Phytochemistry, 1972, Vol. 11, pp. 1455 to 1460. Pergamon Press. Printed in England.

# MONOTERPENE AND SESQUITERPENE HYDROCARBONS OF *PINUS MUGO*

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(*Received* 3 *August* 1971, *in revised form* 24 *September* 1971)

**Abstract**—The monoterpene and sesquiterpene hydrocarbons of a commercial sample of *Pinus mugo* essential oil were isolated by a combination of techniques involving chromatography, distillation and preparative GLC. Eleven monoterpenes were identified by their IR spectra and TLC of their mercuric acetate adducts. IR and mass spectroscopy were employed to identify the following seven sesquiterpenes: $\beta$-caryophyllene, $\alpha$-humulene, $\gamma$-muurolene, $\alpha$-muurolene, $\alpha+\beta$-selinene and $\gamma$-cadinene. Bornyl acetate was also present as a contaminant among these hydrocarbons.

## INTRODUCTION

DESPITE its importance in pharmaceutical preparations, the essential oil of *Pinus mugo* Turra (*P. montana* Mill.) has not been extensively investigated. Early studies[1] are very incomplete. Most recent papers deal with the monoterpene fraction only, studied by TLC[2] and a combination of TLC and GLC,[3] but the sesquiterpene hydrocarbons have not been considered so far.

This study was undertaken to provide further basic information on the *P. mugo* essential oil hydrocarbons. The gas chromatographic survey of the whole oil reveals an unusual complexity. Therefore, in order to allow collection of pure individual components by preparative GLC, a set of preliminary separations, consisting in column chromatography to remove oxygenated compounds from hydrocarbons and high vacuum distillation to isolate monoterpene from sesquiterpene hydrocarbons, was employed. Most of the constituents were identified by IR spectroscopy. Moreover a parallel TLC procedure based on mercuric acetate adduction, was developed for monoterpene hydrocarbons.

## RESULTS

### *Preliminary Separations*

The hydrocarbon fraction was separated from the more polar constituents of the oil by column chromatography on silica gel G (Merck) using *n*-hexane as eluent. After removal of *n*-hexane, the subsequent separation of sesquiterpenes from monoterpenes was performed

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[1] E. GUENTHER, *The Essential Oils*, Vol. 6, p. 243, Van Nostrand, New York (1952).

[2] C. ZANINI, A. DAL POZZO and A. DANSI, *Boll. Chim. Farm.* **100**, 83 (1961).

[3] A. MARTINEK, *Planta Med.* **17**, 201 (1969).

by high vacuum distillation at room temperature. The hydrocarbons were cooled to −190° (liquid $N_2$), the still evacuated to $\simeq 5 \times 10^{-3}$ mm Hg and isolated from the vacuum line. Then the hydrocarbons were heated to 25° and the monoterpenes allowed to distil into a finger dipped in liquid $N_2$, while the sesquiterpenes remained in the distillation flask. Distillation was greatly accelerated by stirring. To improve separation, the process was repeated twice. Monitoring gas chromatograms (Figs. 1a–c) show a very good separation between mono- and sesquiterpenes. Comparison of the GLC curves before and after preliminary separations, indicated that the monoterpenes and the sesquiterpenes suffered no apparent decomposition.

