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The Constituents of Hops. VI[1] Studies of the Volatile Composition of *Humulus lupulus* L. during Ripening

Yoko NAYA and Munio KOTAKE

The Institute of Food Chemistry, Dojimanaka 2-43, Kita-ku, Osaka

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Evidence is presented for the volatile constituents of hops, not only in the resin glands, the so-called lupulin, but also in the stalk with leaves and in the three crops of cones during ripening. Lupulin shows a specific accumulation of myrcene, and the amount of the volatile constituents increases from fifty times to eight hundred times that in any other part of the plant in the course of ripening. While ripe cones contain neither any germacratrienes, the precursors of several sesquiterpenes, nor any aliphatic aldehydes, the stalk with leaves and the young cones have quite high concentrations of them respectively. Observation suggests that some of the volatile constituents are formed at an early stage of growth, whilst others are formed later.

The steam-volatile constituents of the cultivated Japanese hop, "Shinshu-wase" (*Humulus lupulus* L.), have previously been studied.[2-6] In the present paper, the isolation and the identification of volatile compounds from different plant tissues during ripening will be reported.

The part with the highest concentration of volatile components is well-known to be the "lupulin" glands of the cones, whilst the other parts of plant have also been found to include the major constituents to some extent. Germacrene D,[7] which is a key intermediate of cadinene-group compounds, and nonanal are found to be formed at an early stage of growth, whilst myrcene is formed in a remarkable amount at a later stage of growth. This observation suggests the compartmentalization effect of biogenesis in the plant tissue on the accumulation of volatile compounds. However, such a technique of the direct volatilization of individual plant tissue for GLC analysis[8] as will avoid numerous ill effects that the experimental samples will suffer after harvest is required before further detailed study can be done. The positive correlation with the size of the cones, or the formation of lupulin, and the quantity of aroma compounds are shown to be in agreement with the results of the recent study of Hautke *et al.*[9]

Experimental

The seedless Shinshu-wase used for the experiment was all cultivated in the same hop yard in Iwate Prefecture. The experimental samples were twice harvested on July 25th and again on August 31st when the cones were their final size, after dried in an oast below 50°C for several hrs, they were tansported by air each time. The analytical samples of various plant parts at different periods and the contents of the neutral volatile components are listed in Table 1. Lupulin for the sample VI was separated from the other cone parts by grinding in liquid nitrogen.

Each of the six samples was extracted with ether, the extract was then steam-distilled to obtain the essential oil for analysis. The distillate was extracted with ether, and the oil thus obtained was separated into the hydrocarbons and the fraction of the oxygenated constituents by selective adsorption on neutral alumina. The proportions of these two fractions from the six samples examined are also shown

1) Previous paper of this series: Y. Naya and M. Kotake, This Bulletin, **43**, 3594 (1970).

2) N. Shigematsu and Y. Kitazawa, *Bull. of Brew. Sci.*, **8**, 23 (1962).

3) R. G. Buttery, R. E. Lundin, and L. Ling, *Agri. Food Chem.*, **15**, 58 (1967).

4) Y. Naya and M. Kotake, *Nippon Kagaku Zasshi*, **88**, 1302 (1967).

5) Y. Naya and M. Kotake, *ibid.*, **89**, 1113 (1968).

6) Y. Naya and M. Kotake, *ibid.*, **91**, 275 (1970).

7) K. Yoshihara, Y. Ohta, T. Sakai, and Y. Hirose, *Tetrahedron Lett.*, **1969**, 2263.

8) E. von Rudoloff, *J. Gas Chromatogr.*, **3**, 390 (1965).

9) P. Hautke and D. Petricek, *Monatsschrift für Brauerei*, **23**, 241 (1970).

