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A SCHEME FOR THE PREPARATIVE CHROMATOGRAPHIC ISOLATION AND CONCENTRATION OF TRACE COMPONENTS IN NATURAL PRODUCTS USING 4-INCH DIAMETER COLUMNS

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SUMMARY

A general method is demonstrated for effective use of large-scale preparative gas chromatography to isolate and concentrate minor components in natural products. A gas chromatograph equipped with 4 inch O.D. preparative columns was used to collect four fractions enriched in the minor components found in a Bergamot oil sample. The mixed fractions were analyzed directly by a combination gas chromatograph–mass spectrometer, or by subsequent small-scale preparative gas chromatography and collection of individual components. The collected components were characterized by N.M.R., I.R., and mass spectrometry. With the suggested methodology, the time required for characterization of a natural product is significantly reduced, and information not accessible by other means is obtained.

The complete analysis of many natural products is economically important to both the producer and consumer of flavors and essences in which trace components may significantly alter the aroma value of these substances. For this reason, virtually all commercial users of natural oils and essences strive for a complete chemical characterization of these important raw materials. With such definitive information, materials with optimum sensory values can be produced synthetically, or the natural products can be suitably modified to compensate for differences due to the source, climate, time of harvest and other variables affecting the sensory characteristics of the substance.

Of the increasingly sophisticated analytical instruments now available, gas chromatography is the most powerful single technique for examining such complex organic mixtures. As early as 1959, FEARNS[3] made this observation which is equally valid today. Analytical gas chromatography alone provides a highly reliable quan-

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titative determination of the components in such mixtures while preparative gas chromatography offers a rapid convenient means of separating and collecting the individual sample constituents for subsequent qualitative analysis by ultraviolet, infrared, nuclear magnetic resonance or mass spectroscopy.

Trace components—present in quantities too small to be detected in the original sample mixture—can be separated and concentrated by preparative GC. This permits the qualitative identification and, if necessary, the quantitative determination of minor components that may be affecting the sensory characteristics of the natural products.

SMALL-SCALE PREPARATIVE GAS CHROMATOGRAPHY

Generally, spectral analysis requires a larger quantity of a component than can be separated and isolated from a complex mixture in a single GC run. Consequently, many workers in this field resort to GC columns with capacities only slightly greater than the common analytical column and multiple injections of larger than optimum sample volumes. The efficiency of these "small-scale" preparative systems (1/4 to 3/4-in. O. D. columns) is maintained by using columns ranging in length up to 40 ft.[4,7,9]. The capacity of these columns, without resorting to serious overload, is at best not more than 15 times that of normal analytical columns[1]. Therefore, for a product containing about 0.2 % of a component, as many as 100 repetitive runs may be required to obtain a sufficient amount of that constituent for infrared or N.M.R. analysis[2]. Because of the need to achieve the highest possible efficiency, as much as 4 h operating time may be required for each run. Consequently, more than a month of continuous operation may be necessary to obtain a sufficient amount of each important minor component for complete characterization.

Bergamot oil was selected to compare the applicability of "small" and "large" scale preparative GC systems. In these studies, conditions were adjusted to give a reasonable separation of most of the components in the oil. The chromatograms of Fig. 1A–C show the effect of variations in total sample volume on component resolution. It is particularly important to note the increased spreading of the major component peaks as the sample volume was increased from 10 μl to 30 μl. With samples of more than 30 μl, too much peak overlap occurs to make spectral identification meaningful because mixed fractions are obtained. The major components shown in the chromatogram of Fig. 1A comprise 83 % of the total sample, leaving approximately 20 components in the remaining fractions. If these major components have not been previously identified, they can be easily isolated by preparative GC for spectral tests. Most of the problems are encountered in the isolation of the minor components.

With a combination of mass spectrometry and gas chromatography, many of these minor components can be identified if they are known compounds. However, in a number of cases, nuclear magnetic resonance and/or infrared spectrometry must be used to determine the chemical structure. If other tests—such as Raman spectrometry or odor evaluation—are desired in addition to N.M.R., 10 mg or more of a sample component may be required.

Using the maximum sample size of 30 μl, 36 successive samples of Bergamot oil must be chromatographed to collect approximately 10 mg required for subsequent qualitative analyses if the component is originally present in the 1 % range. This

