Volatile Terpenes in California Bay Foliage. Changes in Composition during Maturation

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Volatile components of leaves of California bay, *Umbellularia californica*, isolated by steam distillation and ether extraction, were separated by column and preparative gas chromatography. Individual components were characterized by infrared and mass spectroscopy, and by determination of Kovats indices. Twenty-one compounds were identified, the ones present in greatest abundance being umbellulone, 1,8-cineole, α-terpineol, sabinene, terpinen-4-ol, and methyl eugenol. The compositions of volatile components in the essential oils from California bay new leaf growth and year-old growth were investigated over a growing season. A general trend observed in the analyses of the old growth samples was a continual decrease in the level of volatile terpenes from the first sampling in early April until the middle of June, followed by an increase in amounts until the termination of the study in August. The amounts of all components in new leaf growth, initially very small relative to the amounts of the same components in mature growth, increased slowly through the first 4 weeks and then rapidly thereafter to the point where most components were, by the end of the study, present in greater concentration in new than in old growth. The implications of these results relative to the very low palatability of California bay for browsing ruminants are discussed.

Browsing ruminants (deer, elk, sheep, etc.) are well known to be highly selective in their choice of range forage species. This can result in extensive damage to young plants, which becomes important when the species has economic value—for example, Douglas fir. The immense variation in palatability of many range forage species has been investigated by carefully controlled feeding trials using penned deer at the Hopland Field Station of the University of California (Longhurst *et al.*, 1968). Critical observation of deer both in the field and under penned conditions at Hopland has shown that deer use olfaction to make their initial selection of forage. In investigating the food chain relationships of ruminants, we have studied extensively the volatile components of Douglas fir foliage, a quite palatable forage species (Ellison, 1973; Maarse and Kepner, 1970; Sakai *et al.*, 1967). The possible relationship of the individual constituents of the Douglas fir needle oil to acceptance of different food materials by browsing animals has been investigated (Oh *et al.*, 1967) with the observation that monoterpene alcohols and carbonyl compounds have an inhibitory effect on the activity of the rumen microorganisms. It has also been observed that animals browsing on Douglas fir have strong preference for young growing tips relative to mature needles. The differences in composition of the essential oil from young tips relative to that from mature growth were consequently investigated (Maarse and Kepner, 1970).

In an investigation of palatability preferences of foraging ruminants it is equally important to study species of low palatability as well as palatable forage species. California bay is one of the least palatable forage species, and the essential oil from California bay foliage exhibits strong inhibition to the activity of deer rumen microorganisms (Oh *et al.*, 1968).

Early work on the essential oil of California bay has been reviewed by Guenther (1950). Among the components reported were umbellulone, 1,8-cineole, α-pinene, methyl eugenol, eugenol, safrole, traces of sesquiterpenes, and traces of a mixture of fatty acids containing formic acid.

This paper presents the results of an investigation of the essential oil isolated from the mature leaves of California bay, *Umbellularia californica*. The results of a study of the differences in composition of the volatile terpenes in the new growth relative to the mature growth and the changes in both over a growing season are also presented.

EXPERIMENTAL SECTION

California Bay Mature Leaf Oil. The California bay foliage was collected on the Hopland Field Station, University of California, Hopland, Calif. The essential oil was isolated from 1600 g of mature leaves in approximately 4% yield by steam distillation of the macerated leaves at atmospheric pressure, ether extraction of the steam distillates, drying the combined ether extracts over anhydrous Na_2SO_4, and removal of the ether on a rotary evaporator at 0° and 100 mm pressure. Individual components were isolated from the oil and purified by a combination of column chromatography on basic alumina to separate oxygenated compounds from hydrocarbons and of preparative glc utilizing 10-ft × ¼ in. columns containing Carbowax 20M, SE-30, or 1,2,3-tris(2-cyanoethoxy)propane packings. In the preparative glc runs the fractions were collected at the exit of the thermal conductivity detector utilizing 12-in. long thin-walled glass capillaries cooled with Dry Ice. Individual components were always collected from the SE-30 column for final purification immediately before determination of the infrared spectrum.

Seasonal Variation Studies. Foliage samples were collected periodically from a 10-ft California bay tree growing on the Hopland Field Station, starting a few days after emergence of the new growth in the spring. Collection dates were Apr 10, Apr 23, May 10, June 3, June 26, and Aug 8. Sampling was done from the same general location on the tree each time. The samples were wrapped in aluminum foil, transported to Davis in an ice chest, and kept in a refrigerator until they were analyzed (maximum of 2 days). The leaves were removed from the stems just prior to analysis. The old leaves used were those immediately adjacent to the new growth on the branches.

