Extractives from New Zealand Honeys. 1. White Clover, Manuka, and Kanuka Unifloral Honeys

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Ether extracts were made from aqueous solutions of manuka (*Leptospermum scoparium*), kanuka (*Leptospermum ericoides*), and clover (*Trifolium repens*) honeys with use of a continuous liquid/liquid extractor. The components of the extracts were methylated before being separated and identified by gas chromatography and mass spectrometry, and also by preparative thin-layer chromatography followed by ^1H and ^{13}C NMR analyses. A total of 61 different compounds were detected, and 56 of these were identified. Their concentrations ranged from 0.1 to 4000 $\mu g/g$. Classes of compounds detected included hydrocarbons (C_{21}–C_{33}) and straight-chain monobasic (C_8–C_{28}), dibasic, and aromatic acids. The concentration of aromatic acids in manuka and kanuka honeys was much higher than in clover honey. These acids were not present in a chloroform extract of manuka flowers, which contained many terpenes, none of which were present in manuka honey. Compounds reported for the first time in honey include 2-decenedioic, decanedioic, nonanedioic, and octanedioic acids.

One of the intrinsic features of honey is its natural antibiotic properties. It is known that honey can be kept for long periods of time without becoming spoiled.

Honey is of high osmotic pressure (estimated to be about 2000 mosm), which increases the resistance to spoilage by microorganisms (White, 1975). The acids in honey also contribute to its resistance to spoilage by bacteria (White, 1975). Generally, the pH ranges from 3.4 to 6.1, averaging at about 3.9 (White, 1978). The most studied antibacterial property of honey (Gauhe, 1941; Cocker, 1951; White et al., 1958, 1963) is the action of the enzyme glucose oxidase. Glucose oxidase is virtually inactive in full-density honey but becomes active again in diluted honey, producing hydrogen peroxide (H_2O_2) from glucose.

We have however observed that some New Zealand native honeys, particularly manuka, exhibited a level of additional antibacterial activity greater than that which could be ascribed solely to the three systems listed above (Russell, 1983). Manuka honey typically possessed substantial additional activity whereas honey from white clover was essentially devoid of additional activity (Molan et al., 1987).

Preliminary studies revealed that the antibacterial compounds were unaffected by heat and light and were soluble in organic solvents such as ethanol and ether. Ethanol extraction of manuka honey and subsequent analyses established that two of the major antibacterial components were methyl 4-hydroxy-3,5-dimethoxybenzoate and methyl 3,4,5-trimethoxybenzoate (Russell, 1983).

Total extractable organics from honey have been little studied. Some work has been reported on the volatile constituents of various unifloral honeys (Cremer and Riedmann, 1964, 1965; Wootton et al., 1978; Graddon et al., 1979; Bicchi et al., 1983). Recently the composition of the hydrocarbon fraction of chestnut honey was reported in detail (Bonaga et al., 1986).

In the course of a study to identify the plant-derived antibacterial components of honey, a survey of the extractives in honeys was initiated. The techniques used for the isolation of relatively volatile components are not appropriate for the quantitative recovery of polar phenolic and acidic substances. In this paper we report a procedure for the recovery of these groups of compounds and the application of this technique to a number of white clover, manuka, and kanuka honeys. A preliminary study was also made of the contribution of manuka flower components to honey extractives.

MATERIALS AND METHODS

Samples. A collection of honey samples was obtained from beekeepers throughout New Zealand by the Ministry of Agriculture and Fisheries, Hamilton, New Zealand. All honey samples were collected during the 1982-1986 flowering seasons. The majority were considered to be primarily unifloral specimens. Floral source identification of each honey was based on the flavor, color, and aroma, and also the season and location of its production.

Reagents. All solvents were bulk grade and were redistilled and checked by gas chromatography.

