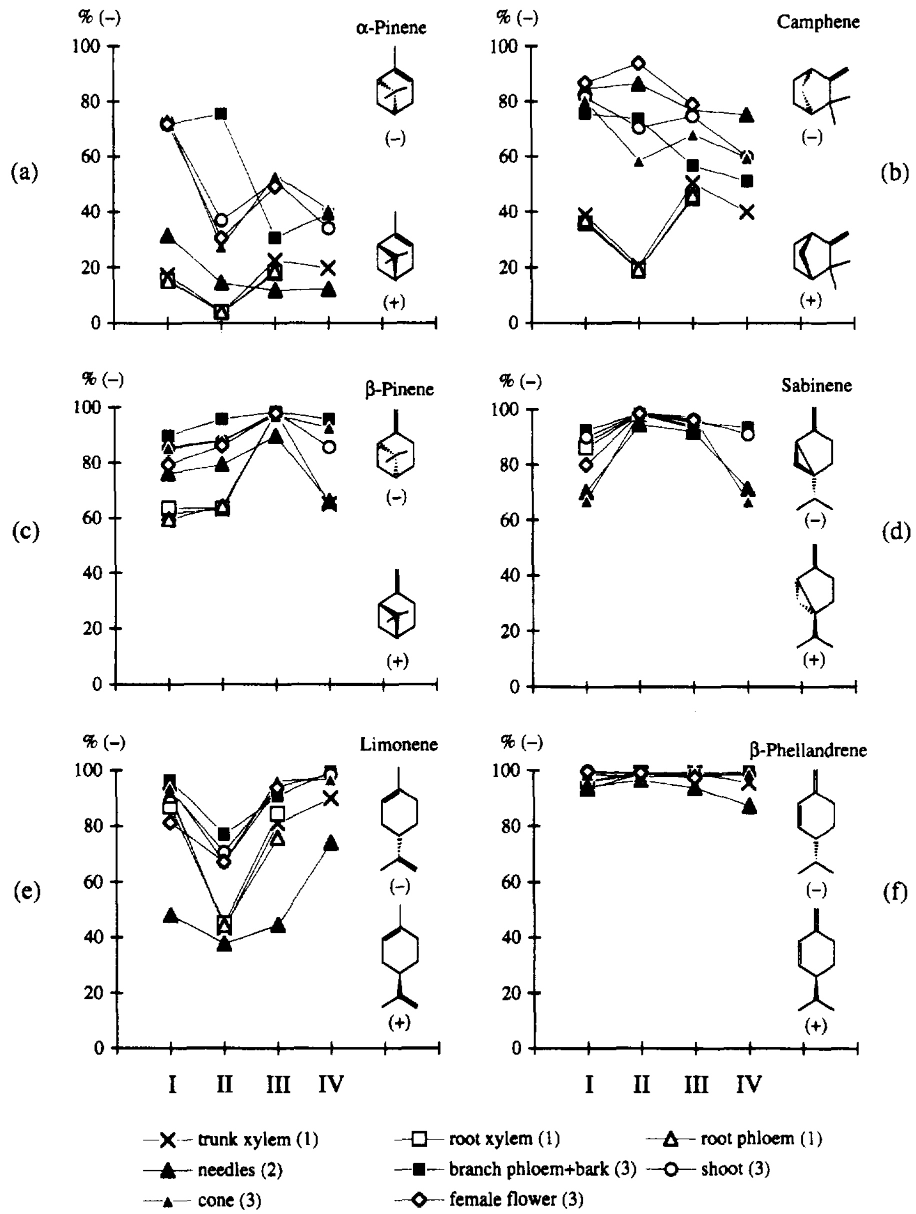

Fig. 1. Enantiomeric compositions (the ratio of the integrated area of the (−)-enantiomer to the sum of the areas of the (+)- and the (−)-enantiomers, in per cent) of (a) α-pinene, (b) camphene, (c) β-pinene, (d) sabinene, (e) limonene and (f) β-phellandrene in different tissues of four *Pinus sylvestris* trees. The enantiomeric compositions of sabinene in the root phloem of tree I and in the trunk xylem of tree IV could not be determined because of the small amounts of sabinene present in these tissues. The figures in parentheses refer to the division of the tissues into three groups on the basis of their monoterpene contents.

