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Gas-liquid chromatography of terpenes. Part XVI. The volatile oil of the leaves of *Juniperus ashei* Buchholz[1]

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The major components of the leaf oil of the Ashe juniper were found to be *d*-camphor (42.1%), *d*-bornyl acetate (22.5%), *d*-limonene (8.4%), tricyclene (4.8%), *d*-camphene (4.4%), *d*-borneol (2.9%), *p*-cymene (2.8%), *d*-myrcene (1.8%), *d*-α-pinene (1.7%), and *d*-camphene hydrate (1.5%). This appears to be the first time that the latter alcohol has been isolated from a natural source. Smaller amounts of linalool, carvone, elemol, and traces of *trans*-2-methyl-6-methylene-3,7-octadien-2-ol were also identified. Several alcohols having terminal methylene groups were isolated in trace amounts.

The monoterpenes found in this oil are not typical for the genus *Juniperus* and this result offers a unique chemical approach to the study of introgression of the Ashe juniper with other juniper species.

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Introduction

The Ashe juniper, *Juniperus ashei* Buchholz, is a member of the section Sabina of the genus *Juniperus* which occurs in a rather limited area of Texas and Oklahoma (1). Its distribution, morphology, and introgression with adjacent juniper species, including *J. virginiana*, *J. monosperma*, and *J. pinchoti*, were studied by Hall (2). It has been shown in recent years that chemical components found in related or supposedly related plants may give information which supplements classical taxonomic data (3, 4). Von Rudloff has shown that the distribution pattern of the terpenes found in the leaf oils of spruce species (5, 6), and possibly also of juniper species (7, 8), may be a useful tool in chemosystematic studies. To carry out such a chemosystematic investigation of the Ashe juniper, and its introgression with *J. virginiana* (9), the chemical composition of its leaf oil was required. A search of the literature failed to yield any reference to chemical analyses of this species, although its wood oil is used in the production of cedarwood oil in areas where the red cedar, *J. virginiana*, is scarce or absent (10).

The analysis of the leaf oils of several juniper species by means of gas–liquid chromatography (g.l.c.) was described earlier (7, 8, 11). However, it has since become apparent that quantitative differences may be significant in studies of introgression or hybridization (5, 12). Hence, improvement of the quantitative results would be desirable as determination of peak areas by the triangulation method or similar techniques can be subject to comparatively large errors (13), especially in partially resolved or minor peaks. A digital computer was employed in this study to obtain such improvement.

It was found that in spruce there exists considerable variation in the quantitative leaf oil composition from one leaf to another, and that several branches had to be collected at different heights of a tree to give a representative leaf oil sample (5). Hence, the direct injection technique (14) was employed to determine what variation, if any, may be encountered in going from one leaf to another and from the green to the brown portions of the scaly leaves of the Ashe juniper.

Experimental

The g.l.c. analyses were carried out with an F & M model 500 instrument (linear temperature programming, thermal conductivity detector: F & M Scientific Instrument Inc.) and a unit of conventional design (isothermal operation). An Aerograph model A-700 gas chromatograph (Wilkens Instrument and Research Inc.) was used for preparative g.l.c. and the experiments employing direct injection (14) were carried out with an Aerograph model 695 inductor and an Aerograph model 600-D gas chromatograph (flame-ionization detector). Integration and summation of the area under each peak was achieved by means of an Aerograph model 471 digital integrator. Helium was used as carrier gas and flow rates were measured by the soap bubble method. Retention times were measured from the time of injection to the initial emergence of the peaks (15). The packing of the g.l.c. columns was prepared by the tray method and columns were packed by means of mild mechanical vibration (excessive stirring and vibration was avoided to minimize fracture of the solid support). The following liquid phases were employed: polyethylene glycol (Carbowax 20M, PEG; 5% and 15% on Gaschrom P, 60–80 mesh); silicone gum polymer (SE-30, 5% on silanized Anakrom ABS,