Honey Extraction. In a typical extraction, 20 g of honey was placed in a beaker with 700 mL of distilled water. The resultant mixture was stirred at room temperature for 5 min by means of a magnetic stirrer. Two aliquots of internal standards, undecane (C_{11}) and methyl heptadecanoate (17:0 fatty acid methyl ester) in chloroform (1 mg/mL), were added at the concentration of 10 $\mu g/g$ of honey. As comparatively higher levels of extractable organics were present in manuka and kanuka honey, internal standards were added at 4 times the usual concentration for these honeys. The honey solution (with the internal standards) was then introduced into a standard continuous liquid/liquid extractor. The beaker itself was washed with diethyl ether (3 × 100 mL), and the washings were introduced to the extractor.

All quick-fit joints were carefully sealed with Teflon tape (John Cropper Ltd.). After 24 h of extraction, the ether extract was dried over anhydrous $MgSO_4$. The extract was then concentrated under reduced pressure in an all-glass rotary evaporator at 25 °C. When the volume was suitably reduced (3–5 mL), the extractive solution was transferred to a 10-mL vial and methylated with etheral diazomethane for 2.5 min. Longer derivatization times resulted in the progressive methylation of some of the phenolic hydroxyl groups. Excess diazomethane was blown off with a stream of nitrogen. After final concentration (air drying) to about

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2 mL, the vial was sealed and stored at 5 °C until required.

Flower Extraction. Fresh whole manuka flowers were soaked in chloroform for 2 h. [Simple soaking may not be the appropriate technique (Erickson et al., 1979).] The filtered solution was concentrated and methylated prior to gas chromatography (GC), preparative layer chromatography (PLC), and gas chromatography/mass spectrometry.

Chromatography. Gas chromatography was performed on a Pye Unicam Model PU4500 chromatograph equipped with a flame ionization detector (FID), modified for capillary column with a split injection system (SGE Unijector, split ratio 200:1). The retention times (in seconds) and peak areas were measured by a Spectra-Physics minigrator. Analyses were performed on a fused silica open tubular (FSOT) column, 0.25 mm (i.d.) × 12 m, coated with methylsilicone (SGE Ltd., Melbourne) with hydrogen as carrier. Injector and FID temperatures were 250 °C.

The column oven was temperature programmed from 40 °C (3-min initial hold) to 250 °C (35-min final hold) at 4 °C/min with a carrier gas linear velocity of 40 cm/s. Between 6 and 10 μL of each concentrated extract was injected. Carbon numbers were determined by interpolation of GC retention times to those of a series of *n*-alkanes run under identical conditions.

Quantification was performed relative to an internal standard of heptadecanoic acid (17:0) methyl ester. Response factors were determined for succinic acid, benzoic acid, caprylic acid (8:0), phenylacetic acid, ethyl phenylacetate, 2′-methoxyacetophenone, 2-methoxybenzoic acid, 2-hydroxy-3-phenylpropionic acid, (*E*)-cinnamic acid, octanedioic acid, 2,6-di-*tert*-butyl-4-methylphenol, 3,5-dimethoxybenzoic acid, 3,4-dimethoxybenzoic acid, 3,4,5-trimethoxybenzoic acid, 4-hydroxy-3,5-dimethoxybenzoic acid, and tetracosane (C_{24} alkane), with 1:1 mixtures of the named compounds and the internal standard. Reasonable linearity in detector response relative to the C_{18} hydrocarbon internal standard was demonstrated over the range of concentrations encountered in the study. Class response factors were applied to the series of obviously related compounds.

The unknown organic components were quantified as "succinic acid equivalents" for peaks with retention time up to that of phenylacetic acid, "phenylacetic acid equivalents" for peaks with retention time up to that of 2-hydroxy-3-phenylpropionic acids, "cinnamic acid equivalents" for peaks with retention time up to that of fatty acid 16:0, and "fatty acid 17:0 equivalents" for peaks with retention time of those of fatty acid 16:0 and above. The results are reported as micrograms/gram of honey (fresh weight).