The analyses of the foliage samples were carried out using the small-scale simultaneous steam distillation–extraction procedure of Maarse and Kepner (1970). A 2.0-g

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sample of leaves was frozen under liquid nitrogen in a smaller mortar, ground to a powder, and then transferred with 50 ml of distilled water into the 100-ml sample flask of the apparatus. *n*-Pentane was placed in the solvent flask and the combined steam distillation–extraction procedure was run for exactly 1 hr. The pentane extract was dried over anhydrous Na_2SO_4 and the pentane removed through a micro-Vigreaux column, using a specially constructed concentration flask, until the volume of extract was less than 0.1 ml. A 50-μl sample of a standard solution of *n*-tetradecane in *n*-heptane (100 μl/100 ml) was added as an internal standard and the volume brought up to the 0.2 ml mark with *n*-heptane. After thorough mixing, a 0.50-μl sample was injected onto the 500-ft × 0.03-in. Carbowax 20M column for analysis.

Chromatographic Conditions. *Preparative Runs.* A Loenco Model 70 gas chromatograph with dual thermal conductivity detection was used. The columns utilized were 10-ft × ¼ in. o.d. stainless steel tubes packed with one of the following: (1) 10% Carbowax 20M on Chromosorb W, HMDS, 60–80 mesh; (2) 10% SE-30 on Chromosorb W, HMDS, 30–60 mesh; (3) 20% 1,2,3-tris(2-cyanoethoxy)propane on Gas Chrom Q, DMCS, 60–80 mesh. Conditions normally used were: injection temperature, 210°; detector temperature, 210°; column temperatures, isothermal or programmed at 4°/min as needed to optimize separation of the peak in question; He flow, 60 ml/min; detector current, 100 mA.

Analytical Runs. An F&M Model 810 gas chromatograph with dual flame ionization detection was used. The instrument was fitted with two 500-ft × 0.03 in. i.d. stainless steel capillary columns coated with Carbowax 20M + Igepal 880 (20:1) and SF 96(50) + Igepal 880 (20:1), respectively; injection temperature, 180°; detector temperature, 200°; He, H_2, and air flows were 8, 23, and 300 ml/min, respectively. For the analyses of the seasonal variation oil samples on the Carbowax 20M column, the column oven conditions were 5 min isothermal at 75°, then programmed at 2°/min to 195° and run isothermal thereafter. Kovats' indices were determined on the two columns under isothermal conditions as follows: Carbowax 20M, 70°, 130°, and 190°; SF 96(50), 100°, 130°, and 170°.

Spectral Analyses. *Infrared Spectroscopy.* Infrared spectra were obtained on thin films on ultramicro demountable sodium chloride cells of our manufacture (Sevenants and Jennings, 1966) requiring approximately 0.1-μl samples, in a Beckman IR8 spectrometer fitted with a beam condenser.

Mass Spectroscopy. Mass spectra were obtained from individual components of the total essential oil or from subfractions obtained by preparative glc, using the 500-ft Carbowax 20M column in a Finnigan Model 1015C quadrupole gc–ms with a Model 6000 data system. Mass spectra of carefully purified components were also obtained with a Consolidated Electrodynamics Corp. mass spectrometer, Model 21-104.

RESULTS AND DISCUSSION

The components identified from the essential oil from California bay leaves are listed in Table I. In this investigation the main emphasis has been on the identification of the major terpenoids present, since these components are most likely to be factors in the low palatability of California bay foliage for browsing ruminants. Umbellulone and 1,8-cineole are present in by far the largest amounts, comprising together over 50% of the essential oil. Camphene, β-phellandrene, terpinolene, cuminal, and cuminyl alcohol, each present in relatively small amounts, are not likely to be factors in the low palatability of California bay unless the sensory response levels of the components for deer are extremely low.

Identifications of the components are based on comparisons of the observed spectral data with spectra obtained on authentic compounds in our laboratory or with literature values for infrared spectra (Mitzner and Mancini, 1969; Mitzner *et al.*, 1965, 1968) and for mass spectra (Ryhage and von Sydow, 1963; von Sydow, 1963, 1964; von Sydow *et al.*, 1970), and determination of Kovats indices on two columns. *trans*-4-Thujanol, a low melting solid, was isolated in a pure state with difficulty and subsequently identified by comparison of the observed properties with literature values for a partial mass spectrum (Ohloff *et al.*, 1966), the infrared spectrum (Lossner, 1965), and glc retention data (Kusomoto *et al.*, 1968; Russell and Jennings, 1970). Separations of β-pinene from sabinene and of β-phellandrene from the large amounts of 1,8-cineole were best accomplished by preparative glc on the 1,2,3-tris(2-cyanoethoxy)propane column. Eugenol and safrole, reported previously as constituents of California bay oil (Guenther, 1950), could not be detected in our sample of bay oil. Analysis of the essential oil by gc–ms indicated the presence of trace amounts of five sesquiterpene hydrocarbons which were not further investigated.