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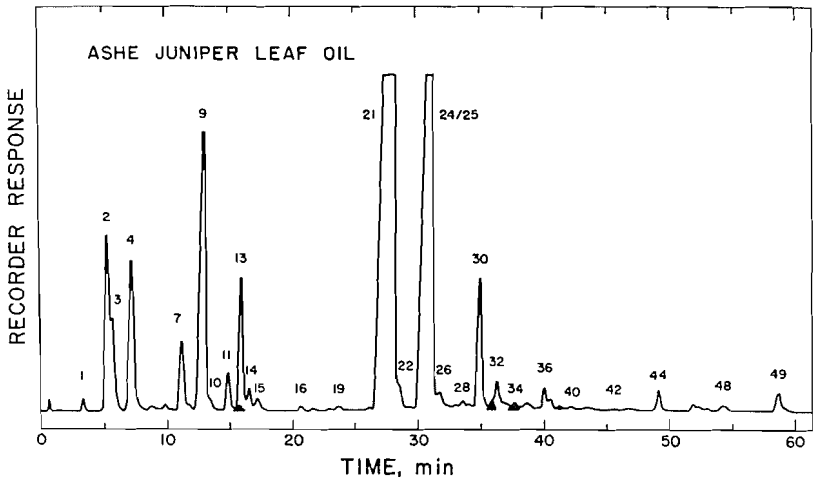

FIG. 1. Gas chromatogram of the leaf oil of *Juniperus ashei* Buchholz. (5% Polyethylene glycol 350 × 0.4 cm column, 50° to 215° at 2.9°/min). Identities of the individual peaks are shown in Table I.

70–80 mesh); fluorinated silicone polymer (QF-1, 5% on Anakrom ABS and 15% on Gaschrom P); rapeseed oil (10% on Gaschrom P); ethylene glycol *bis*propionitrile (15% on Gaschrom P); and a C-modified polyphenyl ether (Monsanto type MCS-562, Monsanto Company, St. Louis, Missouri, 15% on Gaschrom P).

Figure 1 shows a typical gas chromatogram of the leaf oil as obtained in a temperature programmed run (50 to 215° at 2.9° at min) on the 5% PEG (350 × 0.4 cm O.D.) column. The peak numbering used throughout this study is the sequence of elution on this column. In the fractionation procedures the composition of the resulting fractions was determined on this column. The purity of individual components was checked on this, as well as the other analytical columns.

Individual components were isolated by hand collection in glass or teflon tubes (externally cooled with ice or solid carbon dioxide) or in the special collectors of the Aerograph A-700 unit. Fractions which were submitted to nuclear magnetic resonance analysis (n.m.r.) were collected in carbon tetrachloride (0.5 ml). The n.m.r. spectra were recorded with the aid of a Varian HA-100 (100 Hz) spectrometer, using tetramethylsilane as internal standard. Infrared spectra (i.r.) were recorded as films on sodium chloride plates with a Perkin-Elmer model 21 double beam spectrophotometer.

Plant Material and Recovery of Leaf Oil

The foliage (20 kg) of *J. ashei* collected near Austin, Texas, by R. Irving and identified by Prof. B. L. Turner, was steam-distilled in a conventional apparatus. The distillate was extracted with methylene chloride and, after drying, the solvent was removed by distillation; yield 1.1%, $[\alpha]_D^{22}$ +37.3° (*c*, 12.7; $CHCl_3$), n_D^{22} 1.4687. Leaf samples of 15 to 30 g were sent in polyethylene bags from Austin and small scale distillation was carried out as described earlier (12). During the steam-distillation camphor, m.p. 160–175° (sealed tube), $[\alpha]_D^{23}$ +38.0 (*c*, 5.3; $CHCl_3$), crystallized in the condenser of the distillation apparatus. Its identity was confirmed by comparison of the i.r. and n.m.r. spectra with those of an authentic specimen.

Prefractionation

Aliquots of the oil (10 g) were chromatographed on modified silicic acid (120 g) as described previously (3, 5). Elution with petrol (b.p. 40–50°; 500 ml) gave a hydrocarbon fraction *a* (2.4 g, after distilling the solvent (12)) free of oxygenated terpenes (i.r., g.l.c.). A mid-fraction *b* (5.5 g) was obtained by subsequent elution with methylene chloride (400 ml) and a more polar fraction *c* (2.1 g) by elution with methanol (400 ml). The first two fractions could be used as such in preparative g.l.c., but the polar fraction was too complex. It was further fractionated on a 200 × 0.9 cm O.D. QF-1 (15%) column (nonlinear temperature programming from 80 to 200°, 100 µl aliquots) into an alcohol fraction, a mixed fraction, and 3 carbonyl fractions. Final separation of these fractions, as well as the hydrocarbon and mid-fraction, was carried out on 200 × 0.6 O.D. PEG (15%) or MCS-562 columns. The purity of each fraction isolated was tested on analytical g.l.c. columns after recording their i.r. and n.m.r. spectra, and when sufficient material could be isolated the refractive index and optical rotation were determined.