this pine species can be divided into two chemotypes, containing high and low relative amounts of 3-carene in the needles. The phloem [18] and phloem + xylem [19] of these chemotypes show similar differences in their 3-carene contents. One tree of each of these two chemotypes was chosen for this investigation: tree I of the low and tree II of the high 3-carene type. The relative amounts of the constituents of the monoterpene hydrocarbon fraction and the enantiomeric compositions of seven major chiral monoterpenes were determined by two-dimensional gas chromatography (2D-GC). The investigation is a part of our studies on insect–conifer interactions.

RESULTS

The enantiomeric compositions, given as the percentages of the (−)-enantiomers, of the chiral monoterpenes α-pinene, camphene, β-pinene, sabinene, limonene and β-phellandrene showed large variations (Fig. 1a–f). Large differences were found not only between trees but

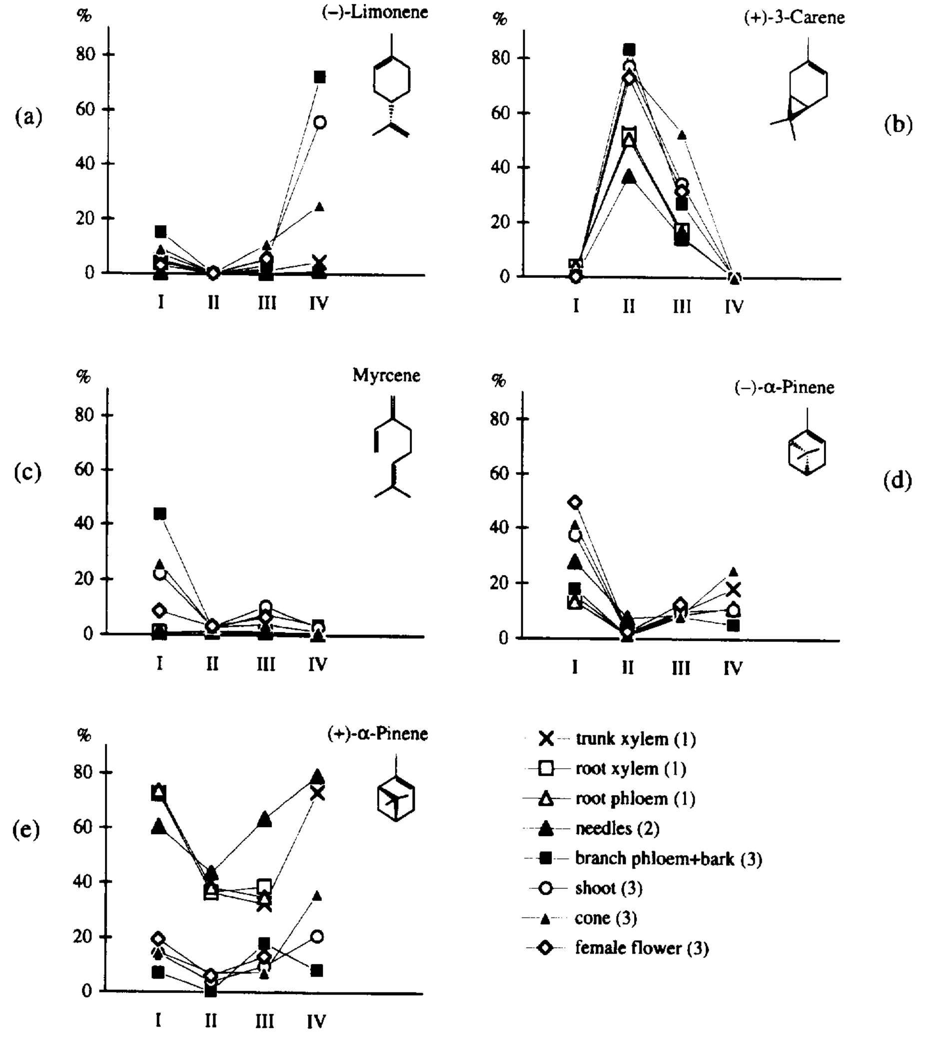

Fig. 2. The relative amounts of (a) (−)-limonene, (b) (+)-3-carene, (c) myrcene, (d) (−)-α-pinene and (e) (+)-α-pinene in different tissues of four *Pinus sylvestris* trees. Here the total integrated area is used as the normalization factor (cf. Experimental: *Multivariate data analysis*). The figures in parentheses refer to the division of the tissues into three groups on the basis of their monoterpene contents.