Table 1. Analytical samples from the different plant parts of "shinshu-wase" at different periods and the contents of the volatile compounds

Sample	Plant parts	Harvested date (1970)	Yield (%)	Constituents (%)	
				HC	Oxy.
I	Stalk with leaves	25/July	0.045	54.0	46.0
II	Stalk with leaves	31/Aug.	0.027	69.0	31.0
III	Cones with no lupulin (1/8 size)	25/July	0.087	54.5	45.5
IV	Cones with lupulin (1/4 size)	25/July	0.11	96.7	3.3
V	Cones with lupulin (final size)	31/Aug.	0.805	93.7	6.3
VI	Lupulin only	31/Aug.	41.4	94.1	5.9

in Table 1. Analysis was carried out by a combination of adsorption chromatography (silica gel), preparative GLC (Carbowax 20M), and the temperature-programmed capillary GC–MS method. The identification of the compounds was verified by the agreement of the mass spectra as well as by that of the retention time on GLC with those of authentic samples or of published standard references. Furthermore, the major constituents were isolated using the combined method described above, and the identity of the constituents was confirmed by comparing their IR spectra with those of authentic samples.

Apparatus and Operating Conditions. The preparative GLC conditions, using a model 90-P Varian Aerograph fitted with a thermal conductivity detector, were as follows: a 10-ft. × 3/8 in. aluminum column containing 20% Carbowax 20M on 60—80 mesh Diasolid-L. Helium was used as the carrier gas, and the column temperature was set at 100—220°C.

For the analytical GLC, a Hitachi Model K-53 apparatus equipped with a flame ionization detector was used. The capillary column used for the analysis was 45m × 0.25 mm, made of stainless steel and coated with HB 2000; the column temperature was programmed non-linearly from 100 to 150°C. The helium-carrier gas-inlet pressure was set at 1 kg/cm² for initiation and at 2.5 kg/cm² at the end.

The GC–MS were measured with the direct combination of GLC fitted with a Golay column (45m × 0.5 mm, HB 2000) and mass spectrometry using a Hitachi Model RMU-6 Mass Spectrometer; the operating conditions were as follows: ionization energy, 80 eV; acceleration voltage, 2000 V. The column temperature was programmed non-linearly at 60—150°C under a gas-inlet pressure of 0.6—2.0 kg/cm².

Results and Discussion

The identified constituents of the examined oils of "Shinshu-wase" are listed in Table 2, together with the peak number on the programmed GLC (Fig. 1—6).

Significant differences in the composition during the course of ripening may be seen in the gas chromatograms (Fig. 1—6) and in Table 2. The proportion of the oxygenated constituents to hydrocarbons is approximately 1: 1—2 in the samples I—III, whilst in the samples IV—VI it appears to be 1: 15—29 (Table 1). In the sample IV, the common major hydrocarbons, *i.e.*, caryophyllene, α- and β-humulenes, α- and β-selinenes, δ-cadinene, and myrcene, increase in almost the same ratio (Table 3), while the amount of myrcene in the samples V and VI increases almost 20 times more than in the other samples. It is very interesting to note that the annual plant *H. japonicus*[1] which has

Table 2. The constituents of the examined six samples(I-VI) with the peak number on the programmed GLC (Fig. 1-6)

Identity of Constituent	I	II	III	IV	V	VI
α-Pinene	4				4	
Isobutyl isobutyrate		5			5	5
β-Pinene	7	7		7	7	7
Sabinene	7					
Myrcene	8	8	8	8	8	8
2-Methylbutyl propionate					8	8
n-Undecane				9		
2-Methylbutyl isobutyrate		10			10	10
Limonene					11	11
β-Phellandrene	12	12			12	12
α-Phellandrene	13				13	13
2,2,7,7-Tetramethyl-1,6-dioxaspiro-[4.4]nona-3,8-diene		14	14	14	14	
Octanal			15	15		
Nona-2,4-dienal[a]	15					
Methyl heptanoate		16		16	16	16
n-Dodecane	18		18	18		
trans-3-Hexenol-1	18	18	18	18		
2-Methylbutyl 2-methylbutyrate		18			18	18
Methyl 4-methyl-2-hexenoate					18	
2-Methylbutyl isovalerate		19			19	19
2-Hexenol-1	20					
Methyl 6-methylheptanoate		21			21	21
2-Nonanone	22	22		22	22	22
Nonanal	23		23	23		