Individual Components

Fractionation of the hydrocarbon fraction *a* on a 200 × 0.6 cm O.D. MCS-562 column operated at 100° resulted in the isolation of fairly pure tricyclene (peak 2), α-pinene (peak 3), camphene (peak 4), myrcene (peak 7), limonene (peak 9), and *p*-cymene (peak 13). The optical rotations of the isolated fractions are shown in Table I. The identity of peaks 5, 6, 8, 10, 11, and 14 could be established only tentatively as β-pinene, 3-carene, α-phellandrene, β-phellandrene, γ-terpinene, and terpinolene respectively by comparison of retention data. Peak 1 appeared to be residual methylene chloride from the extractions of the steam-distilled oil. No sesquiterpene hydrocarbons could be isolated, but trace amounts of peaks 31, 34, and 38 were recorded. Retention times

TABLE I

Percentage composition of the leaf oil of *Juniperus ashei* Buchholz.

Peak No.	Compound*	$[\alpha]_D^{25}$	%
1	(Methylene chloride)	—	0.2
2	Tricyclene	(+1.0°)	4.8
3	α-Pinene	+49.2°	1.7
4	Camphene	+21.0°	4.4
5	(β-Pinene)	—	0.1
6	(3-Carene)	—	0.1
7	Myrcene	+4.8°	1.8
8	(α-Phellandrene)	—	0.1
9	Limonene	+94.9°	8.4
10	(β-Phellandrene)	—	0.2
11	(γ-Terpinene)	—	0.8
12	Unidentified	—	0.1
13	*p*-Cymene	0°	2.8
14	(Terpinolene)	—	0.4
15	Unidentified	—	0.2
16	Unidentified	—	0.1
17	Unidentified	—	trace
18	Unidentified	—	trace
19	Unidentified	—	0.1
20	(Methyl nonanone)	—	trace
21	Camphor	+38.0°	42.1
22	Linalool	—	0.4
23	Unidentified	—	trace
24	Bornyl acetate	+35.0°	22.5
25	Camphene hydrate	+ve	1.5
26	*trans*-2-methyl-6-methylene-3,7-octadien-2-ol	—	0.4
27	Unidentified	—	trace
28	Alcohol B	—	0.1
29	Unidentified	—	trace
30	Borneol	+34.0°	2.9
31	(Bisabolene)	—	0.1
32	Carvone	+ve	0.6
33	Unidentified acetate		0.1
34	(δ-Cadinene)		0.1
35	Alcohol C		0.1
36	Alcohol D		0.4
37	Aromatic alcohol		0.2
38	(Calamenene)		trace
39–43	Unidentified		traces
44	Elemol		0.5
45	Unidentified		0.2
46	Unidentified		0.1
47	Unidentified		trace
48	Unknown ester		0.8

*Names in parentheses refer to tentatively identified compounds.

suggest that these could be bisabolene, a cadinene isomer, and calamenene (16).

The mid-fraction *b* contained only two major and one minor component. Camphor (peak 21), m.p. 160–170°, $[\alpha]_D^{23}$ +35.05° (*c*, 5.1; $CHCl_3$) and bornyl acetate (peak 24), n_D^{25} 1.4619, $[\alpha]_D^{25}$ +34.0° (*c*, 4.8; $CHCl_3$) were readily isolated by fractionation on a 150 × 0.9 cm O.D. QF-1 (15%) column (100 to 200°). The third component, peak 49, was obtained in low yield after many repetitive injections. Its i.r. spectrum had absorption bands at 1735 cm^{-1} (m) ($>$C=O); 3100 (w), 1640 (w), and 912 cm^{-1} (m) with inflexion near 895 cm^{-1} ($>$C=CH_2), and a multiplet in the 1390–1365 cm^{-1} which permitted no conclusion about the presence of isopropyl or gem dimethyl groups. Strong bands were also recorded at 2940, 2880, 1262, 1208, 1120, 1095, 1074, and 1025 cm^{-1} and bands of medium intensity at 1420–1395, 1041, 990, 965, 955, 835, and 795 cm^{-1}. The n.m.r. spectrum showed four single proton signals, viz. three quartets centered near τ5.22, 4.98, and 4.25 with couplings of δ2 and 11, 2 and 17.5, and 17.5 and 11 Hz respectively (—CH=CH_2), and a quartet centered near τ6.65 with couplings of δ6.5 and 13 Hz ($>$CHOR). Five three-proton signals occurred at τ8.74, 8.79, 9.11, 9.18, and 9.20 and less well defined signals in the τ8.0 to 9.1 range. The latter made the integration of the three-proton signals somewhat inaccurate.