Table I. Volatile Terpenes in California Bay Oil

Peak no.[a]	Component	Identification: Spectral	Kovats' indices[b]: CB 20M	Kovats' indices[b]: SF 96(50)
1	α-Pinene	ir, ms	1039	940
3	Camphene	ir, ms	1085	952
5	β-Pinene	ir, ms	1124	980
6	Sabinene	ir, ms	1136	973
7	Myrcene	ir, ms	1173	983
9	α-Terpinene	ms	1194	1012
10	Limonene	ms	1213	1023
11	β-Phellandrene	ir, ms	1223	1027
12	1,8-Cineole	ir, ms	1228	1027
14	γ-Terpinene	ir, ms	1254	1055
15	*p*-Cymene	ir, ms	1280	1017
16	Terpinolene	ir, ms	1290	1082
32	*trans*-4-Thujanol	ir, ms	1483	1058
39	Linalool	ir, ms	1551	1091
48	Terpinen-4-ol	ir, ms	1628	1175
53	Umbellulone	ir, ms	1676	1160
58	α-Terpineol	ir, ms	1708	1184
70	Cuminal	ms	1795	1226
85	Methyl eugenol	ir, ms	2033	1373
90	Cuminyl alcohol	ms	2113	1302
93	Thymol	ir, ms	2173	1283

[a] The peak numbers refer to the numbers on the lower chromatogram of Figure 1. [b] Column temperatures for K.I. determinations: Carbowax 20M, peaks 1–16 at 70°, 32–70 at 130°, 85–93 at 190°; SF 96(50), peaks 1–32 at 100°, 39–70 at 130°, 85–93 at 170°.

Figure 1 presents chromatograms for the essential oils isolated from the first collection (Apr 8) of the old and new growth of California bay foliage. The analyses of the essential oil samples obtained from the new and old growth from all collections are given in Table II. The amounts of the various components are calculated in terms of peak heights relative to the internal standard on each chromatogram. This method of representation is convenient in that it readily permits a comparison of the relative amounts of a given component in the new and old growth at any time and also facilitates observation of the changes in composition over the growing season. Representation of the amounts in terms of peak heights gives only an approximate evaluation of the relative amounts of the various components present since this method tends to underestimate the absolute amounts of the components appearing late on the chromatograms.

The most significant general trend observable in the analyses of the old growth samples is a decrease in amounts of the volatile components from the first collec-