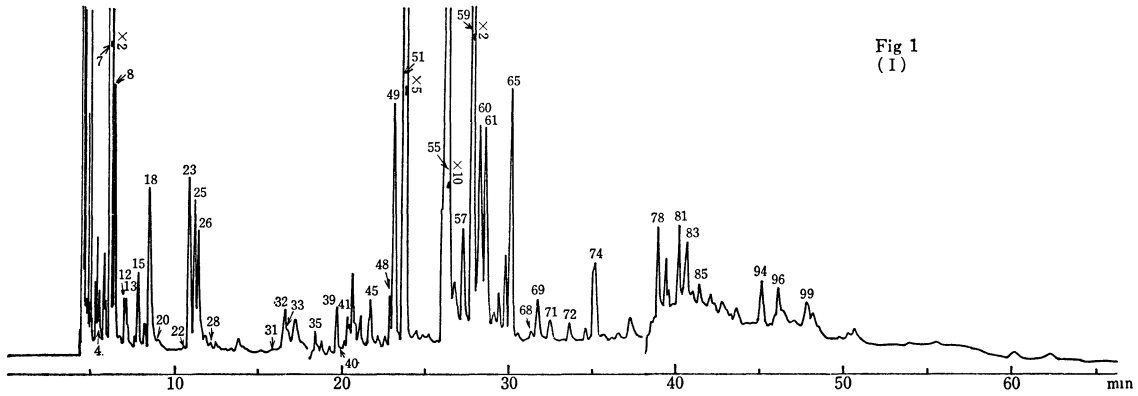
Identity of Constituent	I	II	III	IV	V	VI
Methyl octanoate		24			24	24
1-Octen-3-ol	25	25				
m/e 184, 106(B)[a]	26	26		26		
trans-2-Methyl-2-vinyl-5-hydroxy-isopropyl-tetrahydrofuran		27			27	
n-Tridecane	28	28	28	28		
cis-2-Methyl-2-vinyl-5-hydroxy-isopropyl-tetrahydrofuran		29				
4,4-Dimethylcrotonolactone			30	30		
2-Decanone	31	31			31	31
Linalool	32	32	32	32	32	32
Methyl nonanoate					33	
Decanal	33		33	33		
Decenal[a]			34			
n-Tetradecane	35		35			
Octyl alcohol					36	
9-Methyl-2-decanone	39	39		39	39	39
Terpinen-4-ol	39	39	39			
α-Ylangene	40	40	40	40	40	40
α-Copaene	41	41	41	41	41	41
Methyl 8-methylnonanoate					41	41
2-Undecanone	45	45		45	45	45
4-Undecen-2-one					45	
Undecanal			46	46		
Methyl 4-decenoate					47	

