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Composition of the Essential Oil of Ylang-Ylang (*Cananga odorata* Hook Fil. et Thomson *forma genuina*) from Madagascar

Emile M. Gaydou,* Robert Randriamiharisoa, and Jean-Pierre Bianchini[1]

The essential oil of ylang-ylang was fractionated by distillation and column chromatography, and 17 compounds were isolated in pure form and identified by using spectroscopic methods. The volatile, oxygenated, and hydrocarbon fractions were analyzed, and 52 compounds were identified as far as possible by GC–MS, IR, and ^1H and ^{13}C NMR. Among them, α-cedrene, α-amorphene, γ-bisabolene, and α-, γ-, and δ-cadinols were tentatively identified. γ-Muurolene and (*E,E*)-farnesyl acetate were conclusively identified for the first time in ylang-ylang. The biogenetical relations of the new sesquiterpene hydrocarbons identified are discussed.

INTRODUCTION

The essential oil of ylang-ylang (*Cananga odorata* (Lam.) Hook fil. et Thomson *forma genuina*) is a light to deep yellow liquid having a harsh floral odor. This oil is used in perfumes, shampoos, creams, and lotions but also in ice creams, candies, and baked goods flavors. Approximately 100 tons/year are imported into the United States. The plant is cultivated throughout tropical Asia and some islands of the Indian Ocean, mainly from the Comoro, Nossi Be, and Madagascar islands. This essential oil is obtained by steam distillation from the flowers, with four grades being currently commercially available: extra, first, second, and third. Earlier reports in the literature (summarized by Klein (1975)) have shown that ylang-ylang essential oil contains monoterpenes, terpenic and sesquiterpenic alcohols, sesquiterpene hydrocarbons, acetates, benzoates, and phenols. The investigation of the sesquiterpene hydrocarbon composition of ylang-ylang was used to characterize the authenticity of the oil (Wenninger et al., 1966) or to detect the adulteration with basil essential oil (Teissere and Galfre, 1974). Timmer et al. (1975) reported the free acid composition of ylang-ylang and Duve et al. (1975) the chemical composition of endemic Fijian plants. More recently, ylang absolute and concrete have been analyzed (Buccellato, 1982). While many compounds have been identified, little information on the chromatographic identification and the relative amounts of these compounds has ever appeared in the literature.

In the present study we have developed a fractionation method of ylang-ylang essential oil, first grade obtained from plants grown in Madagascar, for the isolation and the characterization of the main compounds, using spectroscopic methods. Besides this qualitative identification, since the Chemical Society Analytical Methods Committee (1980) recommended a method for obtaining reproducible results in the gas chromatography of essential oils using Carbowax 20 M, we present the quantitative determination of the relative quantities of the components contained in this essential oil.

EXPERIMENTAL SECTION

Materials. The essential oil of ylang-ylang, first grade, was obtained from flowers harvested in 1980, by industrial steam distillation. This oil was supplied by the Service du Conditionnement et du Contrôle de la Qualité des Produits of Antananarivo (Madagascar) who guarantee its authenticity. Furthermore, mature flowers of ylang-ylang, harvested in 1981 from a plantation of Nossi Be (Madagascar), were extracted immediately with hexane for checking the presence of sesquiterpene hydrocarbons found in the industrial essential oil.

General Instrumentation. Fractional distillation was made on a Nester-Faust NF-200 Perkin-Elmer spinning-band column. IR spectra were taken on a Perkin-Elmer 256 using a sealed cell with sodium chloride windows of standard path length 0.1 mm. Proton nuclear magnetic resonance (^1H NMR) spectra were measured in CCl_4 on a Perkin-Elmer R 32 instrument at 90 MHz, with Me_4Si serving as internal lock. Carbon nuclear magnetic resonance (^{13}C NMR) spectra were recorded at 50.3 MHz on a Varian XL-200 spectrometer ($CDCl_3$ as internal lock, pulse with 8 μs, flip angle 45°, acquisition time 1 s). Single-frequency, off-resonance proton-decoupled (sford) ^{13}C NMR spectra were run to aid in the identification of the different types of carbon. Chemical shifts of ^1H and ^{13}C were reported downfield from Me_4Si. Combined gas chromatography–mass spectrometry (GC–MS) was recorded on a Girdel 30 gas chromatograph linked to a Ribermag R-10-10B mass spectrometer and coupled with a Sidar data computer. The GC column was a 0.30 mm i.d. × 50 m fused silica capillary column coated with Carbowax 20 M (0.15-μm phase thickness). The column temperature was programmed from 70 to 210 °C at 2 °C min^{-1} (carrier gas helium, ion source 220 °C, ionizing voltage 70 eV). Recording and searching of mass spectra were done by an integrated disk drive systems (EPA/NIH, 1978).

Gas–Liquid Chromatography Conditions. Analyses by GLC of the various fractions of ylang-ylang essential oil were done either on a FID-type Intersmat 12 DFL gas chromatograph or on a FID-type Girdel 30 gas chromatograph. Detector and injector temperatures were set at 230 and 220 °C, respectively. The GLC columns used were a Carbowax 20 M WCOT glass capillary column (50 m, 0.30 mm i.d., 0.15-μm phase thickness, programmed temperature 70–210 °C at 2 °C min^{-1}, inlet pressure of hydrogen used as a carrier gas 0.4 bar, split 40 mL min^{-1}) and an OV-101 WCOT glass capillary column (100 m, 0.30 mm i.d., 0.15-μm phase thickness, programmed temperature 90–220 °C at 2 °C min^{-1}, inlet pressure of hydrogen used as carrier gas 1.5 bar, split 60 mL min^{-1}).