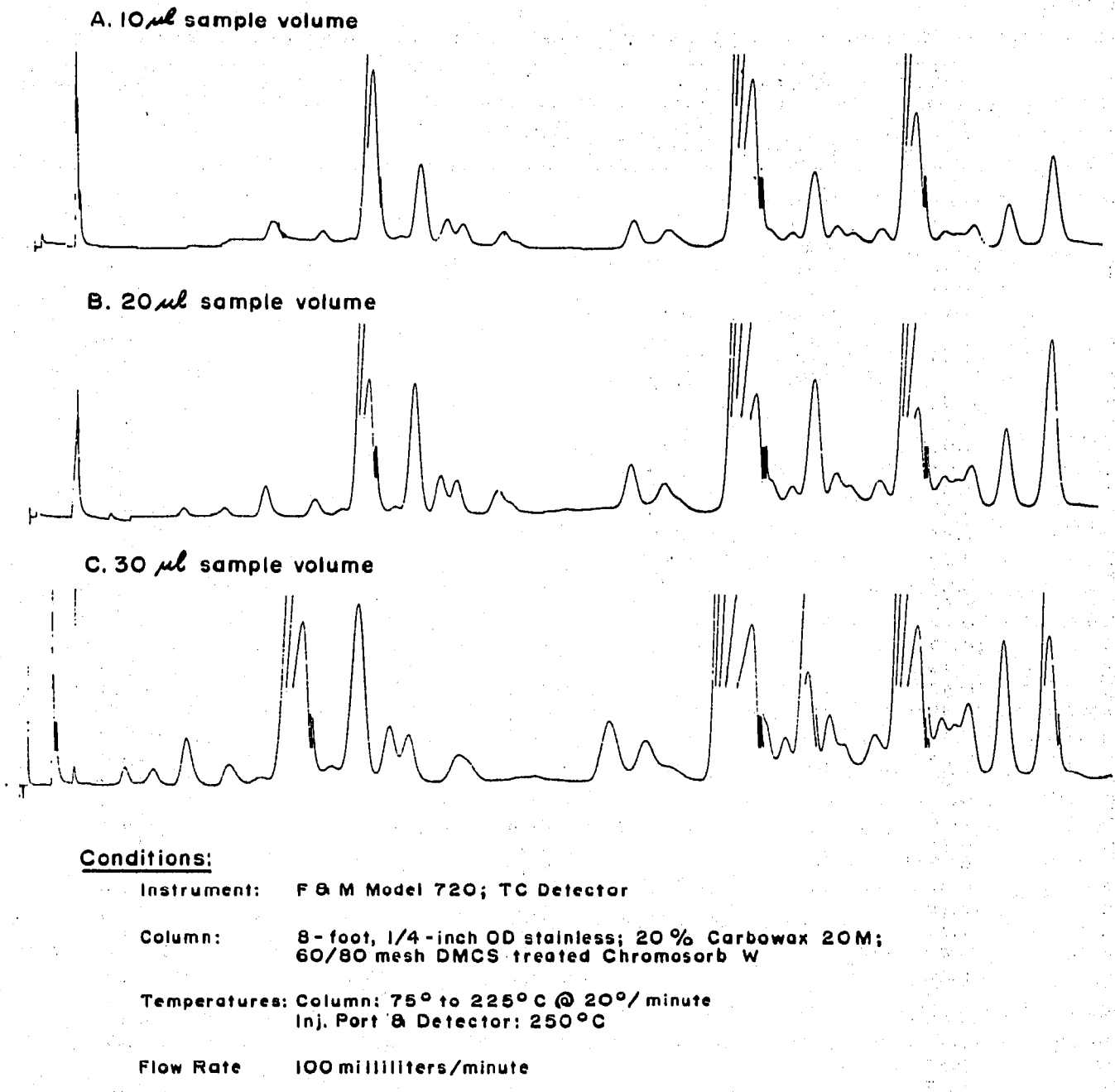

Fig. 1. Response patterns for Bergamot oil showing effect of sample size on component resolution

assumes 100% collection efficiency which is a level seldom attained with these small sample volumes. Thus, approximately one week would be required for the isolation and identification of a component at a 1% concentration level. Actually, the concentration of minor components in this sample varies from 0.1–1.4% with most below 1% (see Table I).

TABLE I

PREPARATIVE GC ENRICHMENT OF MINOR COMPONENTS IN MIXED FRACTIONS OF BERGAMOT OIL

GC peak (No.)*	*Original sample (%)*	*Prep. GC fractions*				*By-pass*	*Enrichment factor****
		I	*II*	*III*	*IV*		
		*(Relative abundance** (%))*					
1	0.15	0.70					4.6
2	0.05	0.30					6.0
3	0.15	1.20					8.0
4	0.63	10.50					16.6
5	0.02	1.00					50.0
6	0.56	6.70	0.23				12.0
7	0.04	0.90	0.03				22.0
8	13.66	75.50	19.70			17.30	5.5
9	0.17	0.70	0.57				4.1
10	0.80		13.70				17.1
11	0.53		1.20				2.3
12	0.64		3.20				5.0
13	0.61		3.60				5.9
14	0.28		1.90				6.8
15	0.57		1.30				2.3
16	0.18		0.90				5.0
17	0.36		0.61				1.7
18	0.24		1.01				4.3
19	0.22		1.10				5.0
20	0.22		—				—
21	46.80		50.30	51.70		61.20	—
22	0.49			0.30			0.6
23	0.52			1.00			1.9
24	0.55			0.24			0.6
25	0.12			0.73			6.1
26	0.53			7.00			13.2
27	0.36			1.40			3.9
28	0.47			0.18			0.3
29	0.08			0.34			4.2
30	0.35			4.50			12.9
31	0.08			0.48			6.0
32	0.21			0.22			1.0
33	0.47			0.63			1.3
34	1.17			1.30			1.1
35	22.80			20.60	45.90	21.40	—
36	0.30				1.20		4.0
37	1.03				3.20		3.2
38	1.00				4.80		4.8
39	1.41				6.50		4.6
40	0.52				6.79		13.0
41	1.07				16.80		15.7

* Chromatogram of Fig. 2.
** Based on peak area derived from chromatograms A–E of Fig. 6.
*** Relative abundance as fraction/percent in original.

LARGE-SCALE PREPARATIVE GAS CHROMATOGRAPHY

Since large-scale preparative gas chromatographs are now commercially available, the applicability of this technique to separating and collecting adequate quantities of minor components in natural products was examined. The large-scale

preparative system should serve admirably to provide concentrated fractions containing mixtures of the minor sample components. By this technique, most of the major components are separated from the minor ones. This eliminates the need to maximize the separation of the individual sample components. The concentrated fractions are then separated into individual components by means of an analytical or small-scale preparative column. By optimizing the operating conditions for each fraction, the desired separations are readily achieved with these high efficiency GC column systems.

At least two attempts to achieve large-scale separation capability are reported in the literature by HUNTER AND VELDHUIS[5] and JOHNS[6]. The former describe an instrument design capable of accommodating up to 60 ft. of 1 1/2 in. O.D. column, but the time and effort required to optimize conditions for achieving maximum separation with maximum sample volumes indicates that this is a less practical approach than the one suggested in this paper. JOHNS described the use of a number of narrow-bore columns operated in parallel to achieve the column cross-section required for large samples. Because of the difficulties encountered in matching flow and other chromatographic parameters in all columns, this technique has not found wide use.

Using high capacity 4-in. O.D. preparative columns, a sample can be readily separated into 3 to 6 mixed fractions from which the major components in the original sample have been removed. Usually, no more than a day or two of an operator's time is required to provide several milliliters, or more, of each fraction. The subsequent separation of the enriched fractions is simplified markedly because each component is present in a substantially higher proportion than in the original sample. Therefore, the need for large numbers of repetitive runs and accumulative collection of each component over a period of time is reduced to a minimum. Depending on the complexity of the original material, the time required to accumulate sufficient amounts of the sample components for characterization might be a week or possibly less. In addition, since the minor components are concentrated in this fractionation process, certain constituents originally present in amounts too low for detection can now be separated and identified. Thus, information becomes available which could not be obtained using only the small column techniques. This paper will demonstrate the use of large-scale preparative GC to separate major from minor components in Bergamot oil and thus provide concentrated fractions containing mixtures of the minor components, plus the subsequent small-scale separation of these minor components for spectral analyses. This work is intended to serve as a model for the investigation of other similar complex natural products.

EXPERIMENTAL DETAILS

Two F & M Analytical Gas Chromatographs, Model 720 and 810, as well as an F & M Model 775 Preparative GC instrument and a Perkin-Elmer Model 226 Capillary Column Gas Chromatograph were used. Operating conditions are shown on the chromatograms in Figs. 2–5. Mass spectra were obtained with a mass spectrograph directly coupled to the capillary-column instrument. For I.R. and N.M.R. measurements, individual samples were collected in appropriate trapping devices and then transferred to the appropriate spectrophotometer for analysis.