also to a similar extent between different tissues of the same tree. The samples from each tree could be divided into three groups: (1) trunk xylem, root xylem and root phloem, (2) needles and (3) branch tissues (except needles). Each group exhibited similarities in the enantiomeric compositions. The group-3-tissues generally showed higher enantiomeric compositions of α-pinene and the group-1-tissues generally showed lower enantiomeric compositions of camphene than the other tissues. The (−)-enantiomers of β-pinene, sabinene and β-phellandrene dominated over their (+)-enantiomers in all samples. For each tissue type, limonene appeared to reach a higher enantiomeric purity, an increasing percentage of (−)-limonene, with increasing relative amounts. Only one of the 3-carene enantiomers, (+)-3-carene, could be detected. The amounts of α-phellandrene were too small to allow a chiral analysis.

The variation in the relative amounts of the monoterpenes, both between and within trees, was also found to be high (exemplified by Fig. 2a–e). The needles contained the highest relative amounts of tricyclene and (−)-camphene and the lowest relative amounts of (−)-limonene (Fig. 2a) and (−)-sabinene. The major constituent in the needles, however, was (+)-α-pinene (Fig. 2e). Along with tree II, tree III should be characterized as a high 3-carene tree (Fig. 2b) based on the limit (>10% in needles) set by Hiltunen *et al.* [17]. In all trees, the relative amounts of (+)-α-pinene are lower in the group-3-tissues than in the other tissues (Fig. 2e).

The differences and similarities between the samples in the compositions of their volatiles can be visualized by PCA (principal components analysis). The PC-plot in Fig. 3 shows that samples representing trees II and III, which contain large amounts of (+)-3-carene, appear to