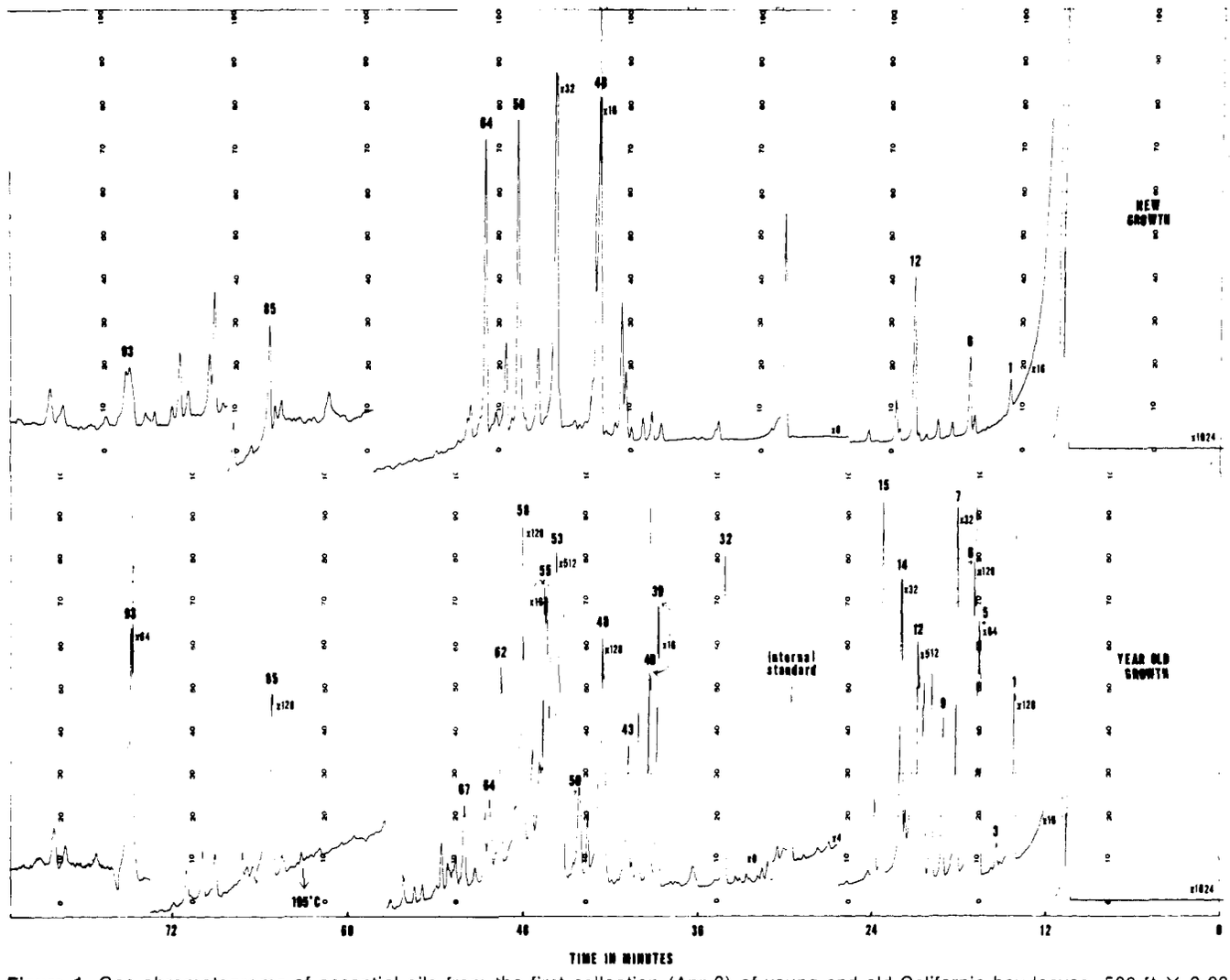

Figure 1. Gas chromatograms of essential oils from the first collection (Apr 8) of young and old California bay leaves. 500 ft × 0.03 in. i.d. Carbowax 20M column; temperature isothermal at 75° for 5 min then programmed at 2°/min to 195° and isothermal thereafter; chart speed, 20 in./hr; range 10, attenuation 4 except when otherwise indicated.

tion in early April until about the middle of June, followed by an increase through the remaining period of the study. The amounts of all components in the new leaf growth in the first collection are very small relative to the amounts of the same components in the year-old growth. The amounts of the components in the new growth increased slowly through the first three collections and then rapidly thereafter to the point where most components were present in greater concentration in the new than in the old growth at the end of the sampling period.

Sabinene, the most abundant monoterpene hydrocarbon in the old growth at the start of the sampling period, was an exception to the general trend noted above in that it decreased in concentration continually to the end of the study. *trans*-4-Thujanol (*trans*-sabinene hydrate) and sabinene show the same trends in changes in composition in both the new and old growth over the period of the study. The ratios of the two components in comparable samples suggest an enzymatically controlled equilibration between the two in California bay foliage. Acid-catalyzed hydration of sabinene gives only traces of *cis*- or *trans*-4-thujanol (Cooper *et al.*, 1973). The pinenes are present in much lower concentrations in mature growth of California bay than in conifers. As a result of the continued decrease in sabinene content, however, both pinenes are present in equal or greater amounts than sabinene at the end of the sampling period. *p*-Cymene, while present in fairly constant amounts in the old growth, was not detectable in the young growth in the first three samples and then increased in amount very slowly to a level much lower than that in the old growth at the end of the study. Terpinen-4-ol increased steadily in amount in both the young and old growth over the sampling period.

In an earlier study (Maarse and Kepner, 1970) of the changes in composition of the volatile terpenes in Douglas fir new tip growth it was observed that acyclic monoterpenes were almost completely absent in the new growth as it first appears, while cyclic monoterpenes were immediately present in amounts comparable to those in year-old growth. No trend of this sort could be detected from the studies on California bay foliage, where all volatile components were present in very low concentration, or not detectable, relative to the amounts in year-old growth.