Retention Indexes. The retention indexes (I_K) determined on Carbowax 20 M and OV-101 glass capillary columns were limited to the Kovats retention index system (Kovats, 1965) in which the retention behavior of a compound was reported relative to that of the *n*-paraffin hy-

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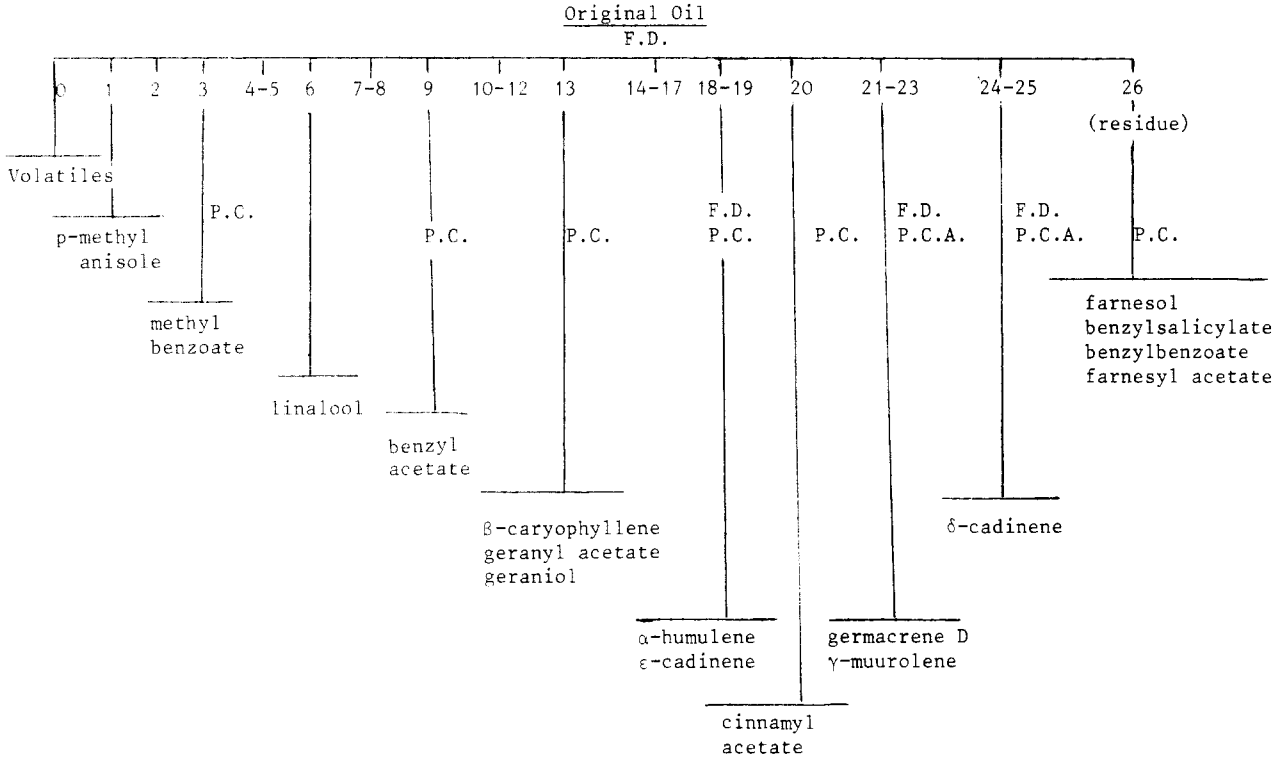

Figure 1. Schematic representation of the subfractional procedure employed in the isolation of pure compounds from ylang-ylang essential oil, first grade: FD, fractional distillation; PC, preparative column chromatography; PCA, preparative column chromatography on silver nitrate.

drocarbons, utilizing a logarithmic scale (Ettre, 1973 and 1974; Jennings and Shibamoto, 1980).

Percentage Composition of the Essential Oil. The relative peak areas in GLC of the unfractionated essential oil and the fractions of this oil (volatiles, oxygenated products, sesquiterpene hydrocarbons) were performed by electronic integration (Shimadzu ICR 1B).

Distillation of the Essential Oil. A portion (117 g) of the ylang-ylang essential oil was fractionally distilled under reduced pressure (0.3–0.7 mmHg) through the spinning-band column. The pot temperature was maintained at 130–140 °C, and the reflux ratio was held around 10:1 with a throughout of 3–4 drop min^{-1}. Twenty-five fractions were collected (Figure 1), the amounts of which were established by GLC: 1, 0.6 g; 2, 1.8; 3, 2.1; 4–5, 4.1; 6, 2.6; 7–8, 6.0; 9, 3.2, 10–12, 7.0; 13, 2.3; 14–17, 22.5; 18–19, 8.5; 20, 3.1; 21–23, 11.5; 24–25, 5.7; residue 26 g. The volatiles (6.5 g) were obtained from the nitrogen trap and were analyzed by GLC and GC–MS techniques.

Column Chromatography. Distilled fractions or original essential oil were fractionated over silica gel 60 (55–70 g, 230–400 mesh, E. Merck), using a column of 15 cm (18 mm i.d.). Elution of the essential oil was carried out by applying a 0–100% gradient of diethyl ether in hexane and collecting a number of fractions of 20 mL. Each fraction was monitored by TLC. Oxygenated compounds and sesquiterpene hydrocarbons were separated from the original oil (1.0 g) employing the same conditions using 300 mL of hexane and 300 mL of diethyl ether. Hydrocarbons (330 mg, 33%) first eluted and oxygenated compound (670 mg, 67%) were analyzed by GLC and GC–MS techniques.

Thin-Layer Chromatography. The purity of the isolated compounds was controlled by using precoated TLC aluminum sheets of silica gel 60 F 254 (E. Merck, 20 × 20 cm, layer thickness 0.2 mm) and eluted with hexane–diethyl ether (80:20, v/v) or benzene–CH_2Cl_2 (50:50, v/v) for heavy esters such as benzyl salicylate, benzyl benzoate, and farnesyl acetate. The compounds were detected by spraying the plates with phosphomolybdic acid and heating them at 70 °C for 10 min. The colors produced were blue or dark blue.