DATA EVALUATION

Prior to the preparative GC separation of the Bergamot oil, an analytical chromatogram (see Fig. 2) was obtained using a high-efficiency capillary column GC system. From this, the retention times of the known major sample components were determined. Since a packed column is used in large-scale preparative separations, a second analytical chromatogram (see Fig. 3) was obtained with a packed column which showed an efficiency comparable to that of the capillary-column system. This provided a reference for detecting any gross changes in the elution pattern.

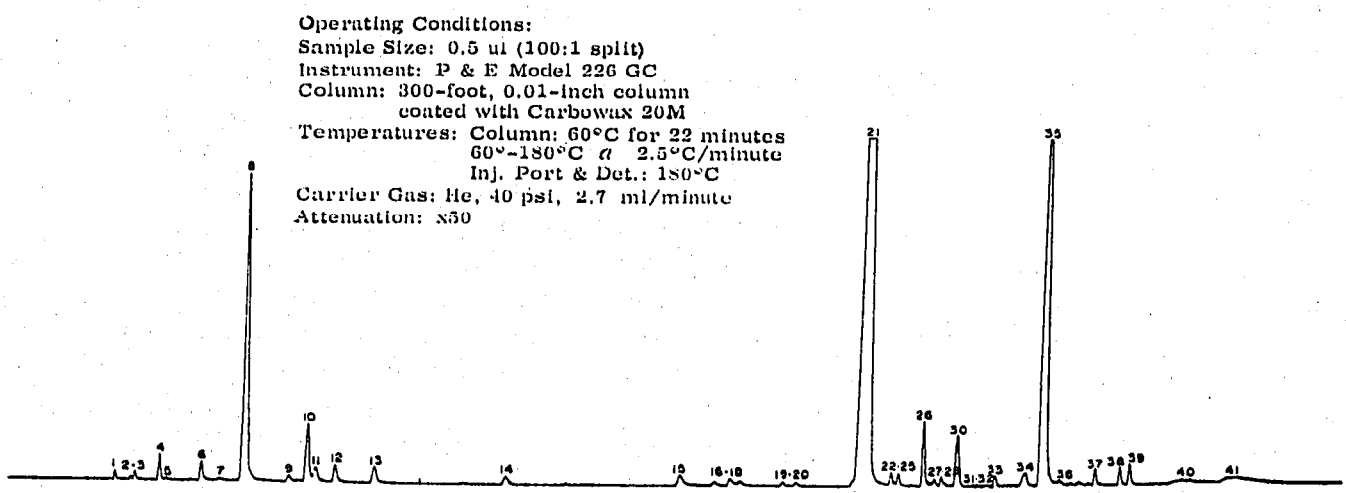

Fig. 2. GC analysis of "as-received" Bergamot oil using a capillary column. (Note: The corresponding peaks in this figure and in Figs. 3–5 are designated by the same numbers.)

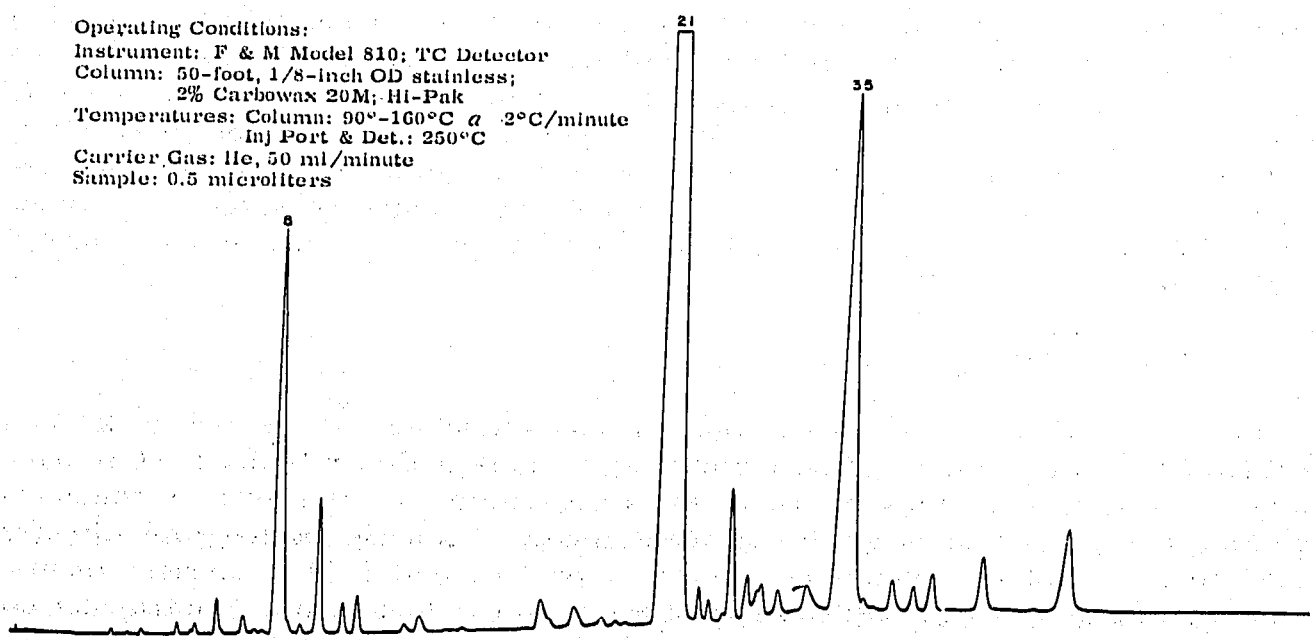

Fig. 3. GC analysis of "as-received" Bergamot oil using high-efficiency packed columns.

An analytical-scale separation was then made using a column system with an efficiency equivalent to that of the large preparative columns. The capillary and high-efficiency packed-column chromatograms indicated that the majority of the sample components were eluted between 130° and 160°. Since programming 4-in. diameter preparative columns is generally not practical, an isothermal operating temperature of 150° was selected. This preliminary analytical-scale separation (see Fig. 4) served two purposes—namely, to establish (1) elution-time limits for five selected mixed fractions and (2) to approximate the maximum sample volume that can be handled with equivalent efficiency using a large-scale preparative GC system.