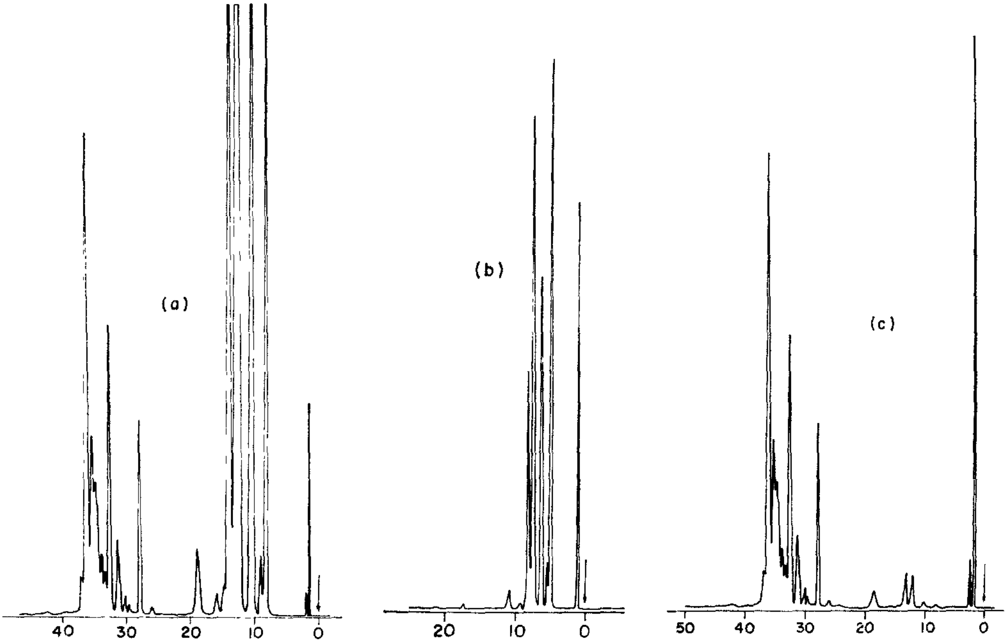

Fig. 1. Low resolution chromatograms of: (a) hydrocarbon fractionbefore distillation, (b) monoterpenes, (c) sesquiterpenes (column type No. 1).

*Monoterpene Hydrocarbons*

In a recent paper,[3] GLC retention times were used for the identification of the monoterpene hydrocarbons in *P. mugo*: since some of the components were not completely resolved, TLC was also used. To confirm these findings, we have identified the monoterpenes, isolated by preparative GLC, mainly by IR spectroscopy.

Several packed columns were tried for the preliminary survey of the monoterpene fraction. Three preparative columns type I and two type II employed in series provided better resolution than a typical analytical Carbowax 20M column (No. 2: isoth. 80°), allowing separation of twelve peaks by operating isothermally at 120° (Fig. 2a and b). With preparative scale injections ($\simeq$100 $\mu$l) it was still possible to collect seven peaks, i.e. 1, 2, 3 + 4 + 5, 7, 8, 11, 12, but not 6, 9 and 10 owing to their low concentration. The collected peaks 1, 2, 7, 8, 11, 12, monitored on the analytical columns No. 2, 3, 4, were found to be

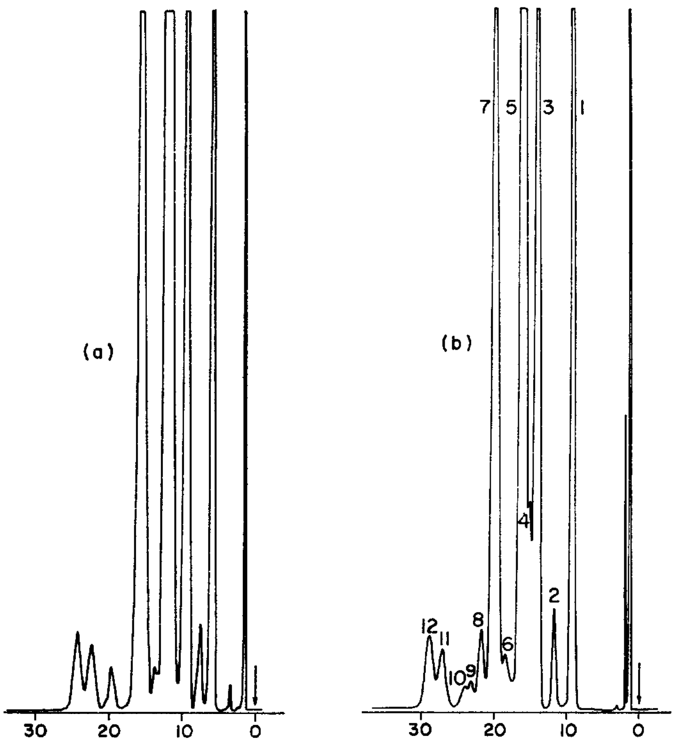

Fig. 2(a). Fig. 2(b).