Caution! Experiments using benzene (human carcinogen) as part of a TLC eluent system should be conducted in a hood.

Isolation of Oxygenated Constituents. All compounds were identified by direct comparison of IR, ^1H NMR, and MS with the data from authentic compounds. The fractions 1 and 6 (Figure 1) contained pure compounds (95–99% by GLC) that were conclusively identified as *p*-methylanisole and linalool, respectively. Fraction 3 (892 mg) was chromatographed on a silica gel column using a 0–100% gradient of diethyl ether in hexane (950 mL), collecting 50 tubes. Tubes 18–28 (320 mg) contained pure methyl benzoate (R_f 0.43). Fraction 9 (2.09 g) was purified by colum chromatography (CC) using a 0–100% gradient of diethyl ether in hexane (1950 mL) and collected in 72 tubes. Tubes 28–36 (920 mg, 44%, purity 94% by GLC) contained benzyl acetate (R_f 0.63). Fraction 13 (1.14 g) was fractionated by CC using a 0–75% gradient of diethyl ether in hexane (800 mL), collecting 42 tubes. Tubes 1–15 (470 mg) contained a pure sesquiterpene hydrocarbon. Tubes 16–23 (240 mg) and 34–42 (340 mg) contained geranyl acetate (R_f 0.31) and geraniol (R_f 0.07), respectively. Fraction 20 (500 mg) was purified by CC using 50 mL of hexane and 50 mL of diethyl ether and collected in five tubes. The last tube contained pure cinnamyl acetate (R_f 0.71). The residue of distillation (fraction 26, Figure 1) was subjected in a first experiment (1.10 g) to CC using 0–100% gradient of diethyl ether in hexane (1350 mL) and collected in 58 tubes. Tubes 41–51 (280 mg) contained farnesol. In a second experiment, fraction 26 (4.44 g) was fractionated by CC using hexane (400 mL), hexane–diethyl ether 95:5 (v/v) (400 mL), 90:10 (400 mL), 80:20 (400 mL), 60:40 (400 mL), and 80:20 (400 mL), diethyl ether (400 mL), and methanol (400 mL) and collected in 118 tubes. Fractions G1 (tubes 31–33) and G2 (tubes 34–37) were

submitted to a second CC separation. The fraction G1 (1.46 g) was fractionated by CC using benzene-CH_2Cl_2 90:10 (v/v) (50 mL), 70:30 (50 mL), 50:50 (50 mL), and 30:70 (50 mL) and collected in 41 tubes. Tubes 5–14 (100 mg) and tubes 15–41 (310 mg) contained pure compounds that were identified respectively as benzyl salicylate (R_f 0.83) and (*E*,*E*)-farnesyl acetate (R_f 0.49).

Isolation of Sesquiterpene Hydrocarbons. The sesquiterpene hydrocarbon isolated from the fraction 13 (470 mg, purity 98% by GLC) was conclusively identified as β-caryophyllene by direct comparison of I_K (Jennings and Shibamoto, 1980), ^1H and ^{13}C NMR spectra (Kashman and Groweiss, 1980), and MS (Stenhagen et al., 1974). The distilled fractions (Figure 1) 18–19 (G3, 8 g), 21–23 (G4, 11 g), and 24–25 (G5, 5 g) were distilled a second time on the spinning-band column to give enriched sesquiterpene fractions that were subjected to CC separation using silica gel 60 (230–400 mesh, 20 g) impregnated with silver nitrate (2 g) according to Gupta and Dev (1963). Elution of the fraction G3 (460 mg) was carried out with hexane (60 mL), hexane–benzene 50:50 (v/v) (150 mL), benzene (60 mL), and benzene–acetone 70:30 (v/v) (350 mL) and collected in 61 tubes. The tubes 27–40 (102 mg) contained a pure sesquiterpene hydrocarbon 98% by GLC) that was conclusively identified as δ-cadinene by IR (Wenninger et al., 1967), ^1H NMR ($CDCl_3$) [δ 4.65 (4 H, m), 2.0 (14 H, m), 0.90 (3 H, d, J = 6.5 Hz), 0.75 (3 H, d, 5 = 6.5 Hz)], and ^{13}C NMR (Randriamiharisoa et al., 1986).

The tubes 47–61 (100 mg) contained a pure sesquiterpene hydrocarbon (99% by GLC) that was conclusively identified as α-humulene by I_K = 1459 on OV-101 [lit. I_K 1465 (Jennings and Shibamoto, 1980)], I_K = 1655 on Carbowax 20 M [lit. I_K 1642 (Toda et al., 1982; Okamoto et al., 1981)], ^1H NMR (Dev, 1960), ^{13}C NMR (Randriamiharisoa et al., 1986), and MS (Stenhagen et al., 1974). Elution of the fraction G4 (275 mg) was carried out with hexane (30 mL), hexane–benzene 20:80 (v/v) (60 mL) and 80:20 (60 mL), benzene (60 mL), and benzene–acetone 10:90 (v/v) (60 mL), 25:75 (30 mL), and 35:65 (180 mL) and collected in 100 tubes. The tubes 7–11 (30 mg) contained a sesquiterpene hydrocarbon (purity 92% by GLC) that was conclusively identified as γ-muurolene by I_K = 1477 on OV-101 [lit. I_K 1476, (Jennings and Shibamoto, 1980)], I_K = 1670 on Carbowax 20 M [lit. I_K 1669, (Toda et al., 1982)], ^{1}NMR ($CDCl_3$) [δ 5.41 (1 H), 4.60 (2 H), 1.70 (12 H, m), 1.61 (3 H, s), 0.90 (3 H, d, J = 6.5 Hz), 0.78 (3 H, d, J = 6.5 Hz)], ^{13}C NMR (Randriamiharisoa et al., 1986), and MS (Kashman and Groweiss, 1980). The tubes 29–41 (107 mg) contained a sesquiterpene hydrocarbon (97% by GLC) that was conclusively identified as germacrene D by ^1H NMR (Maarse, 1973), ^{13}C NMR (Randriamiharisoa et al., 1986), and MS (Maarse, 1973). The fraction G5, enriched in sesquiterpene hydrocarbon (280 mg), was purified by CC using hexane (30 mL), hexane–benzene 10:90 (v/v) (60 mL), 50:50 (v/v) (90 mL), and 80:20 (v/v) (60 mL), benzene (60 mL), and benzene–acetone, 95:5 (300 mL) and collected in 70 tubes. The tubes 6–11 (168 mg) contained a sesquiterpene hydrocarbon (92% purity by GLC) that was conclusively identified as δ-cadinene by I_K = 1521 on OV-101 [lit. I_K 1524 (Jennings and Shibamoto, 1980)], ^1H NMR (Buttery et al., 1967), and ^{13}C NMR (Randriamiharisoa et al., 1986).