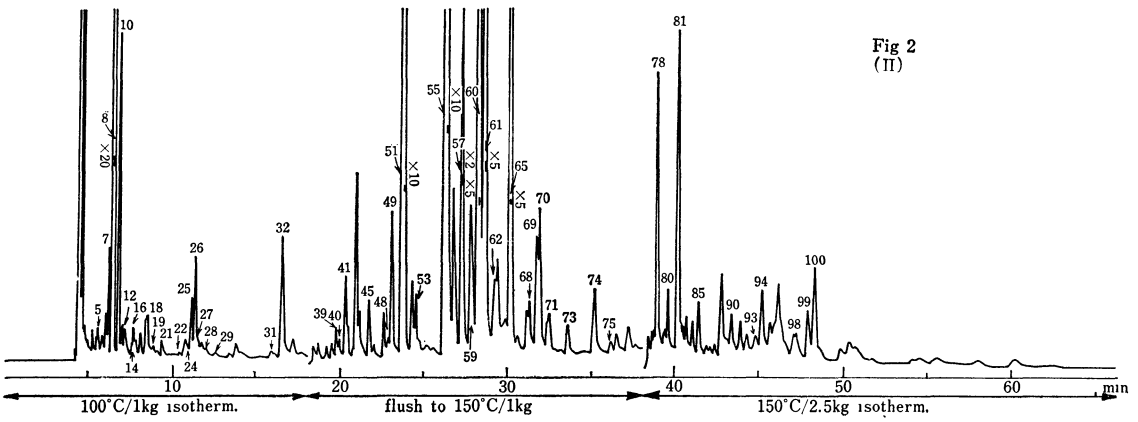
TABLE 2. Continued

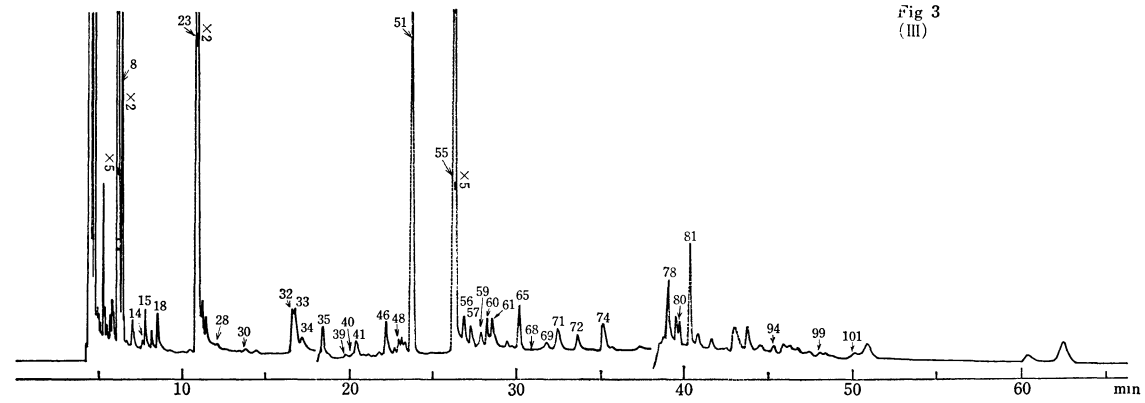
Identity of Constituent	I	II	III	IV	V	VI
α-Terpineol	48	48	48	48	48	48
β-Elemene	49			49		
n-Pentadecane	49	49				
Caryophyllene	51	51	51	51	51	51
β-Farnesene		51		51		
Methyl geranate		53			53	53
α-Humulene	55	55	55	55	55	55
β-Humulene		55	55	55	55	55
Benzyl alcohol	55	55	55	55		
α-Farnesene		55				
Dodecanal			56			
γ-Muurolene	57	57	57	57	57	57
Nerol	57					57
Geranyl acetate					58	58
Germacrene-D	59	59	59			
n-Hexadecane	59		59			
Phenethyl alcohol	60		60	60		
β-Selinene	60	60		60	60	60
α-Selinene	61	61	61	61	61	61
Geraniol		62			62	62
δ-Cadinene	65	65	65	65	65	65
γ-Cadinene				65	65	65
δ_2-Cadinene[a)]		68	68	68	68	68
Heptadecane (branched)[a)]	68		68			
α-Cadinene		69	69	69	69	69
2-Tridecanone	69			69	69	69
Geranyl isobutyrate		70	70		70	70