The various oxygenated fractions obtained in the prefractionation (see above) were resolved on a 180 × 0.6 cm O.D. PEG (15%) column operated either isothermally (120 and 140°) or programmed from 120 to 200° at 4°/min. The alcohol fraction was resolved into 5 components which had spectral properties corresponding to linalool (peak 22), camphene hydrate (peak 25), *trans*-2-methyl-6-methylene-3,7-octadien-2-ol (peak 26) (17), and unknown alcohol (*a*), with a terminal methylene group (peak 28) and borneol (peak 30). Terpinen-4-ol, α-terpineol, and isoborneol were not present. The identity of camphene hydrate (m.p. 145–149°) was confirmed by synthesis (18) (m.p. 147–149°) and co-injection on all analytical columns, when identical retention times were recorded.

The mixed fraction was found to contain a trace of a methyl ketone (possible methyl nonanone; peak 20), camphor (peak 21), camphene hydrate (peak 25), the unidentified alcohol *a* (peak 28), borneol (peak 30), three unidentified alcohols (*b*, *c*, and *d*; peaks 33, 35, and 36), and an unknown aromatic alcohol (peak 37). The methyl ketone (peak 20) had the retention time of 2-decanone, but the weak infrared spectrum did not show the band near 700 cm^{-1} expected for an aliphatic chain of more than four carbon atoms. Only alcohol *d* (peak 36) was obtained in sufficient amount to record a satisfactory i.r. spectrum with strong bands near 3360, 2980–2880, 1642, 1450–1440, 1375, 1255, 1163, 1151, 1050, 1025, 955, 937, 880 (inflexion near 898), and 810 (inflexion near 803) cm^{-1} and weaker ones near 3090, 1212, 1110, 1090, 858–850, and 715 cm^{-1}. Only a weak n.m.r. spectrum with signals near τ8.30, 8.73, 8.78, 9.00, 9.12, and 9.18 and very weak ones near τ4.2, 5.0, and 5.2 could be recognized. The infrared spectrum of component 37 showed absorption bands characteristic of a hydroxyl group (3370 cm^{-1}) and an aromatic ring (1515 cm^{-1}). The other alcohols were not obtained in sufficient amount for further characterization. The i.r. spectrum of the aromatic alcohol differed from those of *p*-cuminyl alcohol, carvacrol, thymol, and the common ethers of the eugenol type. The three carbonyl fractions consisted mainly of camphor, bornyl acetate, and carvone respectively. Each compound was purified by g.l.c. on the 150 × 0.6 cm O.D. PEG (15%) column. The amount of purified carvone isolated was not sufficient for accurate determination of the optical rotation. The values for camphor (m.p. 165–175°) and bornyl acetate were +37.6° and +35.0° respectively. The third carbonyl fraction contained some higher boiling

components in small amounts, but these could not be obtained in sufficient amount for further identification.

Single Leaf Injection

Leaf portions (5–20 mg) were placed into the boat-shaped cavity of the Aerograph inductor and were injected for 15 to 30 s into the pyro-oven (at 250°) with the helium flow turned on 5 s after the injection. The released volatiles were analyzed on a 180 × 0.3 cm O.D. SE-30 (5% on Anakron ABS) column. For comparative purposes the leaf oil (0.1 μl) was enclosed in indium tubing (0.02 in. I.D.) and injected in the same manner. The green and brown portions of the same leaf stalks from the same and neighboring branchlets gave the same qualitative chromatogram. The quantitative differences were relatively small in the monoterpene range (±1 to 5%), but were more pronounced in the sesquiterpene range (±5 to 20%). When the bark from a branchlet was injected in the same manner, virtually the same chromatograms were obtained, but the amount of sequiterpenes was invariably low.

Results and Discussion

The quantitative data, as calculated from the gas chromatograms obtained with the PEG, SE-30, and MCS-562 columns are shown in Table I. The peak numbering is that of the sequence of elution on the PEG column, which provided the best overall resolution (see Fig. 1). The accuracy of determining the percentage composition with the digital integrator was ±0.1 unit % on well resolved peaks and ±0.1 to 0.5 unit % on poorly resolved peaks or when comparing data obtained on different columns. This is a noticeable improvement over the accuracy obtained previously (6, 7) by the triangulation method (13).