RESULTS AND DISCUSSION

The essential oil of ylang-ylang, first grade, from Madagascar was examined initially by routine temperature-programmed GLC using glass capillary columns coated with Carbowax 20 M or OV-101 stationary phases. The complex mixture of components was separated into 27 fractions (including volatiles and residue) using fractional distillation. Each fraction was examied by GLC and two compounds *p*-methylanisole, linalool) were obtained in high purity. Other components were isolated from the fractions using preparative column chromatography and in some cases a second fractional distillation, as shown in Figure 1. It can be seen that 17 components were isolated in pure form, and their identifications were based upon spectroscopic data. The other constituents were identified as far as possible by using GC–MS by comparison with literature spectra. In all instances where positive identities are quoted, agreements between sample spectra and literature spectra were nearly perfect, within instrumental error. For the identification of components in small amount, GC–MS analyses were performed on the volatile fraction obtained by fractional distillation, which represents 5.6% of the essential oil, the oxygenated fraction and the hydrocarbon fraction obtained by column chromatography representing 67% and 33%, respectively, of the ylang ylang essential oil.

Table I. Compounds Identified from the Oxygenated Fraction[a] of the Ylang-Ylang Essential Oil, First Grade, and Their Percentage Compositions and Kovats Indexes

compd	I_K[b] CW-20M	I_K[b] OV-101	%[c]	identification MS	identification ^1H NMR	identification IR
3-methyl-2-buten-1-yl acetate	1200	900	0.2	+		
1,8-cineol	1217	1026	0.6	+		
2-methyl-3-buten-2-ol	1257	920	0.6	+		
3-methyl-3-buten-1-ol	1274	1075	0.2	+		
3-methyl-2-buten-1-ol	1318	996	0.2	+		
p-methylanisole	1434	1003	13.0	+	+	+
p-menthone (?)	1440		tr[d]	+		
benzaldehyde	1486		tr	+		
oxygenated compd	1512		tr			
linalool	1520	1087	28.4	+	+	+
oxygenated compd	1546		tr			
methyl benzoate	1586	1072	5.5	+	+	+
α-terpineol	1672		0.4	+		
benzyl acetate	1693	1136	6.9	+	+	+
geranyl acetate	1734	1360	10.0	+	+	+
methyl salicylate	1734		2.0	+		
nerol	1761		0.2	+		
geraniol	1821	1252	2.3	+	+	+
benzyl alcohol	1834		0.2	+		
2-phenylethanol	1876		0.1	+		
safrole	1903		0.1	+		
oxygenated compd	1909		0.2			
methyleugenol	1978		0.2	+		
oxygenated compd	2006		0.7			
(*E*)-nerolidol	2009		0.1	+		
sesquiterpene ester	2018		0.9			
p-cresol	2050	1182	0.1	+		
cinnamyl acetate	2100	1419	1.5	+	+	+
eugenol	2113	1354	0.5	+		
δ-cadinol[e]	2123	1625	1.3	+		
α-cadinol[e]	2137	1632	1.2	+		
γ-cadinol[e]	2150	1635	0.6	+		
muurolol T[e]	2178	1635	2.3	+		
(*E*,*E*)-farnesyl acetate[f]	2222	1672	2.8	+	+	+
(*E*,*E*)-farnesol	2300	1614	2.7	+		
benzyl benzoate	2496	1724	11.0	+	+	+
benzyl salicylate	2619	1703	3.0	+	+	+

[a] The oxygenated fraction represent 67.0% of the essential oil. [b] Experimentally determined Kovats indexes. [c] Percentages were calculated from the peak area determined on the Carbowax 20M column. [d] Traces. [e] Tentatively identified for the first time in ylang-ylang by their MS. [f] Identified for the first time in ylang-ylang.