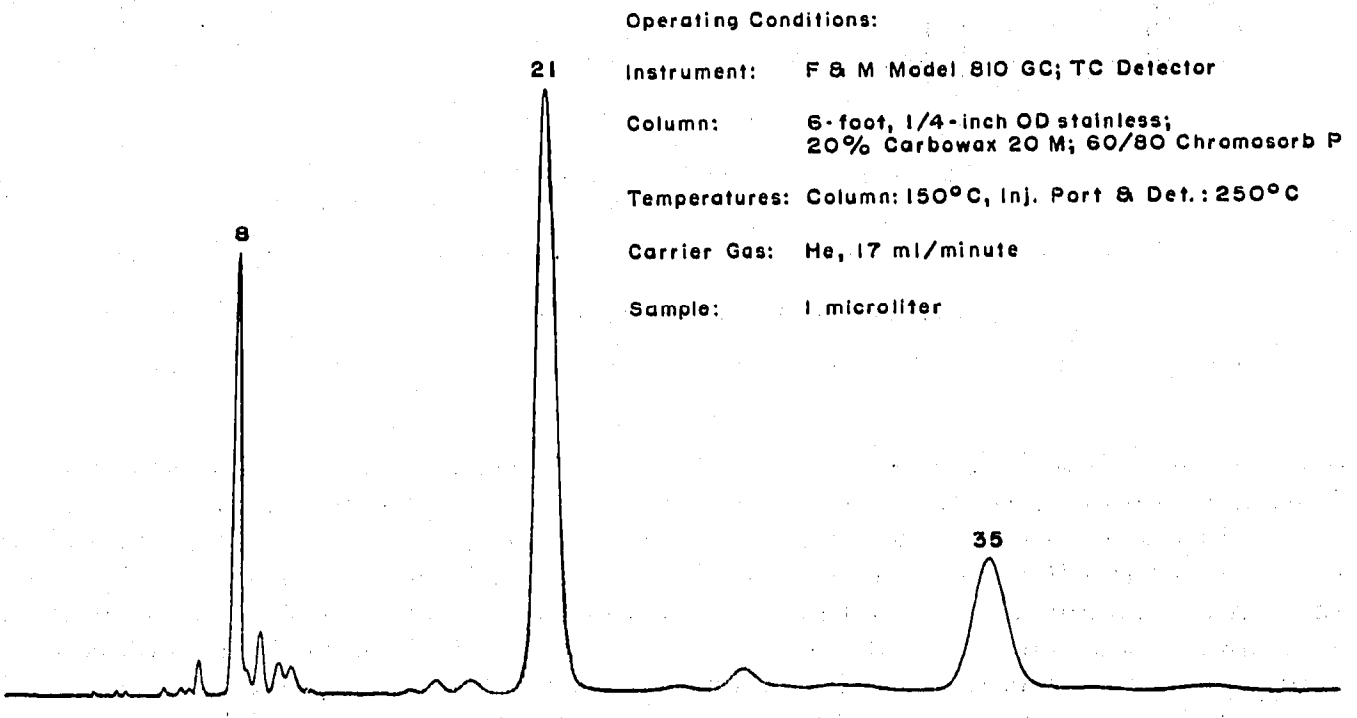

Fig. 4. Analytical-scale preparative GC separation of "as-received" Bergamot oil components.

The approximate sample volume was determined by direct extrapolation from the ratio of the cross-sectional areas of the 1/4-in. O.D. analytical columns and the 4-in. O.D. preparative columns (*i.e.* 1:480 respectively)[1]. Since we were interested in collecting mixed fractions rather than individual components from the 4-in. column, the only restriction on sample volume was that of avoiding serious spreading of the major peaks. As indicated previously, only moderate spreading occurred with a 30-μl sample volume (see Fig. 1). Furthermore, the slower linear flow rate in the preparative column permits more effective use of the stationary phase capacity. Therefore, we estimated that a 6 ft. 1/4 in. O.D. column could handle a 60-μl sample without excessive overload. This number was then multiplied by 500—the approximate cross-sectional area ratio of the 1/4 and 4-in. columns—to establish the sample size (*i.e.* 30 ml) for the preparative GC separation and collection of Bergamot oil components.

As shown in the chromatogram of Fig. 5, mixed fractions of minor components in the Bergamot oil were collected in the regions indicated. The major sample com-

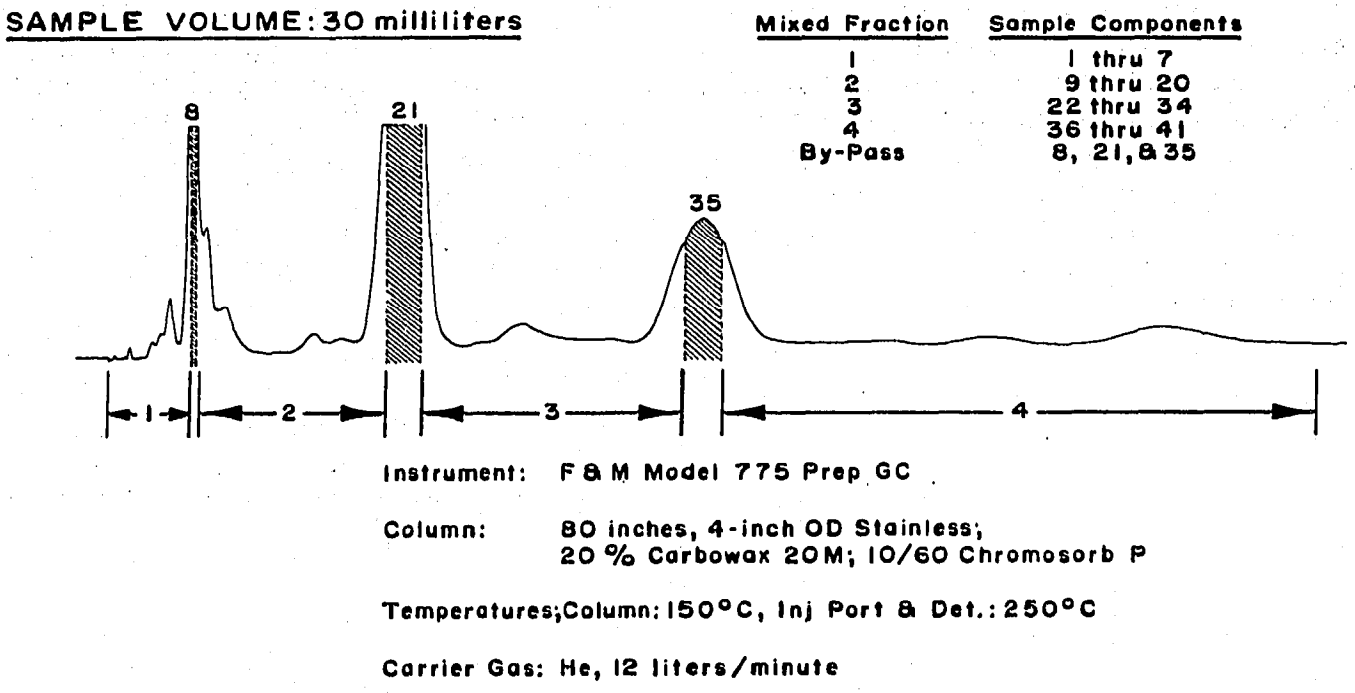

Fig. 5. Preparative-scale collection of Bergamot oil fractions using 4 in. diameter columns.

ponents were vented to a by-pass trap (Fraction V) where they were collected together. The corresponding peaks in Figs. 2—5 are designated by the same numbers.