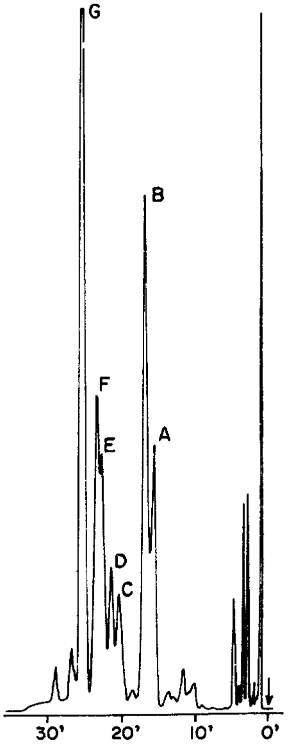

Fig. 3.

Fig. 2. Gas chromatographic separation of monoterpene hydrocarbons.
(a) on Carbowax 20M, (b) on the column series (see text): 1, α-pinene; 2, camphene; 3, β-pinene; 4, myrcene; 5, 3-carene; 6, α-terpinene; 7, limonene; 8, β-phellandrene; 9, γ-terpinene; 10, not identified; 11, p-cymene; 12, terpinolene.

Fig. 3. Gas chromatogram of sesquiterpene hydrocarbon fraction.
A, bornyl acetate; B, β-caryophyllene; C, α-humulene; D, γ-muurolene; E, α-muurolene; F, α + β-selinene; G, γ-cadinene.

sufficiently pure (>95%) and identified by their IR spectra as α-pinene, camphene, limonene, β-phellandrene, *p*-cymene and terpinolene respectively.[4]

Products corresponding to the peaks 3, 4 and 5, not resolved in this preparative procedure, were collected together and afterwards rechromatographed isothermally at 100° on a series of three type I columns. The two major peaks, 3 and 5, were then collected and, after GLC purity check, identified by their IR spectra as β-pinene and 3-carene respectively. The minor peak 4, not resolved under these conditions, seems to behave like myrcene. Evidence for this monoterpene will be given during the parallel TLC procedure described later. On the other hand the identification of the trace components represented by peaks 6 and 9 was achieved by GLC retention data (columns No. 2, 4, 5). So peak 6 was recognized as α-terpinene and peak 9 as γ-terpinene. The pure standards for retention measurements were obtained by GLC purification of the isomerization products of (+)-limonene on silica gel, according to Hunter.[5]

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[4] B. M. Mitzner, E. T. Theimer and S. K. Freeman, *Appl. Spectry.* **19**, 169 (1965).

[5] G. L. K. Hunter and W. B. Brogden, Jr., *J. Org. Chem.* **28**, 1679 (1963).

### *TLC of Mercuric Acetate-monoterpene Hydrocarbons Adducts*

In order to avoid the troubles involved with the use of $AgNO_3$ impregnated layers, we have developed a method based on mercuric acetate adducts prepared before chromatography in contrast to the silver complexes which are formed on the plates during chromatography. The adducts, obtained by the same procedure[6,7,8] for lipids, were very stable compounds. No decomposition was observed after 3 months. To improve separation, diverging lines (angle 30°) were drawn with a pin on the layers (silica gel G, Merck, 20 × 20 cm, thickness 350 $\mu$m), according to Stahl. A 15-cm run was then accomplished in 90 min using a mixture of $Me_2CO$–MeOH–AcOH (75:25:1·5) as eluent in presaturated chambers. The $CHCl_3$ solution of the adducts was applied (10–20 $\mu$l), as a 0·5-cm band, to the starting line situated at 3 cm from the triangle apex. After visualization with diphenylcarbazone, the spots were identified by comparison of their $R_f$ values with those of authentic monoterpene adducts developed simultaneously in parallel runs. For this purpose two adjacent triangles (10 cm wide at the top, 20 cm in height) were drawn in the 20 × 20 cm layer. Due to the great sensitivity of the method and to the fact that some terpenoids may give more than one spot,[8,9] very pure specimens of standard adducts were prepared using GLC purity grade monoterpenes. Therefore, in order to save time and avoid too many manipulations, the standard adducts were obtained by bubbling each monoterpene eluted from the gas chromatograph directly into the adducting solution. The mobilities relative to $\gamma$-terpinene (1·0) were camphene (0·88), $\alpha$- and $\beta$-pinene (0·75), limonene (0·65, 0·59, 0·53), 3-carene and $\beta$-phellandrene (0·41) and myrcene (0·37). Good resolution of $\gamma$-terpinene, camphene and myrcene was found, while poor separation was observed for the $\alpha$-pinene, $\beta$-pinene and 3-carene, $\beta$-phellandrene couples. The existence of three well resolved spots was the remarkable feature of limonene, whereas *p*-cymene, $\alpha$-terpinene and terpinolene did not produce adducts.