Gas Chromatography/Mass Spectrometry. A Varian MAT CH5 mass spectrometer was coupled with a Varian 2700 gas chromatograph via an open-split interface. An OV-1 FSOT column, 0.32 mm × 25 m (diameter), was employed with helium as carrier gas and the temperature programmed from 40 to 280 °C at 8 °C/min (no initial hold, 10-min final hold). The mass spectra were recorded in electron ionization (EI) mode at 70 eV, ion source temperature 200 °C. The scan repetition rate was 4 s over a mass range of 35 amu to 700 amu. Chemical ionization (isobutane) GC/MS was carried out on a Hewlett-Packard HP5985 mass spectrometer.

^{1}H and ^{13}C NMR. Sufficient quantities were recovered where possible, isolated by PLC on silica gel for ^{1}H and ^{13}C NMR to be recorded. ^{1}H NMR spectra were determined in $CDCl_3$ on a Jeol FX90Q (89.6-MHz) FT NMR spectrometer or Bruker AC200 FT NMR spectrometer using tetramethylsilane as an internal reference. ^{13}C NMR spectra were determined in $CDCl_3$ on a Jeol FX90Q (22.5-MHz) FT NMR spectrometer or a Bruker AC200 (50 MHz) FT NMR spectrometer using tetramethylsilane as an internal standard.

RESULTS

Various extracting solvents were investigated in the liquid/liquid extractor: ethyl acetate, chloroform, ether, hexane. The volume of solvent used in each extraction was about 300 mL. Each of the solvents gave extractive mixtures for which generally similar profiles were observed. The higher the extraction temperature, the greater the level of (hydroxymethyl)furfural (HMF). The use of ether substantially reduced the level of HMF and ethyl ester analogues of the parent acids. It is concluded that HMF arises from an acid-catalyzed thermal elimination of water from glucose and fructose. Crane (1980) reported that every extra 10 °C increases HMF production by about 4.5 times.

Additionally, these studies revealed that at the higher extraction temperatures the ethyl ester levels increased at the expense of the parent acids, particularly for aromatic acids. Presumably the higher levels of esters reflect an increased rate of acid-catalyzed esterification of these acids of ethanol, which had probably arisen from fermentation of glucose and fructose by yeast cells in the honey samples supplied.

Although higher temperatures facilitated dissolving of the honey in water prior to extraction, significant losses of more volatile components occurred. Accordingly, dissolution was carried out at room temperature with stirring for 5 min. Adjusting to pH 2 did not increase the quantity of extracted material. Extraction was therefore carried out at natural pH to avoid possible degradation of some compounds. Liquid/liquid extraction was carried out for 24 h as no increase in yields was obtained at longer extraction times.

The identification of individual components was based on a combination of the GC carbon number and published mass spectra. Identification was confirmed by comparison with authentic samples in most cases. In the case of uncertain or absent molecular ions, CI (isobutane) mass spectra were used to deduce molecular weight. Examination of blanks (from methylation and from ether) under the conditions employed did not show any interfering components.

The acidic compounds were identified and quantified as their methyl esters. However, gas chromatography of unmethylated extracts demonstrated that the majority of the acids were present in the free form.

Table I summarizes the compounds present in the clover, manuka, and kanuka honeys examined, together with the prominent ions in the EI mass spectra.

White Clover Honey. A representative GC trace of white clover honey is depicted in Figure 1a. All eight clover honey samples gave remarkably similar GC profiles. Some samples showed extra peaks, which were identified by GC/MS as the ethyl esters of the major parent acids. Presumably these arose by acid-catalyzed esterification with ethanol. Quantitative data for the eight clover honeys examined are given in Table II.