Oxygenated Fraction. The 37 main oxygenated com-

Table II. Monoterpenes and Sesquiterpenes Identified from the Hydrocarbon Fraction[a] of the Ylang-Ylang Essential Oil, First Grade, and Their Percentage Composition and Kovats Indexes

compd	I_K[b] CW-20M	I_K[b] OV-101	%[c]	identification MS	IR	NMR ^1H	NMR ^{13}C
α-pinene	1038	940	1.1				
β-pinene	1123	980	0.6				
myrcene	1169	988	0.5				
α-ylangene	1460		0.1	+			
α-copaene	1474	1500	3.3	+			
β-cubebene	1514	1516	0.2	+			
α-cedrene[d]	1574	1434	0.1	+			
β-caryophyllene	1577	1426	32.0	+	+	+	+
α-humulene	1655	1459	8.4	+	+	+	+
ϵ-cadinene	1655	1459		+	+	+	+
γ-muurolene[e]	1670	1477	3.3	+	+	+	+
α-amorphene[d]	1670		0.2	+			
γ-bisabolene[d]	1674			+			
germacrene D	1678	1486	31.0	+	+	+	+
α-muurolene[d]	1685	1505	3.0	+			
δ-cadinene	1730	1521	7.1	+	+	+	+
γ-cadinene	1734	1497	8.1	+		+	
α-farnesene	1734	1498		+			
calamenene	1800		0.1	+			

[a] The hydrocarbon fraction represent 33.0% of the essential oil. [b] Experimentally determined Kovats indexes. [c] Percentages were calculated from the peak area determined on the Carbowax 20 M column. [d] Tentatively identified for the first time in ylang-ylang by their MS. [e] Identified for the first time in ylang-ylang.

pounds are listed in Table I. Among them, 32 have been positively identified, five of which were identified for the first time in ylang-ylang. The main components *p*-methylanisole, methyl and benzyl benzoates, benzyl, geranyl, cinnamyl, and (*E*,*E*)-farnesyl acetates, linalool, geraniol, and benzyl salicylate have been isolated in pure form and their structures determined from MS, IR, and ^1H NMR data. Methylbutenols and some of their acetates were identified in ylang-ylang by Naves (1971). Linalool is the main component of this fraction (28%) that gives the floral odor character of ylang-ylang. Benzyl and geranyl acetates are responsible of the fruity floral odor of this essential oil. Methyl and benzyl salicylates provide the medicinal note of ylang-ylang (Buccellato, 1982).

Hydrocarbon Fraction. The hydrocarbon fraction of ylang-ylang contains mainly sesquiterpenes since monoterpenes represent only 2.2% of this fraction. The hydrocarbons α- and β-pinenes and mycrene are not important odor contributors of ylang-ylang. The 16 main sesquiterpene hydrocarbons positively identified are listed in Table II. Among them, γ-muurolene was identified, using ^1H and ^{13}C NMR and mass spectra, for the first time in ylang-ylang. α-Muurolene, α-cedrene, α-amorphene, and γ-bisabolene were tentatively identified on the basis of their mass spectra. Germacrene D and β-caryophyllene represent 63% of this fraction and α-humulene, γ-, δ-, and ϵ-cadinenes, and α-farnesene represent 23.6%. The sesquiterpenes and specifically cadinenes and β-caryophyllene are responsible for the woody aroma of ylang-ylang. On the other hand α-farnesene exhibits a floral character (Buccellato, 1982).

Composition of Ylang-Ylang Essential Oil, First Grade. Among the 60 peaks obtained by GLC, 52 components were identified and are indicated in Table III. The other peaks could not be identified because their mass spectra were those of mixed state or indistinct state. Peak numbers on the left side of Table III show the elution order on the Carbowax 20 M column (Figure 2A); peak numbers on the right side show the elution order on the OV-101 column (Figure 2B). Those peak areas (from the Carbowax 20 M column) that had value of less than 0.1% and had not been identified are not listed. The main components of ylang-ylang, first grade, are linalool (19%), β-caryophyllene (10.7%), germacrene D (10.3%), geranyl acetate (7.8%), benzyl acetate (4.6%), *p*-methylanisole (8.4%), benzyl benzoate (7.6%), and methyl benzoate (3.6%). The presence of these components and others in less amount are characteristic of the complex aroma of ylang-ylang that is a combination of floral, fruity, woody, and medicinal notes.

Table III. Composition and Peak Identification of Compounds Identified in Ylang-Ylang Essential Oil, First Grade, from Madagascar

peak no. on CW-20M	peak area,[a] %	compd	peak no. on OV-101
2	0.4	α-pinene	5
3	0.2	β-pinene	6
4	0.1	myrcene	7
5	0.3	3-methyl-2-butenyl-1-yl acetate	3
6	0.7	1,8-cineol (eucalyptol)	10
7	0.5	2-methyl-3-buten-2-ol	4
8	0.2	3-methyl-3-buten-1-ol	8 bis
9	0.2	3-methyl-2-buten-1-ol	8
10	8.4	*p*-methylanisole	9
11	0.2	*p*-menthone (?)	
12	0.1	α-ylangene	
13	0.7	α-copaene	34
14	tr[b]	benzaldehyde	
15	tr	β-cubebene	
16	19.0	linalool	12
17	0.2	oxygenated compd	
17 bis[e]	tr	α-cedrene[c]	
18	10.7	β-caryophyllene	25
19	3.6	methyl benzoate	11
19 bis[e]	0.4	furfuryl alcohol	
20	2.8	α-humulene + ϵ-cadinene	27
22	1.5	γ-muurolene[d] + α-amorphene[c]	29
22 bis[e]	0.6	α-terpineol	
22 ter[f]	tr	γ-bisabolene[c]	
23	10.3	germacrene D	
25	0.6	α-muurolene[c]	23
26	4.6	benzyl acetate	13
27	2.3	δ-cadinene	37
28	2.6	γ-cadinene + α-farnesene	32 + 33
29	7.6	geranyl acetate	19
29 bis[e]	0.2	methyl salicylate	
30	0.1	nerol	
31	0.3	calamenene	33 bis[e]
32	1.6	geraniol	16
33	0.1	benzyl alcohol	
34	tr	2-phenylethanol	
35	tr	safrole	
37	tr	oxygenated product	
38	tr	methyl eugenol	
39	0.4	oxygenated product	
40	0.1	(*E*)-nerolidol	
41	0.1	sesquiterpenic ester	n
41 bis[e]	0.4	*p*-cresol	14
42	1.1	cinnamyl acetate	24
43	0.4	eugenol	17
44	0.8	δ-cadinol[c]	44
45	0.6	α-cadinol[c]	45
46	0.4	γ-cadinol[c]	46 bis[e]
47	1.7	muurolol T	46
48	1.8	(*E*,*E*)-farnesyl acetate	47
49	1.3	(*E*,*E*)-farnesol	47 bis[e]
50	7.6	benzyl benzoate	50
51	1.9	benzyl salicylate	49

[a] Calculated from the peak area determined on the Carbowax 20M column. [b] Traces (peak area <0.1 %). [c] Tentatively identified for the first time in ylang-ylang. [d] Identified for the first time in ylang-ylang. [e] Second compound in the corresponding peak. [f] Third compound in the corresponding peak.