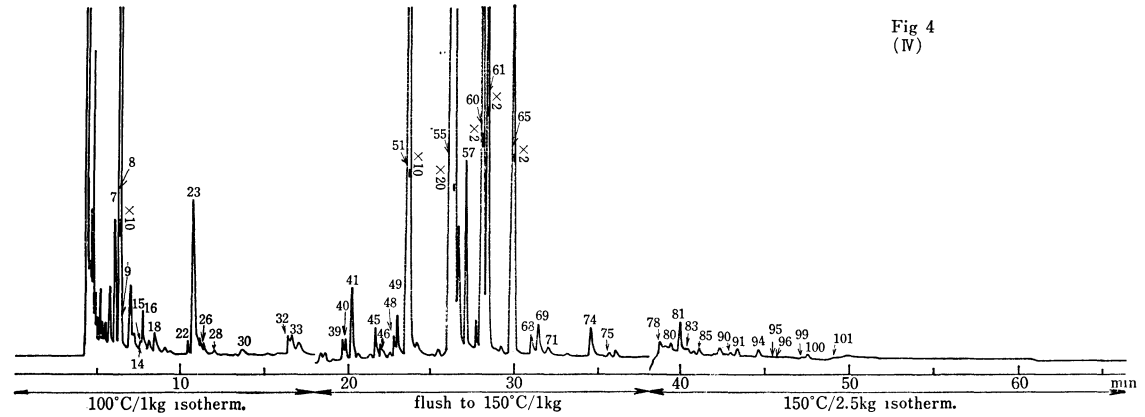
Identity of Constituent	I	II	III	IV	V	VI
Calamenene	71	71	71	71	71	71
n-Heptadecane	72		72			
m/e 210, 126(B)[a)]		73				
m/e 220, 205 (B)[a)]	74		74	74		
m/e 220, 177(B)[a)]	74	74	74	74		
γ-Calacorene					74	74
α-Calacorene		75		75	75	75
Perrillyl alcohol					76	
Caryophyllene-oxide	78	78	78	78	78	78
Humulene-epoxide-I		80	80	80	80	80
Humulene-epoxide-II	81	81	81	81	81	81
Caryophyllene-alcohol					82	82
Nerolidol	83			83	83	83
Junenol						84
epi-Cubenol	85	85		85	85	85
m/e 222, 43(B) (ketone)[a)]						89
m/e 222, 43(B) (juniper camphor type)[a)]		90		90	90	90
Humulol				91	91	91
γ-Eudesmol		93				
T-Cadinol	94	94	94	94	94	94
T-Muurolol				95	95	95
δ-Cadinol	96			96	96	
α-Eudesmol		98			98	
α-Cadinol	99	99	99	99	99	99
Juniper camphor		100		100	100	100
Humulenol-II			101	101	101	101

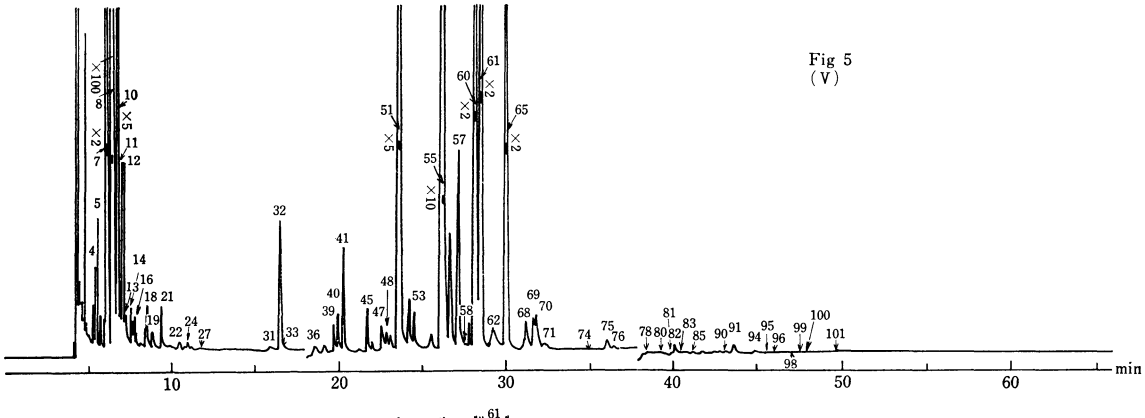
a) tentatively identified or unknown compunds.