Chromatograms for the mixed fractions (see Fig. 6A–E) were then compared with that of the Bergamot sample (see Fig. 2) to establish the relationship between these fractions and the original material. From this comparison, it is apparent that even greater concentration of the minor components could have been achieved by venting a larger proportion of the major constituents to the by-pass trap.

Chromatograms A–E of Fig. 6 were quantitatively evaluated by integrating the area under each peak with an Infotronics® Model 11-HSB Integrator. The percentage area of each peak was then computed, using the internal normalizing technique, and enrichment factors (see Table I) were calculated by dividing each value by the percentage of that component in the original sample. Examination of these data indicates the presence of small amounts of certain low-boiling components that were not detectable in the original sample. Apparently, all components can not be collected with the same absolute efficiency. However, the overall efficiency for the total collection from the large scale preparative system was 98 %. This is based on a total injected volume of 200 ml and collection of 197 ml with 6, 35, 66, and 23 ml, respectively, in Fractions 1–4 and 67 ml in the by-pass (*i.e.* Fraction V).

Using the combination of a gas chromatograph and a mass spectrometer, the components of Fraction I (Fig. 6A) were identified. As shown by the data in Table II, peaks designated as I-A and I-G—undetected in the original sample—were present in concentrations of 0.1 % and 0.8 %, respectively, in Fraction I.

These components were subsequently identified by mass spectrometry as α-pinene and ocimene (see Figs. 7 and 8). The identity of these components was verified by adding small amounts of these compounds to Fraction I and measuring changes in

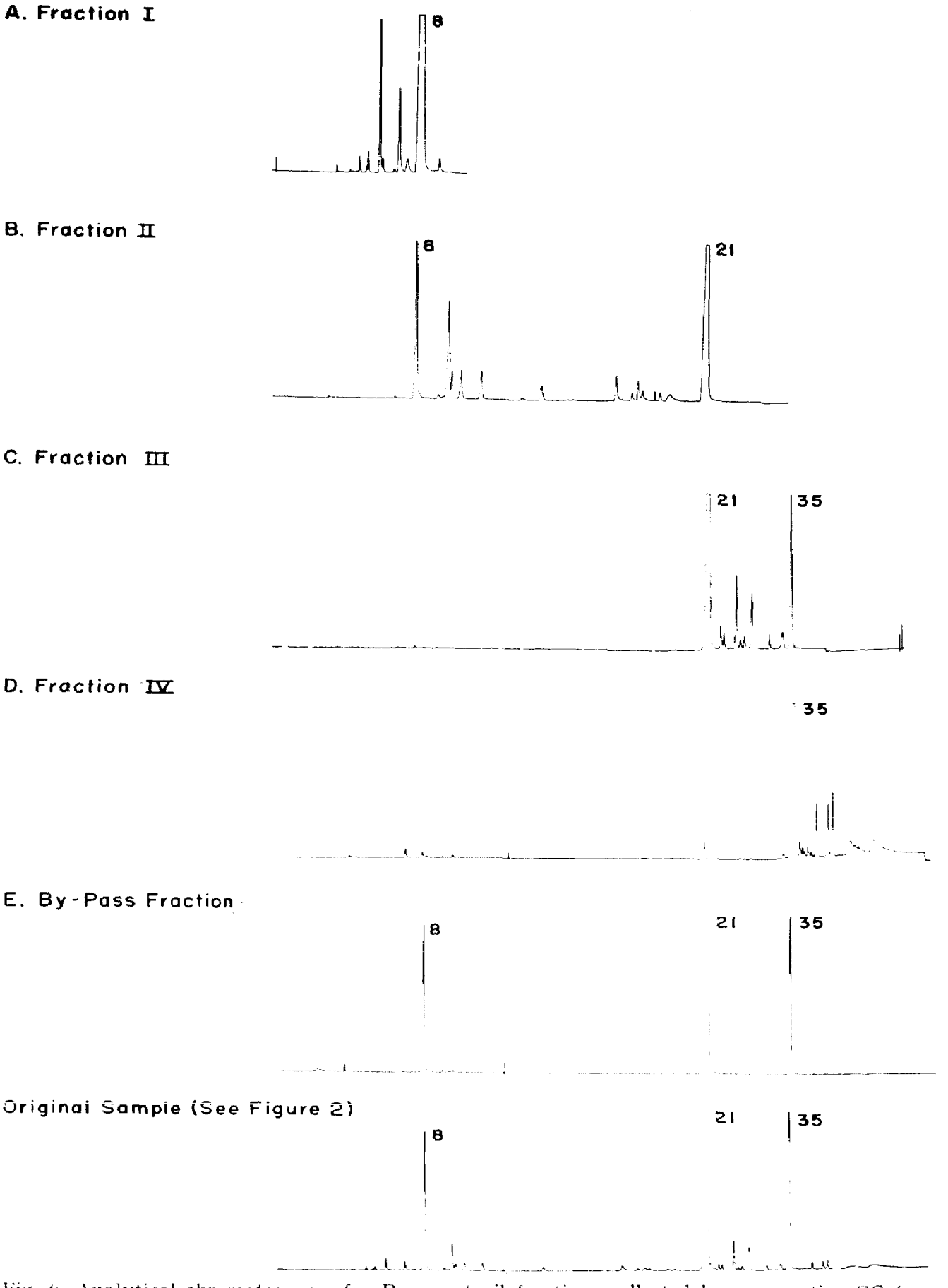

Fig. 6. Analytical chromatograms for Bergamot oil fractions collected by preparative GC (see Fig. 2 for conditions).