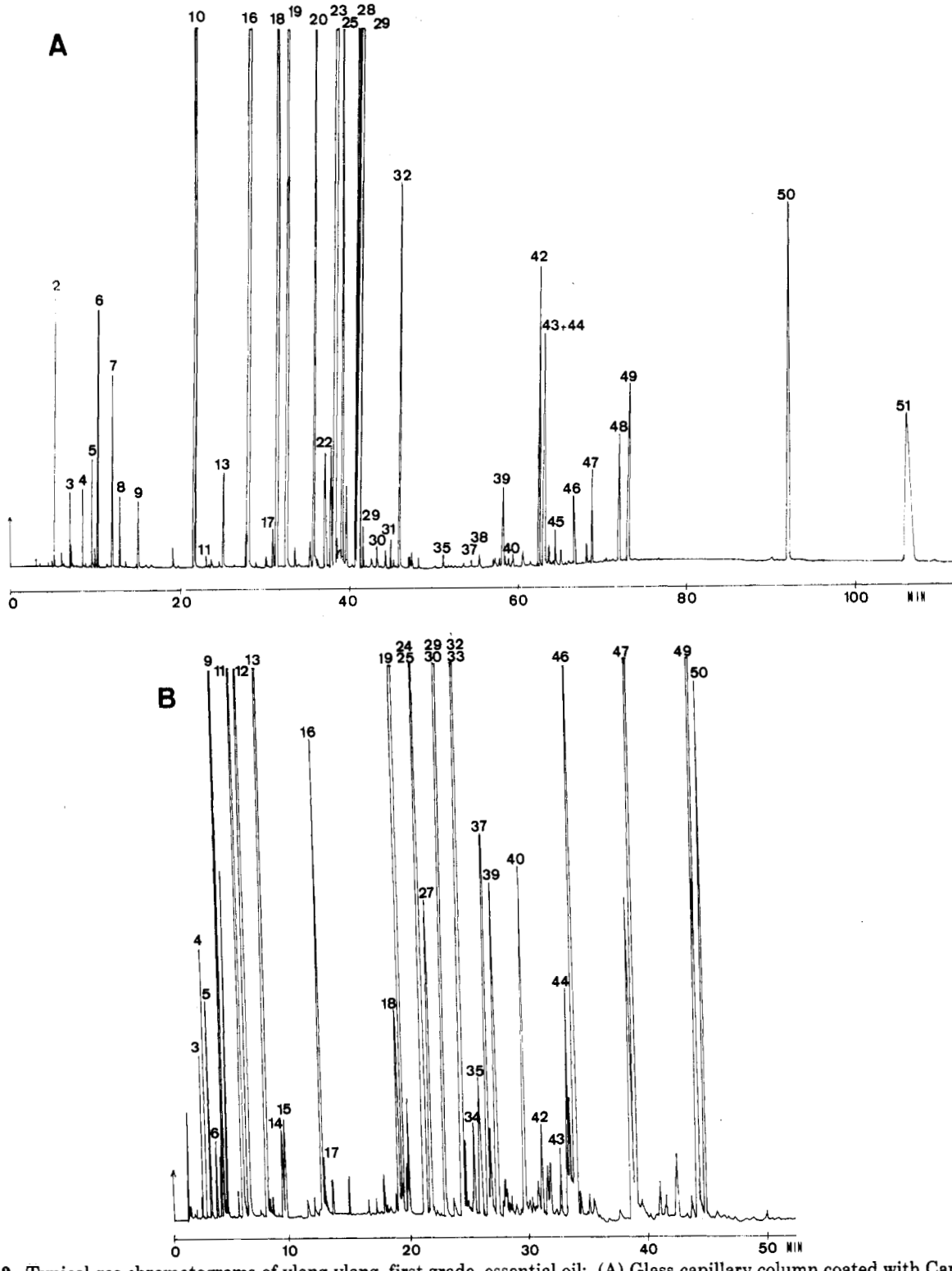

Figure 2. Typical gas chromatograms of ylang-ylang, first grade, essential oil: (A) Glass capillary column coated with Carbowax 20 M (50 m, 0.30 mm i.d., 0.15-μm phase thickness); column temperature programmed from 70 to 210 °C at 2 °C min^{-1}; carrier gas hydrogen, 0.4 bar, split 40 mL min^{-1}. (B) Glass capillary column coated with OV-101 (100 m, 0.30 mm i.d., 0.15-μm phase thickness); column temperature programmed from 90 to 220 °C at 2 °C min^{-1}; carrier gas hydrogen, 1.5 bar, split 60 mL min^{-1}. See Table III for peak identification.