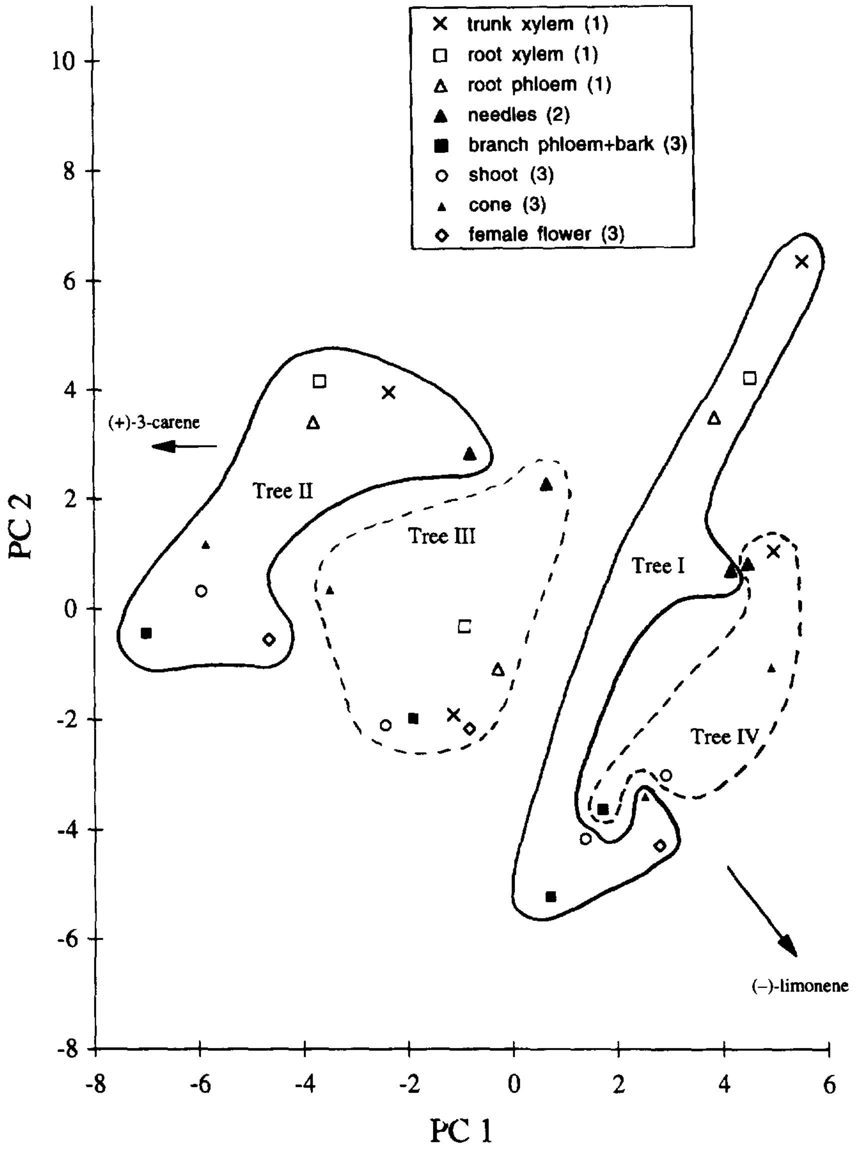

Fig. 3. Principal components analysis, score plot, based on selectively normalized raw data (see Experimental) obtained by GC-analysis of extracts of tissue samples from four *Pinus sylvestris* trees. A total of 49% of the variance in the data is explained by the first (PC 1) and the second (PC 2) principal components, both of which were found to be significant as judged by cross-validation. The figures in parentheses refer to the division of the tissues into three groups on the basis of their monoterpene contents. The arrows indicate the directions of increasing relative amounts of (+)-3-carene and (−)-limonene, respectively.

the left. The samples representing the low 3-carene trees I and IV, are found to the right in the plot. The samples from each tree could, based on their relative amounts of volatiles be divided into three groups: trunk and roots (phloem and xylem), needles and branches (phloem, shoot, cone and female flower), i.e. the same groups that were formed on the basis of the enantiomeric compositions. Within each of the two 3-carene classes the needle samples were similar in their composition of volatile compounds. The needle samples of the high 3-carene trees were found to have greater similarity (were found closer) to the samples from the low 3-carene trees, in their relative amounts of volatiles, than the more extreme samples originating from the branches. The corresponding loading plot (not shown) showed that, except for (+)-3-carene, the following combination of constituents was important for separating the samples of the high 3-carene class of trees from the samples of the low-3-carene class: 2-carene, α-phellandrene, α-terpinene, γ-terpinene, terpinolene and (−)-sabinene. Similarly, (+)-

and (−)-α-pinene, (+)- and (−)-camphene, (+)- and (−)-limonene, (+)-β-pinene, (+)-β-phellandrene, and tricyclene were important for the low-3-carene class. In most cases, analyses showed close similarity between duplicate samples.

DISCUSSION

The relative amounts, determined by conventional gas chromatography analysis, of monoterpene hydrocarbons in *P. sylvestris* needles [20], cortical oleoresin [21–23], twigs [24], xylem oleoresin [25], and phloem, shoots and needles [18] are known to vary considerably within the species. The differences in composition between the tissues are large but mainly quantitative [18, 20–25]. This within-tree tissue variation is well known in conifers [26], but the reasons for these differences have not been established. Hanover [26] states: "Clearly, genes regulate the concentrations of terpenes in each tissue system but the factors causing their differential expression within the tree are largely unknown". In our investigation the differences found within and between trees mainly concerned the relative amounts of the constituents (Fig. 2a–e). This agrees with the results obtained through the nonchiral analyses mentioned above. Moreover, as the two enantiomers of a chiral monoterpene hydrocarbon have to be considered as two separate constituents [3], the large variation in enantiomeric composition between the tissues within a tree as well as between trees is in accordance with the large variation in the relative amounts of the individual constituents in general.