### *Sesquiterpene Hydrocarbons*

Very little is known about the composition of the sesquiterpene hydrocarbon fraction of *P. mugo* and only the presence of a 'cadinene' was reported by early investigators.[1] The fraction isolated by us with the techniques described previously, represents a minor part ($\simeq$8%) of the whole oil. The best gas chromatographic separation (Fig. 3) was obtained using column No. 6 (oven temp.: 6 min at 110→160°, 2°/min). The peaks A, B, C, D, E, F, G constitute the subject of this investigation, while the minor peaks were neglected.

In order to confirm the sesquiterpenic nature of the fraction, mass spectra of each peak were recorded before isolating the components. The GLC/MS instrument, having an electron beam energy of 70 eV, was fitted with the column No. 7 (oven temp.: 12 min at 90→175°, 4°/min), so that a GLC curve similar to that of Fig. 3 was achieved.

Molecular weights (MW) of the materials corresponding to B, C, D, E, F, G bands were all in agreement with $C_{15}H_{24}$ formula (204), while for the peak A a 196 MW was observed.

Identification from MS data was unsuccessful. In fact, according to Hill,[10] it was evident that, in the case of sesquiterpenes, the assignment of the molecular structure is a

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[6] E. JANTZEN and H. ANDREAS, *Chem. Ber.* **92**, 1427 (1959).
[7] E. STAHL, *Thin-Layer Chromatography*, p. 403, Springer-Verlag, New York (1969).
[8] H. K. MANGOLD and R. KAMMERECK, *Chem. & Ind.* 1032 (1961).
[9] L. SYPER, *Dissertationes Pharm.* **17**, 33 (1965).
[10] H. C. HILL, R. I. REED and M. T. ROBERT-LOPEZ, *J. Chem. Soc.* **C**, 93 (1968).

matter of considerable difficulty, due to the similarity of cracking patterns. On the other hand the lack of a large variety of pure sesquiterpene standards did not allow identification on the field by MS comparison. Therefore we returned to the combined use of GLC and IR spectroscopy and a two-step procedure was set up consisting first of a large scale preparative process for coarse separation, followed by a micropreparative refinement carried out on 6 mm analytical columns.

Two type No. I plus one type No. III columns were used for the coarse separation, operating isothermally at 155°. The resolution obtained allowed the collection of the peaks A, B and G, while peaks C, D and peaks E, F were collected together. After GLC purity check (columns No. 6, 8, 9), IR spectra of the materials corresponding to the peaks A, B and G were registered. It was shown that the peak A was due to bornyl acetate, in agreement with the MW 196 measured by MS. This oxygenated compound was not completely removed from hydrocarbons during preliminary separation on silica gel, probably because of its abundance in whole oil. The sesquiterpene represented by the peak B was identified as $\beta$-caryophyllene by comparison of its IR spectrum with that published by Wenninger.[11] The material corresponding to the peak G is the major sesquiterpenic constituent of the oil. Its IR spectrum was shown to be identical with that of $\gamma$-cadinene,[11] thus confirming the 'cadinene' reported by early investigators.