The identification of the major components *d*-camphor (peak 21) and *d*-bornyl acetate presented no difficulties and tricyclene (peak 2), *d*-α-pinene (peak 3), *d*-camphene (peak 4), *d*-myrcene (peak 7), *d*-limonene (peak 9), *p*-cymene (peak 13), linalool (peak 22), *d*-borneol (peak 30), carvone (peak 32), and elemol (peak 44), were also obtained in sufficient amounts to permit positive identification. Camphene hydrate (peak 25) was only identified after synthesizing this tertiary alcohol (18) and comparing g.l.c. and spectral properties. Under all g.l.c. conditions normally employed in conifer leaf analysis this labile alcohol was found to form from 10 to 30% camphene. This facile dehydration was already observed by Ashan (18) and may account for camphene hydrate not having been found in nature before. The dehydration during g.l.c. leads to a single peak with the retention time of camphene and this may serve as a convenient means of detecting camphene hydrate. The retention time of camphene hydrate is similar to that of 4-terpinenol (PEG, MCS-562, QF-1, polyester columns) except on the SE-30 column, where it coincides with isoborneol. In oils which contain these two alcohols it is difficult to distinguish camphene hydrate. Thus, alcohol **1** of red and black spruce leaf oil (6), was found to be a mixture of camphene hydrate and 4-terpinenol. If camphor is present in large amounts, it is not clearly resolved from camphene hydrate on the latter column.

Noteworthy is the finding of small amounts of alcohols all having terminal methylene groups (peaks 27, 29, 35, and 36). Of these, alcohol *a* (peak 27) had the same spectral properties of *trans*-2-methyl-6-methylene-3,7-octadien-2-ol, which was recently isolated from the frass produced by *Ips confucus* feeding on ponderosa pine (17). Silverstein *et al.* (17) report this alcohol as well as 2-methyl-6-methylene-7-octen-4-ol, to attract this beetle to the pine. Hence the alcohols found in the leaf oil of *J. ashei* may also be insect attractants. Component 36 had the retention time and spectral properties expected for *cis*- or *trans*-carveol, but neither isomer was available for comparison.

The most interesting unidentified compound from a chemical point of view appears to be component 49. The i.r. suggest it is an acetate or ester (1735, 1262 cm^{-1}) with a terminal methylene group of the type —CH=CH_2 (3100, 1640, 912–895, and possibly 990 cm^{-1}). This is confirmed by the n.m.r. spectrum. The single proton signals (quartets) near τ 5.22, 4.98, and 4.25 are typical of the ABX system in —CH_2—C(CH_3)OH—CH=CH_2 as found in nerolidol and manool (19). The presence of a secondary alkoxy group is confirmed by the single proton signal near τ6.65 and since this is a quartet it must have an adjacent methyl group (—CH(CH_3)—OCOR). The five methyl signals in the range τ8.74 to 9.19 are more difficult to interpret but these signals as well as the fingerprint region of the i.r. spectrum differed markedly from those of nerolidol, nerolidyl acetate, manool (19), manoyl oxide, and 13-epimanoyl oxide (20). Hence, component 49 must have a structure different from the terpenes normally found in the volatile oils of conifers.

The separation and isolation of the trace com-

ponents of the oil of *J. ashei* presented difficulties owing to overlaps which could not be resolved and some of these components could only be identified tentatively by comparison of retention data. Thus, the three trace components 31, 34, and 38 which are obtained in the hydrocarbon fraction may be bisabolene, a candinene isomer, and calamenene respectively. The former two sesquiterpenes were identified in the leaf oils of spruces (6), with which the leaf oil of *J. ashei* shows remarkable similarities. In fact, all of the components except elemol of this oil are not typical of the members of the genus *Juniperus* as well as other members of the family Cupressaceae. This finding makes the leaf oil of the Ashe juniper well suited for chemosystematic studies, especially with regard to introgression with related juniper species. The results obtained with direct injection of small portions of the leaves of *J. ashei* indicate that no significant differences exist between the green and brown portions of the leaf stalks. Hence, for chemosystematic studies the branchlets may be steam-distilled without danger of contamination of the leaf oil. These findings are being employed in a detailed investigation of the introgression of *J. ashei* with *J. virginiana* (9).

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