The monoterpene alcohols, linalool, terpinen-4-ol, and α-terpineol, are each present in much greater concentration in California bay mature foliage than in Douglas fir mature needles. The overall larger amounts of monoterpene alcohols in addition to the very large amount of umbellulone, a monoterpene ketone, in the mature California bay foliage, are thus consistent with our observation of the much lower palatability of California bay relative to Douglas fir for browsing ruminants. The essential oil from California bay has been found to exhibit the strongest inhibition to the activity of deer and sheep rumen microorganisms of eight relatively unpalatable range forage species investigated (Oh *et al.*, 1968). 1,8-Cineole, the second most abundant component of California bay oil, does not appreciably inhibit the activity of deer rumen micro-

Table II. Changes in Terpene Composition in California Bay Foliage Oil[a]

Peak no.[b]	Component	Leaf age[c]	Collection dates: Apr 10	Apr 23	May 10	June 3	June 26	Aug 8
			Relative peak heights[d]					
1	α-Pinene	Y	0.2	0.3	0.7	4.4	26.6	60.1
		O	41.2	36.3	40.4	30.6	31.8	42.8
3	Camphene	Y					0.4	1.0
		O	0.7	0.6	0.6	0.5	0.5	0.7
5	β-Pinene	Y	0.1	0.2	0.4	2.9	16.7	39.0
		O	28.5	24.7	26.6	21.0	22.8	28.4
6	Sabinene	Y	0.4	0.7	1.6	9.0	64.6	135.5
		O	65.8	60.3	52.8	31.2	33.7	29.2
7	Myrcene	Y	0.1	0.1	0.3	2.6	15.2	34.2
		O	18.9	17.1	17.7	14.5	15.7	18.3
9	α-Terpinene	Y	0.2	0.2	0.4	1.7	5.7	10.6
		O	3.7	3.7	3.6	3.2	3.4	4.9
10	Limonene	Y			0.1	0.7	3.2	7.3
		O	5.3	4.7	4.9	4.2	4.3	5.3
11	1,8-Cineole	Y	1.3	1.5	3.6	25.7	120.6	244.7
		O	197.9	188.1	174.0	159.3	189.9	193.1
14	γ-Terpinene	Y	0.5	0.4	0.7	3.6	12.3	24.8
		O	16.3	13.9	16.4	14.2	14.3	20.2
15	*p*-Cymene	Y				0.5	0.8	3.7
		O	11.4	8.5	11.6	16.9	14.0	16.1
16	Terpinolene	Y	0.1	0.1	0.2	0.9	3.1	5.3
		O	2.0	1.8	1.9	1.6	1.9	2.3
32	*trans*-4-Thujanol	Y			0.2	0.4	2.0	4.1
		O	3.5	3.8	3.4	1.3	2.0	1.1
39	Linalool	Y		0.1	0.4	1.7	5.6	10.5
		O	7.2	6.5	7.3	7.1	7.4	6.6
48	Terpinen-4-ol	Y	2.7	3.0	2.4	10.0	23.1	49.4
		O	52.3	47.3	53.6	58.6	63.9	68.6
53	Umbellulone	Y	5.4	6.5	17.9	37.6	147.4	297.0
		O	259.4	252.8	223.7	201.3	242.4	242.4
58	α-Terpineol	Y	1.3	1.4	3.7	14.0	43.7	93.6
		O	71.5	67.2	64.9	47.1	69.0	66.7
85	Methyl eugenol	Y	0.5	0.5	1.1	5.2	20.4	49.4
		O	44.7	36.9	35.3	24.0	28.5	32.4
90	Cuminyl alcohol	Y						0.6
		O	0.7	0.5	0.6	0.6	0.4	0.7
93	Thymol	Y	0.2	0.3	0.4	1.9	1.7	4.6
		O	25.8	24.4	28.3	20.8	21.8	23.7

[a] Essential oil isolated by combined steam distillation–extraction procedure; analyzed on 500 ft × 0.03 in. Carbowax 20M column, column oven isothermal at 75° for 5 min, then programmed at 2°/min to 195° and isothermal thereafter. [b] Peak numbers refer to the numbers on the lower chromatogram of Figure 1. [c] Y = new leaf growth; O = year-old growth. [d] Listed peak heights were calculated relative to the peak height of the internal standard, all at range 10, attenuation 32.

organisms (Oh, 1972). The effects of various fractions of California bay oil and pure umbellulone on the activity of rumen microorganisms have been investigated but will be reported as part of a separate publication.

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