The hydrocarbons (peaks 35, 41, 42, 44, 45, 48, 49, 52, 53, 56, 57, 58, 59, and 61) consisted mainly of *n*-alkanes typically ranging from C_{21} to C_{33}, with C_{27} most abundant. Even-numbered alkanes were in much lower concentrations. Some unsaturated analogues were also detected (see Table II). Despite their variation from sample to sample, the relative concentrations of C_{23} alkane, C_{25} alkane, C_{27}

Table I. GC/MS Summary of Honey Extracts (Components in Order of Elution Time)

peak no.	C no.	prominent ions	compound	basis for ident[a-c]
1	9.74	43, 57, *69*, 87	unknown (manuka)	MS
2	9.76	54, 57, *90*	unknown (manuka)	MS
3	10.04	55, 59, 87, 114, *115*	dimethyl succinate	GC, MS
4	10.64	51, 77, *105*, 136 M^+	methyl benzoate	GC, MS
5	10.70	55, 59, 87, 101, *115*	ethyl methyl succinate	MS
6	11.07	*74*, 87, 158 M^+	methyl caprylate (8:0)	GC, MS
7	11.44	65, *91*, 150 M^+	methyl phenylacetate	GC, MS
8	12.24	65, *91*, 105, 164 M^+	ethyl phenylacetate	GC, MS
9	12.50	77, 91, 105, *135*, 150 M^+	2′-methoxyacetophenone	GC, MS
10	12.95	77, 92, *135*, 151, 166 M^+	methyl 2-methoxybenzoate	GC, MS
11	13.36	65, *91*, 121, 162, 180 M^+	methyl 2-hydroxy-3-phenylpropionate	GC, MS, NMR
12	13.50	77, 103, *131*, 162 M^+	methyl (*E*)-cinnamate	GC, MS
13	14.07	69, *74*, 97, 107, 138, 171	dimethyl octanedionate	GC, MS
14	14.15	55, *71*, 97, 117, 165, 180 M^+	unknown (clover)	MS
15	14.45	*91*, 103, 121, 176, 194 M^+	ethyl 2-hydroxy-3-phenylpropionate	MS
16	14.78	*74*, 87, 119, 214	methyl laurate (12:0)	GC, MS
17	14.85	*55*, 74, 83, 111, 152, 185	dimethyl nonanedioate	MS
18	15.10	107, 122, 138, 165, *196* M^+	methyl 3,5-dimethoxybenzoate	GC, MS
19	15.20	69, *165*, 181, 196 M^+	methyl 3,4-dimethoxybenzoate	GC, MS
20	15.40	91, *121*, 151, 192, 210 M^+	methyl 2-hydroxy-3-(4-methoxyphenyl)propionate	MS
21	16.20	59, *74*, 98, 125, 157, 199	dimethyl decanedioate	MS
22	16.60	87, 95, *136*, 164, 196, 197	dimethyl 2-decenedioate	MS, NMR
23	16.78	59, 195, 211, *226* M^+	methyl 3,4,5-trimethoxybenzoate	GC, MS
24	16.92	74, 98, *125*, 166, 199, 213	ethyl methyl decanedioate	MS
25	17.15	*74*, 87, 107, 121, 242 M^+	methyl myristate (14:0)	GC, MS
26	17.25	153, 181, 197, *212* M^+	methyl 4-hydroxy-3,5-dimethoxybenzoate	GC, MS
27	17.35	81, 108, 119, *136*, 164, 197	ethyl methyl 2-decenedioate	MS
28	17.53	73, 103, 223, *254*, 269, 284 M^+	unknown (manuka)	MS
29	18.30	75, *103*, 167, 227, 242 M^+	unknown (mamuka)	MS
30	19.04	*74*, 87, 143, 239, 270 M^+	methyl palmitate (16:0)	GC, MS
31	19.70	*88*, 101, 157, 284 M^+	ethyl palmitate (16:0)	MS
32	20.61	59, 74, *81*, 95, 109, 294 M^+	methyl linoleate (18:2)	GC, MS
33	20.69	59, 67, *79*, 95, 108, 292 M^+	methyl α-linolenate (18:3)	MS
34	20.76	55, 69, *74*, 83, 97, 264, 296 M^+	methyl oleate (18:1)	GC, MS
35	21.00	*57*, 71, 85, 113, 296 M^+	C_{21} alkane	GC, MS
36	21.16	59, *74*, 87, 225, 298 M^+	methyl stearate (18:0)	GC, MS
37	21.39	50, *74*, 83, 95, 109, 308 M^+	ethyl linoleate (18:2)	MS
38	21.55	59, 67, *79*, 95, 108, 306 M^+	ethyl α-linolenate (18:3)	MS
39	21.60	69, *83*, 97, 101, 264, 310 M^+	ethyl oleate (18:1)	MS
40	22.02	59, *74*, 88, 101, 312 M^+	ethyl stearate (18:0)	MS
41	22.00	*57*, 71, 85, 99, 310 M^+	C_{22} alkane	GC, MS
42	23.00	*57*, 71, 85, 324 M^+	C_{23} alkane	GC, MS
43	23.02	*74*, 87, 354 M^+	methyl arachidate (20:0)	GC, MS
44	24.00	*57*, 71, 85, 338 M^+	C_{24} alkane	GC, MS
45	25.00	*57*, 71, 85, 352 M^+	C_{25} alkane	GC, MS
46	25.10	*74*, 87, 354 M^+	methyl behenate (22:0)	GC, MS
48	26.00	*57*, 71, 85, 99, 366 M^+	C_{26} alkane	GC, MS
49	27.00	*57*, 71, 85, 99, 380 M^+	C_{27} alkane	GC, MS
50	27.10	*74*, 87, 143, 382 M^+	methyl lignocerate (24:0)	GC, MS
52	28.00	*57*, 71, 85, 99, 394 M^+	C_{28} alkane	GC, MS
53	29.00	*57*, 71, 85, 99, 408 M^+	C_{29} alkane	GC, MS
54	29.04	*74*, 87, 143, 410 M^+	methyl cerotate (26:0)	GC, MS
56	30.0	*57*, 71, 85, 422 M^+	C_{30} alkane	GC, MS
57	30.62	55, *57*, 69, 83, 97, 434 M^+	$C_{31:1}$ alkene	MS
58	30.85	55, 57, 69, 83, *97*, 434 M^+	$C_{31:1}$ alkene	MS
59	31.00	*57*, 71, 85, 99, 436 M^+	C_{31} alkane	GC, MS
60	31.25	*74*, 87, 143, 438 M^+	methyl montanate (28:0)	GC, MS
61	32.20	57, 69, 83, *97*, 111, 462 M^+	$C_{33:1}$ alkene	MS