Biogenesis of Sesquiterpenes in Ylang-Ylang Essential Oil. The ylang-ylang essential oil contains α-humulene, β-caryophyllene, muurolenes, cadinenes, germacrene D, α-copaene, α-farnesene, and other sesquiterpene hydrocarbons in minor amounts. The biogenesis of some of these compounds has been investigated by biogenetic-type rearrangement studies and has been summarized (Cordell, 1976; Coates, 1976). Both (*Z*,*E*)- and (*E*,*E*)-farnesyl-PP may be transformed into α-humulene and β-caryophyllene by 1,11 cyclization, which represent 40.4% of the sesquiterpene fraction, and into γ-bisabolene (0.2%) by a 1,6 cyclization (Ruzicka, 1963). The stereospecific cyclization of the three double bonds of γ-bisabolene lead to α-cedrene (Parker et al., 1967). As shown in Figure 3, (*Z*,*E*)- and (*E*,*E*)-farnesyl-PP may be transformed via an alternative 1,10 cyclization to germacrene cation A that gives germacrene D (31%). The cation A, involving 1,3 hydride shift or two 1,2 hydride shifts, leads

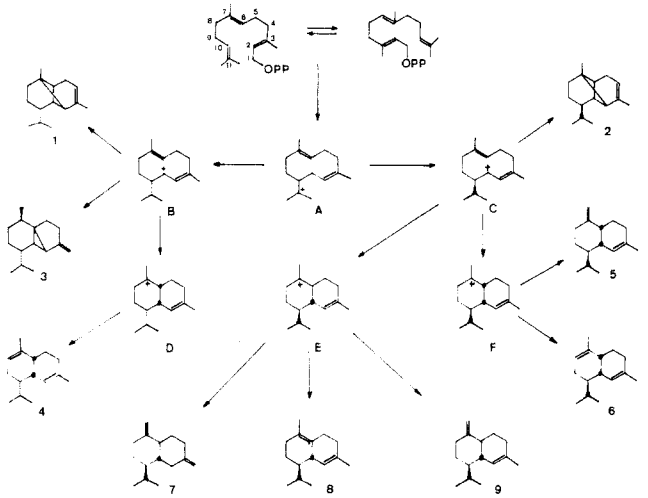

Figure 3. Simplified pathway to explain the formation of some sesquiterpene hydrocarbons found in ylang-ylang essential oil, first grade: **1** α-copaene; **2**, α-ylangene; **3**, β-cubebene; **4**, α-amorphene; **5**, γ-muurolene; **6**, α-muurolene; **7**, ϵ-cadinene; **8**, δ-cadinene; **9**, γ-cadinene. A–C are germacrene cations, the D cation leading to the amorphane group, the E cation leading to the cadinane group, and the F cation leading to the muurolane group.

Table IV. Comparison of Sesquiterpene Quantities of Ylang-Ylang Flowers Extracted by Two Methods

	extractn with hexane[a]		steam distillation[b]	
compd	peak area,[c] %	ratio,[d] %	peak area,[c] %	ratio,[d] %
α-ylangene	tr[e]		0.1	0.3
α-copaene	0.4	2.1	0.7	2.2
β-caryophyllene	5.6	29.3	10.7	33.5
α-humulene + ϵ-cadinene	1.6	8.2	2.8	8.8
γ-muurolene + α-amorphene	0.6	3.1	1.5	4.7
germacrene D	7.3	38.1	10.3	32.3
α-muurolene	0.3	1.5	0.6	1.8
δ-cadinene	1.0	5.1	2.3	7.2
γ-cadinene + α-farnesene	2.4	12.5	2.6	8.2
calamenene	tr		0.3	0.9
total	19.2	99.9	31.9	99.9

[a] Freshly extracted ylang flowers at ambient temperature. [b] Values taken from Table III. [c] Percentages were calculated from the peak area determined on Carbowax 20M. [d] Ratio expressed against the sum of sesquiterpenes. [e] Traces (peak area <0.1%).

to the two isomeric cations B and C according to the orientation of the ispropyl group (Figure 3). The isomeric cations B and C may be transformed into four cadalene type cations according to the nature of ring fusion. The cis-oriented hydrogen cyclization leads to amorphane D and muurolane F groups. Ylang-ylang essential oil contains α-amorphene and α- and γ-muurolenes. The trans-oriented hydrogen cyclization leads to cadinane E and bulgarane groups. We have found in ylang-ylang essential oil γ-, δ-, and ϵ-cadinenes, but we have not detected sesquiterpenes belonging to the bulgarane group. Calamenene may be formed in the same way by the loss of two hydrogens. The acid-catalyzed isomerization of α-cubebene, α-copaene, and α-ylangene has shown the mutual relation among these closely related tricyclic compounds with the cadalene type ones (Ohta et al., 1968). The intermediate cation B may explain the formation of α-copaene and β-cubebene and the cation C, the formation of α-ylangene, found in ylang-ylang essential oil. The direct correlation between germacrene cations and tricyclic skeletons has been also recently proposed (Berger et al., 1983). Since it is known that germacrene D is transformed into γ-muurolene, α-amorphene, and δ- and γ-cadinenes in contact with silica gel (Yoshihara et al., 1969), we have rapidly analyzed by GLC a sample of ylang-ylang essential oil freshly extracted with hexane from mature flowers. The sesquiterpene fractions (Table IV) have about the same composition as that given in Table III, showing therefore that some of the sesquiterpenes characterized are not artifacts formed during the steam distillation or the laboratory fractionation.