TABLE II

FRACTION I TRACE COMPONENT ENRICHMENT DEMONSTRATES PRESENCE OF CONSTITUENTS ORIGINALLY PRESENT IN QUANTITIES TOO SMALL TO DETECT

Peak designation	*Percent of total Fraction I*
I-A	0.10
I-B	0.10
I-C	<0.02
I-D	<0.02
I-E	0.10
I-F	0.40
I-G	0.80
I-H	0.10
I-I	0.50

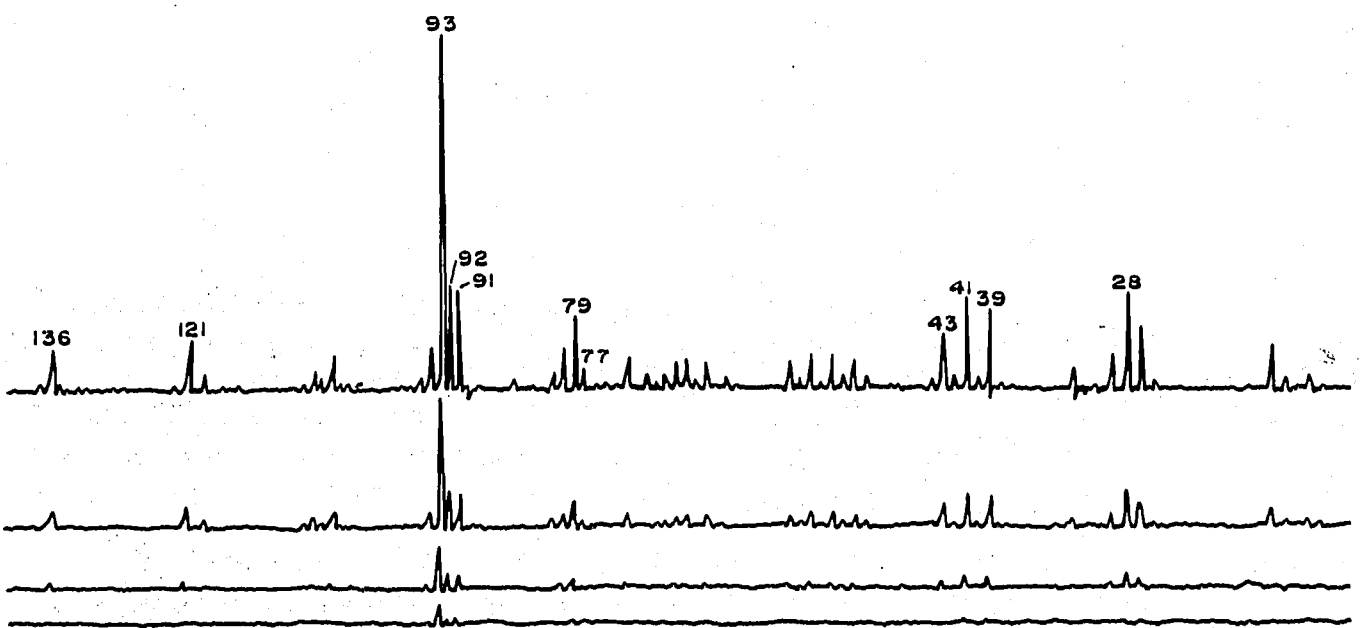

Fig. 7. Bergamot oil Fraction I, peak I-A: α-pinene component identification by mass spectrometric analysis.

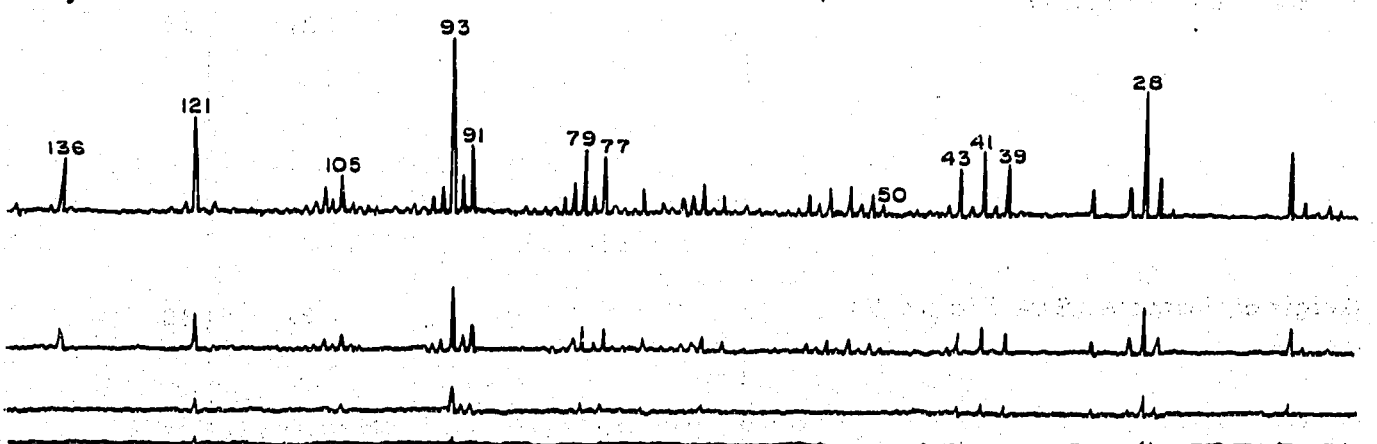

Fig. 8. Bergamot oil Fraction I, peak I-G: ocimene component identification by mass spectrometric analysis.

the I-A and I-G peak heights on the resultant chromatograms. These results were obtained with a Hitachi RMU-6B mass spectrometer and a 50 ft. 0.02 in. stainless steel support-coated open tubular column (Carbowax 20M) operated at a flow rate of 6 ml/min with a 5:1 effluent split (5 parts going thru a WATSON–BIEMANN separator[10,11] to the mass spectrometer and 1 part to the flame ionization detector of the GC system).