[a] GC, gas chromatographic data. [b] MS, mass spectral data. [c] ^1H and ^{13}C NMR data.

alkane, C_{29} alkane, C_{31} alkene, C_{31} alkene, C_{31} alkane, and C_{33} alkene in each sample remained consistently in the approximate ratios of 1:2:6:3:1:1:2:3.

GC investigation of the ether-extractable lipids revealed the presence of dibasic as well as monobasic acids. The first to elute was succinic acid (peak 3). Other dibasic acids detected were octanedioic (peak 13), decanedioic (peak 21), and 2-decenedioic (peak 22), and a trace of nonanedioic (peak 17) acids. None of the diacids gave molecular ions in EI mass spectra.

The identification of 2-decenedioic acid dimethyl ester was initially based on a match of retention index and mass spectrum to that reported for a urinary acid (Spiteller and Spiteller, 1979) and a component of royal jelly (Lercker et al., 1981, 1982). It is the only decenedioic acid isomer with a carbon number higher than decanedioic acid on a nonpolar phase.

The position and configuration of the double bond were confirmed as 2(*E*) by NMR analysis of a diacid fraction isolated by PLC fractionation of clover honey sample 3 (Table II). The structure of the methylated diacid was further substantiated by a comparison of the ^1H and ^{13}C NMR spectral data determined for a specimen of 2(*E*)-dodecenedioic acid dimethyl ester (Sigma, St. Louis, MO).