The micropreparative procedure was applied to peaks C, D previously unresolved, using column No. 8 (oven temp.: 110→165°, 2°/min). The separated individual constituents C and D were collected by condensation in small glass vials (1·8 mm i.d., 20 cm long) kept at room temp. After GLC purity check (columns No. 6, 8, 9) their IR spectra, obtained using a microcell suitable for submicroliter samples,[12] were found to be identical with those reported by Wenninger for $\alpha$-humulene (C)[11] and $\gamma$-muurolene (D).[13]

TABLE 1. GAS CHROMATOGRAPHIC COLUMNS

| No. | Dimensions | Packing |
|---|---|---|
| 1 | 3 m × 6 mm o.d. (coiled glass) | 5% Silicone grease DC on Chromosorb G AW-DMCS, 60/80 mesh |
| 2 | 2·75 m ,, ,, ,, | 10% Carbowax 20M on Anakrom ABS, 80/90 mesh |
| 3 | ,, ,, ,, ,, | 20% Apiezon L on Chromosorb A, 60/80 mesh |
| 4 | 3·7 m ,, ,, ,, | 10% Silicone XE-60 on Anakrom ABS, 80/90 mesh |
| 5 | 3 m ,, ,, ,, | 20% Carbowax 20M on Chromosorb A, 60/80 mesh |
| 6 | 2·75 m ,, ,, ,, | 5% Carbowax 20M on Anakrom ABS, 80/90 mesh |
| 7 | 3 m × 3 mm o.d. (stainless steel) | ,, ,, ,, ,, |
| 8 | 2·75 m × 6 mm o.d. (coiled glass) | 5% Apiezon L on Anakrom ABS, 80/90 mesh |
| 9 | 2·75 m × 6 mm o.d. (coiled glass) | 5% Silicone XE-60 on Anakrom ABS, 80/90 mesh |
| I | 1 m × 9 mm o.d. (inox, U shaped) | 20% Carbowax 20M on Chromosorb A, 60/80 mesh |
| II | 1 m × 9 mm o.d. (inox, U shaped) | 20% Silicone XE-60 on Chromosorb A, 60/80 mesh |
| III | 1 m × 9 mm o.d. (inox, U shaped) | 5% Silicone XE-60 on Anakrom ABS, 70/80 mesh |

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[11] J. A. WENNINGER, R. L. YATES and M. DOLINSKY, *J. Ass. Offic. Anal. Chem.* **50**, 1313 (1967).

[12] *GC Newsletter* Vol. 3, No. 1, Perkin–Elmer Corp. Norwalk, Conn. U.S.A. (1967).

[13] J. A. WENNINGER and R. L. YATES, *J. Ass. Offic. Anal. Chem.* **53**, 949 (1970).

The same procedure was applied to the peaks E, F. Good separation was attained with column No. 4 (oven temp.: 90→150°, 2°/min). The substance constituting peak E was pure (columns No. 4, 6, 8) and was identified as α-muurolene by comparison of its IR spectrum with that reported by Wenninger.[13]

The material corresponding to peak F was found to be a mixture. In fact the IR spectrum was indicative of two selinene isomers,[11] α and β, with comparable concentrations as estimated from bands intensity. Further, by a TLC run carried out on silica gel (Merck, $GF_{254}$) with *n*-hexane as eluent, two well resolved spots were found (UV light). Attempts to resolve the two isomers by GLC were ineffective with our columns.

## EXPERIMENTAL

*Apparatus.* Preparative scale GLC utilized a Perkin–Elmer Mod. F21 instrument with FID detector. Analytical work was performed by the same instrument fitted with 6 mm coiled glass columns. Mass spectra (MS) were recorded on a Perkin–Elmer Mod. 270 B (GLC/MS). Gas chromatographic columns used throughout this work are summarized in Table 1.

*Materials.* The *Pinus mugo* essential oil was obtained commercially. Our material (DAB VI suppl.) came originally from the Inn valley (Austria) and was found qualitatively in agreement with authentic samples prepared in this laboratory, from trees growing in the Arboreti Sperimentali di Vallombrosa (Istituto Sperimentale di Selvicultura, Arezzo, Italy) by steam distillation.

*Acknowledgements*—The authors are grateful to Professor L. Boniforti (Istituto Superiore di Sanità, Roma) for making the mass spectral equipment available. Thanks are also due to E. Calvani, Miss M. P. Carbognin, R. Conti, G. Gangarossa and Mrs. S. Coran for technical support.

*Key Word Index—Pinus mugo*; Pinaceae; essential oils; monoterpene; sesquiterpene.