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Registry No. α-Pinene, 80-56-8; β-pinene, 127-91-3; myrcene, 123-35-3; 3-methyl-2-buten-1-yl acetate, 1191-16-8; 1,8-cineol, 470-82-6; 2-methyl-3-buten-2-ol, 115-18-4; 3-methyl-3-buten-1-ol, 763-32-6; 3-methyl-2-buten-1-ol, 556-82-1; *p*-methylanisole, 104-93-8; *p*-methone, 89-80-5; α-ylangene, 14912-44-8; α-copaene, 3856-25-5; benzaldehyde, 100-52-7; β-cubebene, 13744-15-5; linalool, 78-70-6; α-cedrene, 469-61-4; β-caryophyllene, 87-44-5; methyl benzoate, 93-58-3; furfuryl alcohol, 98-00-0; α-humulene, 6753-98-6; ϵ-cadinene, 27542-04-7; γ-muurolene, 30021-74-0; α-amorphene, 20085-19-2; α-terpineol, 10482-56-1; γ-bisabolene, 495-62-5; germacrene D, 23986-74-5; α-muurolene, 10208-80-7; benzyl acetate, 140-11-4; δ-cadinene, 483-76-1; γ-cadinene, 483-74-9; α-farnesene, 502-61-4; nerol, 106-25-2; calamenene, 483-77-2; geraniol, 106-24-1; benzyl alcohol, 100-51-6; 2-phenylethanol, 60-12-8; safrole, 94-59-7; methyl eugenol, 93-15-2; (*E*)-nerolidol, 40716-66-3; *p*-cresol, 106-44-5; cinnamyl acetate, 103-54-8; eugenol, 97-53-0; δ-cadinol, 36564-42-8; α-cadinol, 481-34-5; γ-cadinol, 50895-55-1; muurolol T, 19912-62-0; (*E,E*)-farnesyl acetate, 4128-17-0; (*E,E*)-farnesol, 106-28-5; benzyl benzoate, 120-51-4; benzyl salicylate, 118-58-1; geranyl, 105-87-3.

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Fractionation, Characterization, and Protein-Precipitating Capacity of the Condensed Tannins from *Robinia pseudo acacia* L. Leaves

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Condensed tannins (proanthocyanidins) from black locust (*Robinia pseudo acacia*) leaves were fractionated into five different molecular sizes on a column of Sephadex LH-20 using 70% aqueous acetone as eluent. All five fractions yielded four anthocyanidin pigments upon acid hydrolysis, and their degree of polymerization measured by the vanillin assay in acetic acid ranged from 1.53 to 4.12. Both the percentage of protein-precipitable phenolics and the protein-precipitating capacity of the tannin fractions increased with the increase in the degree of polymerization.

INTRODUCTION

The leaves of *Robinia pseudo acacia* (black locust) are being utilized as fodder in some countries (Negi et al., 1979; Bonciarelli, 1980; Papageorgiou et al., 1981; Sheikh and Khan, 1983). The trees are heavily lopped for fodder as the black locust leaves are rich in crude protein (17–25%), calcium (1.93–3.49%), and phosphorus (0.14–0.22%) (Singh, 1982). The supplementation of black locust leaves with rice straw (Lee and Kang, 1980), barley (Kang and Yoo, 1978), and maize and defatted soybean meal diets (Takada et al., 1980) have been found useful for the sheep, goat, and poultry, respectively. However, when offered as a sole feed the black locust leaf meal was found to have the low digestibility for crude protein, organic matter, and phosphorus (Horton and Christensen, 1981; Negi et al., 1979). The leaves have also been found toxic when eaten in excess (Everist, 1969). Horigome et al. (1984) and Negi et al. (1979) reported that the low nutritional value of the black locust leaves was due to the presence of condensed tannins. Since the most relevant antinutritional property of condensed tannins is their ability to interact and precipitate feed and enzyme proteins, this largely depends upon the chemical nature of the tannins (Kumar, 1983; Porter and Woodruffe, 1984). This paper describes the fractionation, characterization, and protein-precipitating capacity of the condensed tannins from black locust leaves.

EXPERIMENTAL SECTION

The tannin from four separate batches of leaves collected in June 1984 was purified, with identical results each time. The tannin was extracted from 400 g of leaves with 1.4 L of acetone–water (70:30, v/v) containing 0.5% ascorbic acid (Jones et al., 1976). The acetone and water were separated into immiscible phases by saturating the extract with NaCl. Acetone was removed in vacuo at less than 40 °C, and the extract was diluted with an equal amount of water. The aqueous phase was extracted three times with diethyl ether followed by ethyl acetate to remove the pigments, lipids, and low molecular weight polyphenolic compounds, and the solution of crude tannins was dialyzed against water containing 0.5% ascorbic acid. To the dialyzed solution was added an equal amount of methanol. The 50% methonolic solution of crude tannins was applied to a column of Sephadex LH-20 (2.6 × 7.9 cm) preswollen in 50% methanol. The adsorbed tannins were washed with 800 mL of the same solvent and were eluted with 50% aqueous acetone as a discrete visible band. The acetone was removed in vacuo and water removed by freeze-drying to yield light, tan, fluffy solids that gave a single spot at the origin when two-dimensional paper chromatography was carried out in butanol–acetic acid–water (6:1:2) and 2% acetic acid.

For fractionation, a sample of tannin (0.55 g) in 3.0 mL of 70% acetone was loaded onto a column of Sephadex LH-20 (2.6 × 61 cm) that had been equilibrated with 70% acetone. The column was eluted at a flow rate of 1.4 mL/min with 70% acetone and 3.2-mL fractions were collected. The absorbance at 350 nm was monitored for each fraction. The fractions 29–35 (A), 38–47 (B), 53–60 (C), 64–71 (D), and 74–81 (E) were pooled. After removal of the acetone, pooled fractions were lyophilized to yield light, white fluffy solids that were used in the following tests.

The degree of polymerization of fractions A–E was determined by the vanillin assay in acetic acid (Butler et al., 1982). One milliliter of tannin solution (1 mg/mL in MeOH) was diluted at 25 mL with glacial acetic acid. The 1-mL diluted solution was mixed with 4.0 mL of freshly prepared vanillin reagent (4% concentrated HCl and 0.5% vanillin in acetic acid), and the absorbance was determined at 510 nm. Catechin (Sigma) dissolved in acetic acid was used as standard for the calculation of degree of polymerization.

For anthocyanidin analysis, 10-mg tannin fractions were mixed with 1.0 mL of 5% HCl in butanol in sealed tubes and the resultant mixtures heated in a boiling water bath for 2 h. The digest was applied as a streak to a sheet of

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