The monobasic acids (peaks 6, 16, 25, 30, 32, 33, 34, 36, 43, 46, 50, 54, and 60) revealed a great variation in terms of concentration and carbon chain length. In general, palmitic acid (16:0) (peak 30), lignoceric acid (24:0) (peak

Table II. Concentration (μg/g) of Methylated Components in Clover Honey

peak	compound	sample number 1	2	3	4	5	6	7	8	mean
3	dimethyl succinate	13.1	7.1	36.9	43.8	13.6	62.8	5.1	24.7	25.9
4	methyl benzoate	0.4	1.0	1.1	0.5	0.8	0.9	0.2	24.7	3.7
6	methyl caprylate (8:0)			0.2				0.1		0.2
7	methyl phenylacetate	1.2	1.0	1.1	0.5	0.8	0.9	0.2	0.4	0.8
9	2′-methoxyacetophenone		1.5							1.5
10	methyl 2-methoxybenzoate	1.3	11.3	2.8				0.6	1.1	3.4
11	methyl 2-hydroxy-3-phenylpropionate	2.5	66.9	4.5	4.7	9.3	10.6	33.5	44.6	22.1
12	methyl (*E*)-cinnamate	3.9	1.6	3.3	3.6	2.3	1.8		1.7	2.6
13	dimethyl octanedioate	0.9	1.5	9.3	0.8	0.9	0.7	0.8	1.2	2.0
14	unknown	12.5	30.2		12.0	5.9	9.1		1.3	11.8
16	methyl laurate (12:0)	0.1	0.2	0.4	0.9		0.1	0.2	0.5	0.3
17	dimethyl nonanedioate	0.3	0.5	0.8	0.2	0.1	0.2	0.9	0.2	0.4
18	methyl 3,5-dimethoxybenzoate		0.3				0.1	0.3	0.2	0.2
19	methyl 3,4-dimethoxybenzoate	0.4	0.5	2.9	0.3	0.3	0.3		0.5	0.7
20	methyl 2-hydroxy-3-(4-methoxyphenyl)propionate		0.2			0.2			20.6	7.0
21	dimethyl decanedioate	4.0	8.7	50.9	4.9	5.0	4.7	4.7	7.5	11.3
22	dimethyl 2-decenedioate	14.9	30.3	181.2	17.1	19.8	18.0	14.3	26.9	40.3
23	methyl 3,4,5-trimethoxybenzoate		1.6						1.0	1.3
25	methyl myristate (14:0)	0.3	0.8	1.2	0.1	0.3	0.1	0.6	0.5	0.5
26	methyl 4-hydroxy-3,5-dimethoxybenzoate		2.6						1.8	2.2
30	methyl palmitate (16:0)	3.9	12.2	16.0	3.9	11.4	7.4	6.2	17.5	13.6
32	methyl linoleate (18:2)	0.7	1.5					1.3		
				11.9[a]	4.1[a]	15.0[a]	9.6[a]		12.9[a]	8.4[a]
33	methyl α-linolenate (18:3)	2.6	4.7					2.6		
34	methyl oleate (18:1)	2.0	10.7	11.2	3.2	4.6	3.8	5.0	14.3	6.9
35	heneicosane (C_{21})	0.2	0.3	0.4	0.1	0.1	0.2	1.6	0.4	0.4
36	methyl stearate (18:0)	0.5	2.2	2.7	0.5	0.8	0.7	0.9	2.6	1.4
41	docosane (C_{22})	0.3	0.3	0.2				0.2		0.3
42	tricosane (C_{23})	0.9	1.8	3.1	0.8	1.3	1.1	3.0	1.8	1.7
43	methyl arachidate (20:0)	0.1	0.1	0.4		0.1		0.1	0.1	0.2
44	tetracosane (C_{24})	0.6	0.1	0.2	0.1	0.1	0.1	0.1	0.2	0.2
45	pentacosane (C_{25})	1.9	4.1	7.5	1.8	2.9	2.1	5.0	4.3	3.7
46	methyl behenate (22:0)	0.3	0.9	1.2	0.3	0.8	0.5	2.3	1.2	0.9
48	hexacosane (C_{26})	0.1	0.4	0.4	0.1	0.2	0.1	0.3	0.3	0.2
49	heptacosane (C_{27})	4.9	12.8	12.2	4.7	8.1	5.9	13.7	12.8	9.4
50	methyl lignocerate (24:0)	2.6	8.1	7.3	3.3	6.9	4.3	9.8	10.9	6.7
52	octacosane (C_{28})	0.3	0.5	1.9	0.1	0.3	0.1	0.5	0.3	0.5
53	nonacosane (C_{29})	2.8	6.3	7.0	2.4	3.9	3.1	6.3	6.1	4.7
54	methyl cerotate (26:0)	0.8	2.5	2.1	1.0	2.2	1.6	2.8	3.3	2.0
56	triacontene (C_{30})	0.1	0.1	0.3		0.1	0.1	0.1	0.1	0.1
57	hentriacontene ($C_{31:1}$)	1.6	2.4	5.6	0.9	1.6	1.4	1.4	2.0	2.1
58	hentriacontena ($C_{31:1}$)	1.0	1.7	3.4	0.6	1.2	0.9	1.0	1.5	1.4
59	hentriacontane (C_{31})	1.7	3.3	4.3	1.3	2.4	1.8	3.4	3.5	2.7
60	methyl montanate (28:0)	0.8	2.6	2.2	0.8	1.9	1.2	2.8	2.5	1.9
61	tritriacontene ($C_{33:1}$)	3.0	4.6	12.1	2.2	4.4	2.9	3.8	5.8	4.9