The high relative amounts of (−)-limonene found in the branch phloem + bark, in the shoot and to some extent in the cone of tree IV were not found in the xylem or the needles of that tree (Fig. 2a). Similarly, the largest relative amounts of (+)-3-carene in tree II (Fig. 2b) as well as the higher relative amounts of myrcene in tree I (Fig. 2c) were found in the branch phloem, the shoot, the cone and the flower. The large within-tree differences found in the relative amounts of these constituents emphasize the importance of specifying the tissue when the characterization of a conifer is based on its composition of volatiles.

In all samples, β-phellandrene was found in high enantiomeric purity with a dominating (−)-enantiomer (Fig. 1f). The same predominance has been found in wood and foliage of *P. radiata* D. Don [27], in *P. sylvestris* xylem [16] and in xylem oleoresin of four Cuban pine species [28], and has been indicated also for *P. peuce* Griseb. needles [29]. The predominance of (−)-β-pinene over (+)-β-pinene reported here (Fig. 1c) has also been found in the above-mentioned investigations on pines. In contrast to the predominance of the (−)-enantiomer for sabinene, reported here for *P. sylvestris* (Fig. 1d) and previously for *P. radiata* [27], the sabinene occurring in the four Cuban species was recently found to be racemic or to consist mainly of the (+)-enantiomer [28].

No (−)-3-carene was detected in the samples. This is in accordance with earlier results regarding *P. sylvestris* [16] and *Picea abies* [12]. König [30], however, claimed, in 1991, to have detected the (−)-enantiomer of 3-carene in *P. sylvestris* needle oil. Our loading plot (not shown here) showed that, along with 3-carene and 2-carene, a number of structurally similar monoterpene hydrocarbons were important in the samples of high 3-carene trees, namely, α-phellandrene, α-terpinene, γ-terpinene and terpinolene. When performing multivariate data analysis of gas chromatography data from *P. sylvestris* needles, Hiltunen *et al.* [15] also found that the amounts of γ-terpinene and terpinolene, among other constituents, were strongly dependent on the amounts of 3-carene. In our investigation, the two enantiomers of β-phellandrene, although biosynthetically related to α-phellandrene [31, 32], were found to be important for the low-3-carene trees. Trees II and III, which contained high relative amounts of (+)-3-carene in their tissues (Fig. 2b), contained low amounts of (−)-limonene (Fig. 2a). A low content of (+)-3-carene, as in the tissues of trees I and IV, was accompanied by a high relative amount of (−)-limonene only in tree IV.

The maximum value of 32% (−)-α-pinene (enantiomeric composition, Fig. 1a) found in the needles was far lower than the maximum value of 84% found by Hiltunen *et al.* [15] for needles of the same pine species. The enantiomeric composition of α-pinene did not exceed 23% in the samples of trunk xylem from any of the four specimens now investigated (Fig. 1a). In an earlier investigation a maximum value of 29% (−)-α-pinene was found in the trunk xylem [16] which corresponds well with the maximum value of 28% obtained in an investigation unpublished. This is to be compared with the values, ranging from 23% to 89%, found in *Picea abies* branch xylem (close to the trunk) [12]. Only the (−)-enantiomer is transferred to the active pheromone component (*S*)-*cis*-verbenol by the bark beetle *Ips typographus* (L.) [33]. The fact that the proportion of (−)-α-pinene to (+)-α-pinene is higher in *Picea abies* than in *Pinus sylvestris* might partly explain why the bark beetle predominantly attacks the spruce.

As has been pointed out by Cool and Zavarin [27], chiral analyses give increased information for chemotaxonomical studies. Such analyses also provide a new tool for investigations of biosynthetic routes. The information obtained through extensive chiral analyses will make it possible in the future to widen the scope of investigations on insect–plant interactions.

EXPERIMENTAL

Experimental trees. Four *Pinus sylvestris* trees, all about 40 years old, were chosen to represent pines containing widely different relative amounts of 3-carene and limonene. The trees were growing in (trees I, II and IV) or, in one case (tree III), close to a progeny trial stand at the Bogesund experimental park, 30 km east of Stockholm, in Central Sweden.