The components of Fraction II (fraction between major peaks 8 and 21) were subsequently separated using a 20 ft. 3/8 in. O.D. column with operating conditions adjusted to give maximum separation. From this fraction, peak numbers 10, 12, and

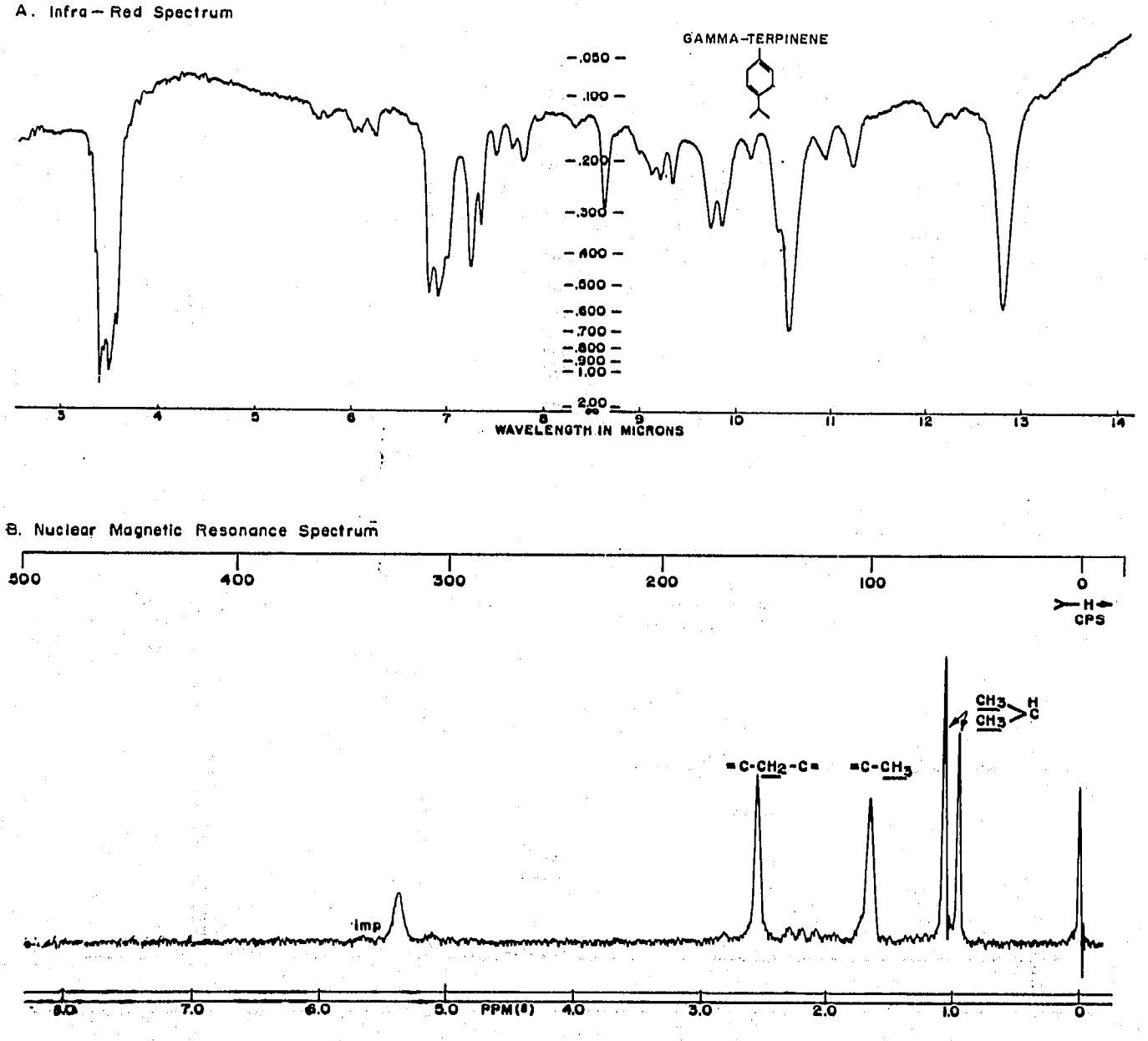

Fig. 9. Bergamot oil Fraction II, peak 10: γ-terpinene component identification by I.R. and N.M.R. analysis.

18, as designated in Table I, were trapped separately. The concentrations of these components in the original oil were 0.80 %, 0.64 %, and 0.24 %, respectively. In Fraction II, these concentrations were 13.7 %, 3.2 %, and 1 %. Enough of each component was collected for N.M.R., I.R., and mass spectrometry. The time required for collection was less than a day.

Peak 10 of Fraction II—(*γ-terpinene*): Pertinent features of the N.M.R. spectrum are the olefinic proton signal (2H) at 5.35 p.p.m.; the doubly allylic methylene signal (2H) at 2.52 p.p.m., the allylic methyl signal at 1.66 p.p.m., and the isopropyl doublet signal at 1.00 p.p.m. (cg), which were confirmed by I.R. (see Fig. 9). The mass