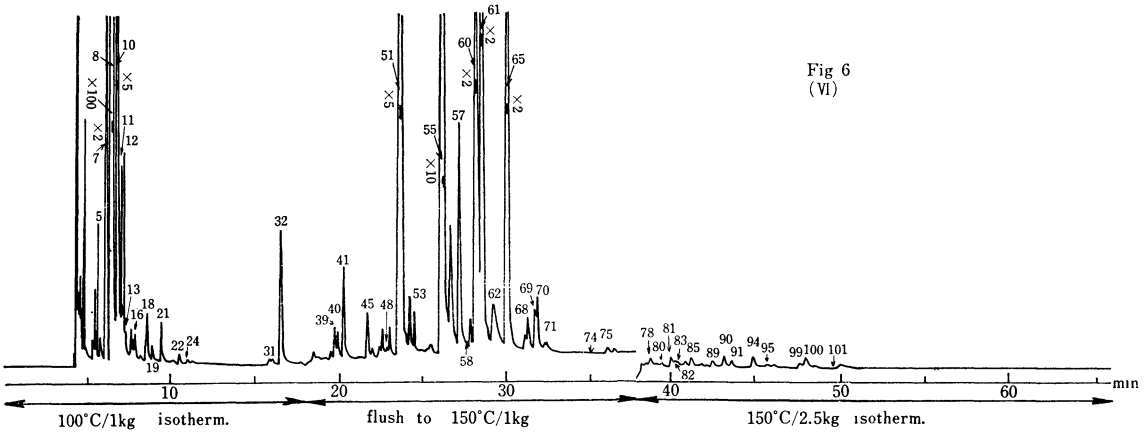


Fig. 1—6. Gas chromatograms of the steam-volatile oil from the examined samples (I—VI). Column 45 m × 0.25 mm coated with HB 2000.

Table 3. Relative abundance of characteristic constituents calculated from the peak area of GLC

Sample	C	H	β-S	α-S	Cd	M	N	G-D
I	1	1.24	0.15	0.15	0.19	0.20		0.45
II	1	1.36	0.42	0.54	0.42	2.32		0.08
III	1	2.50	0.08	0.08	0.13	0.36	1.92	+
IV	1	1.84	0.24	0.26	0.22	0.97	0.09	
V	1	1.45	0.45	0.49	0.38	18.2		
VI	1	1.42	0.42	0.50	0.38	20.6		

C: caryophyllene, H: humulene, β-S: β-selinene, α-S: α-selinene, Cd: cadinenes (δ,γ), M: myrcene, N: nonanal G-D: germacrene-D.

no lupulin, contains only a little amount of myrcene although in other respects it is similar to the volatile constituents of *H. lupulus* previously reported. Therefore, myrcene might be a characteristic compound in the lupulin of fully-ripened hops. Thus, the size of the cones is naturally correlated to the quantity of aroma compounds. The most predominant sesquiterpenic hydrocarbon is humulene, and the next is caryophyllene, in all the examined samples (I—VI), in the ratio of 2: 1. These two hydrocarbons are characteristic of the whole hop plant and are not connected with the differences in the plant tissue and the course of ripening.

A considerable amount of germacrene-D is detected in the sample I at an earlier stage of growth, and a little of it in the samples II and III, but none of it is detected in the samples IV—VI. The contents of selinenes and cadinenes increase considerably at later stages of growth. These compounds are known also to be produced chemically[7] through the germacratrienes. In addition, β-elemene produced by a Cope rearrangement of germacrene-A[10] is found a notable amount in the sample I. The above evidence strongly suggests that the labile germacrene-A, the precursor of β-elemene, α-, and β-selinenes, is present at an earlier stage of growth.

Aliphatic aldehydes, particularly nonanal are specifically accumulated in young cones (the sample III). It is interesting to consider the biological meaning of this compound at that stage of growth. It might be a repellent against insects, with its homologues the stink substances of *Pentatomidae* and *Coreidae*.[11]

10) A. J. Weinhaimer, W. W. Youngblood, P. H. Washecheck, T. K. B. Karns, and L. S. Ciereszko, *Tetrahedron Lett.*, **1970**, 497.

11) T. Tsuyuki, Y. Ogata, I. Yamamoto, and K. Shiomi, *Agr. Biol. Chem.* (Tokyo), **29**, 419 (1965).