Sampling. Samples were taken from eight different tissues of each of the four pines. The tissues sampled were trunk xylem, root xylem, root phloem, 1-year-old needles, branch phloem + bark of the previous year, shoot, cone and female flower. The samples were collected between 26 May and 11 June, 1992, during the flowering season, at an outdoor temperature of about 20°. The xylem was sampled, about 1 m above the ground, using a 5 mm i.d. increment borer. Duplicate samples were taken. No samples of the root were taken from tree IV and no female flower was found on that tree. The samples were cut in 1–3 mm pieces, put into glass vials, and covered with hexane. The solutions were then filtered through silica gel (Matrex silica 90–130 mm, pore size 30 Å, Amicon). The flower samples were extracted in 0.5 ml hexane and the small amounts of extracts obtained were not filtered.

The flower extracts and some of the other, filtered, extracts were immediately analysed by GC, but the majority of the extracts were kept at − 25° for 10 months before analysis. Comparisons of the results of analyses performed direct after sampling, with the results found for the same samples after storing for 10 months revealed only minor compositional differences of the volatiles as a result of storing.

GC analysis. GC-analyses were based on the method for two-dimensional GC presented in [10]. The relative amounts of the volatiles eluted between tricyclene and terpinolene were determined in the first GC, GC(A), by a DB-WAX capillary column (J&W Scientific Inc., CA, 30 m, 0.25 mm id; film thickness 0.25 μm; carrier gas, He; inlet pressure 30 psi; temp. programme, 40° isothermal 2 min, increase to 60° at 50° min^{-1} after 1 min increase to 95° at 4°/min^{-1}, thereafter rapid increase of temp. to 180°; injector temp., 180°, 0.8 μl injected, splitless mode for 6 s; FID detector temp., 200°).

The enantiomeric compositions of α-pinene, camphene, β-pinene, sabinene, limonene and β-phellandrene were determined using two Cyclodex-B (permethyl-β-cyclodextrin/DB-1701) columns in the second GC, GC(B), (30 m, 0.25 mm id; film thickness 0.25 mm; carrier gas, He; inlet pressure 20 psi in GC(B), 23 psi in GC(A); temperature programme, 55° isothermal for 11 min, increased to 75° at 1° min^{-1}. Detector temperature: 150°). The enantiomeric analyses of 3-carene were carried out by means of a Lipodex E (dipentylbutyryl-γ-cyclodextrin) column (25 m, 0.25 mm id; carrier gas, He; inlet pressure 23 psi; 30° isothermal; Detector; 120°). A small amount of (+)-3-carene was added to a portion of the sample. In accordance with [34] we used a hexane extract of black pepper oil as a reference for (−)-3-carene which was the first eluted enantiomer of 3-carene. The enantiomeric compositions are presented in Fig. 1 as percentages of the (−)-enantiomer and are calculated from the integrated areas, A, of the (−)- and (+)-enantiomer, $A(-) \times 100/[A(+) + A(-)]$.

Multivariate data analysis. Selectively normalized raw data from the GC analyses were subjected to principal components analysis (PCA) using the program CODEX (Chemometric Optimization and Design for Experimenters, available from Sum IT Systems AB, P.O. Box 1936, S-17119 Solna, Sweden) which is installed as an add-in module to Microsoft Excel. The normalization factor used was the total sum of the integrated areas minus the sum of the areas of the major peaks—those caused by (−)-α-pinene, (+)-α-pinene, 3-carene and (−)-limonene. In that way negative correlations between large peaks, one effect of closure, were reduced [35]. As in an earlier investigation on pines [19] the normalized data were subjected to a logarithmic transformation, whereupon each variable was scaled to unit variance (autoscaling). The number of significant components was determined by cross-validation [36].

The enantiomeric compositions of sabinene in the root phloem of tree I and in the trunk xylem of tree IV could not be precisely determined because of the small amounts of sabinene present in these tissues. Thus, these values are not included in Fig. 1. For use in the PCA, however, these two values were estimated partly on the basis of the gas chromatograms and partly on the basis of the values determined for tissues within the same tree that are known to exhibit similar enantiomeric compositions.

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