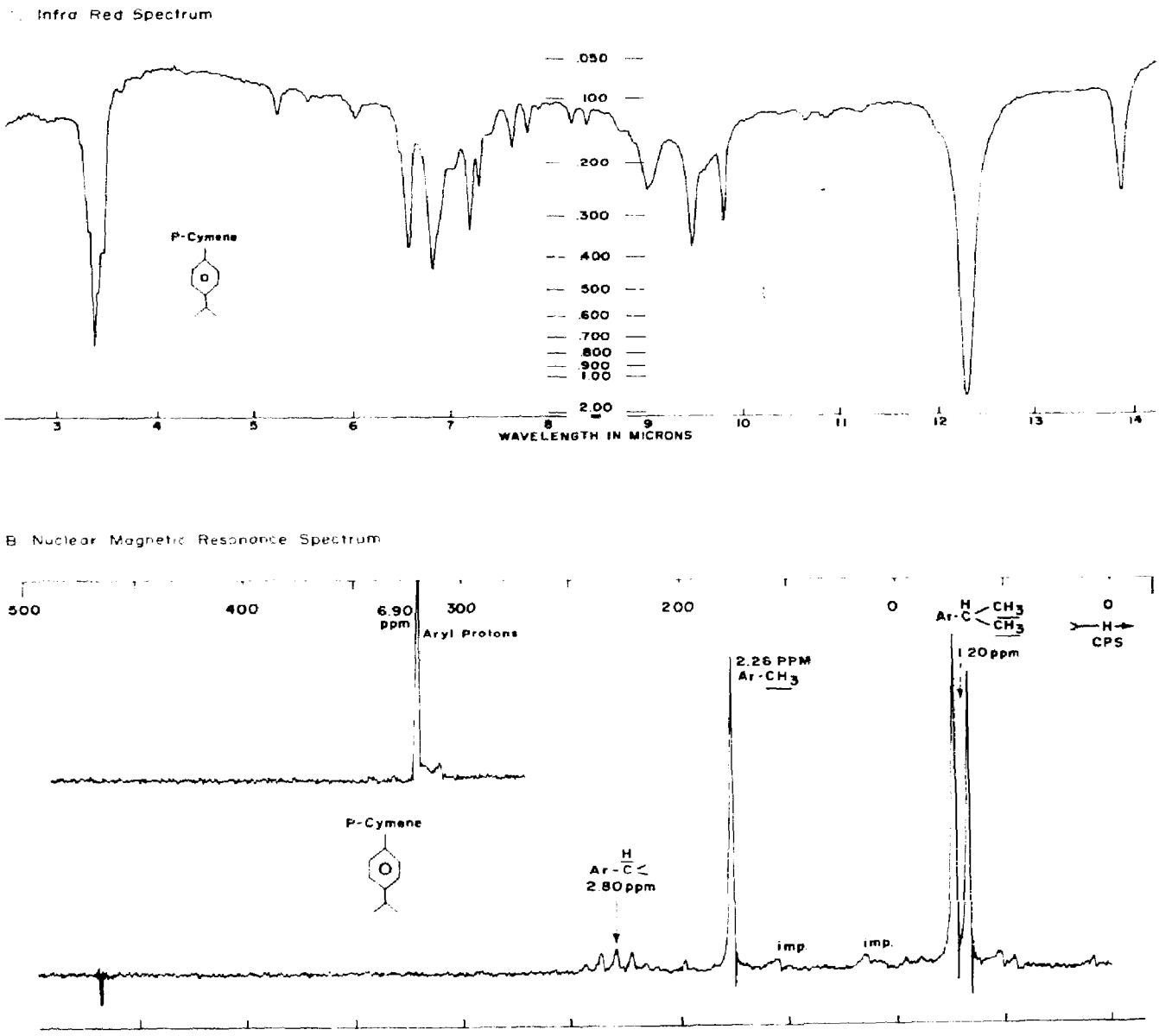

Fig. 10. Bergamot oil Fraction II, peak 11: *p*-cymene component identification by I.R. and N.M.R. analysis.

spectrum obtained with a CEC 21–103 mass spectrometer confirmed the identification.

Peak 12 of Fraction II—(*p-cymene*): Spectral features of this component include the aryl proton signal at 6.90 p.p.m.; the signal at 2.80 p.p.m. assigned to ArCH<; the singlet at 2.28 p.p.m. assigned to Ar-$\underline{CH}_3$ and the doublet at 1.20 p.p.m., assigned to $ArC(CH_3)_2$ by N.M.R. (see Fig. 10).

Peak 18 of Fraction II—(*linalool oxide*). The gem dimethyl protons appear at 1.03 p.p.m., and 1.14 p.p.m. The signal at 1.26 p.p.m. is assigned to the methyl protons on the carbon α to the oxygen. The signal at 3.63 p.p.m. is assigned to the $\underline{H}$-C-OH. The vinyl protons appear at 5.98 p.p.m.–4.81 p.p.m. (see Fig. 11).

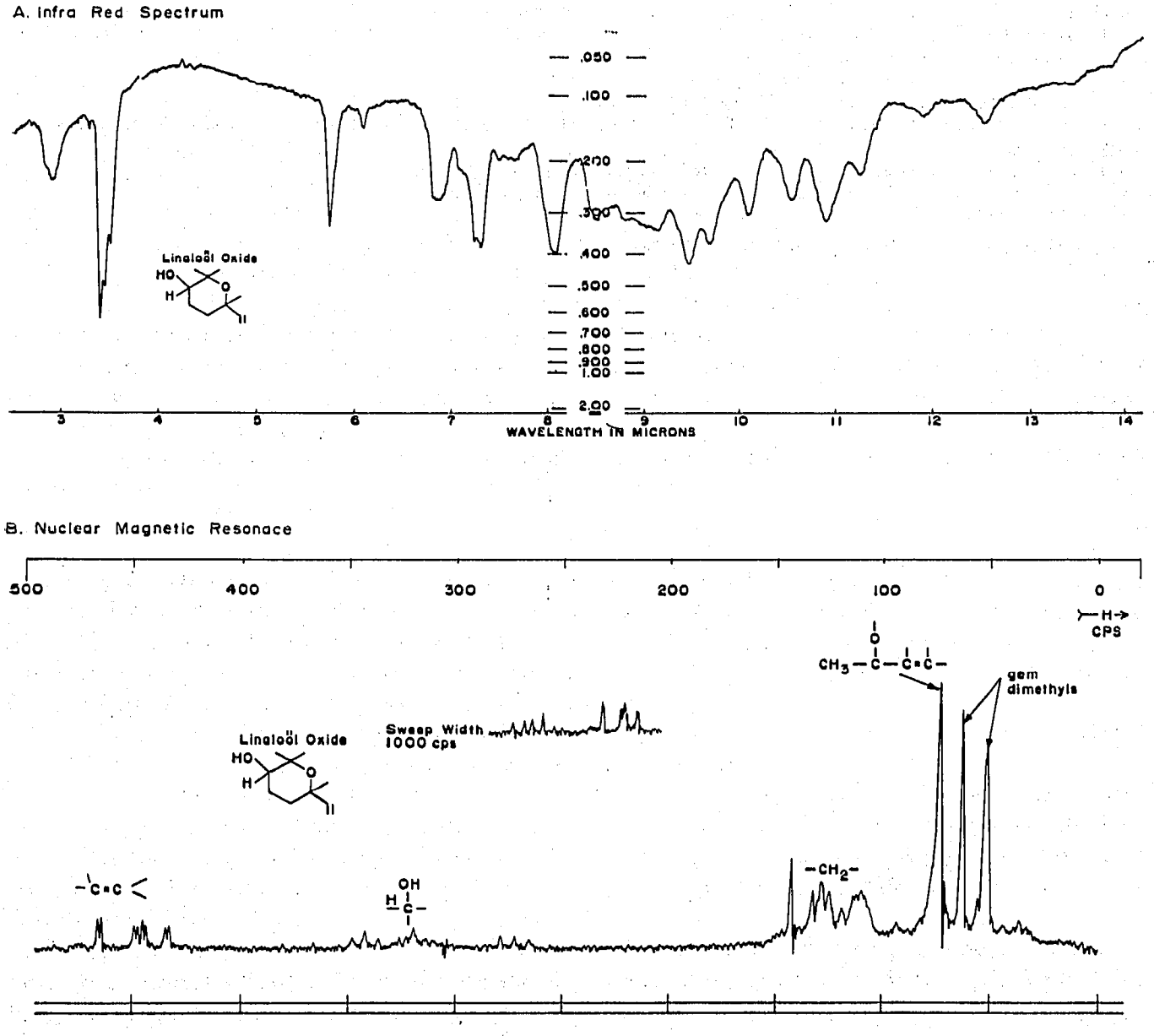

Fig. 11. Bergamot oil Fraction II, peak 18: linaloöl oxide component identification by I.R. and N.M.R. analysis.

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