[a] Fatty acids 18:2 and 18:3 unresolved.

Table III. Relative Percent Composition of Straight-Chain Fatty Acids in Clover Honeys

chain length	sample number 1	2	3	4	5	6	7	8	mean
8:0	0.1	0.1	0.4				0.5		0.3
12:0	0.5	0.4	0.7	0.5	0.1	0.2	0.7	0.7	0.5
14:0	1.7	1.6	2.1	0.9	0.6	0.5	2.2	0.8	1.3
16:0	26.4	26.3	27.6	22.7	26.8	25.2	21.7	26.3	25.3
18:3	17.5	10.2					9.0		
			20.5[a]	23.6[a]	34.1[a]	32.7[a]		19.5[a]	21.9[a]
18:2	4.7	3.2					4.4		
18:1	13.8	22.9	19.4	18.6	10.5	13.1	17.7	21.6	17.2
18:0	3.8	4.7	4.7	2.7	1.9	2.5	3.3	3.9	3.4
20:0	0.7	0.3	2.5	0.2	0.3	0.1	0.4	0.2	0.6
22:0	2.3	2.0	2.0	1.9	1.8	1.6	8.0	1.8	2.7
24:0	17.6	17.4	12.6	18.9	15.8	14.7	34.5	16.4	18.5
26:0	5.5	5.4	3.7	5.8	4.9	5.4	9.8	5.0	5.7
28:0	5.4	5.5	3.9	4.3	4.2	4.0	9.9	3.8	5.1

[a] Fatty acids 18:2 and 18:3 unresolved.

50), oleic acid (18:1) (peak 34), and α-linolenic acid (18:3) (peak 33) dominated the GC traces. Certain pairs were only partially resolved, e.g. linoleic (18:2) (peak 32) and α-linolenic acids. Table III shows the relative percentage composition of these acids in the eight clover honey samples studied.

A wide variety of aromatic substances were detected. In clover honey, among the more dominant aromatic compounds was 2-hydroxy-3-phenylpropionic acid (peak 11). Its identity was confirmed by direct comparison with an authentic standard. The amount of 2-hydroxy-3-phenylpropionic acid varied greatly in different honey samples,

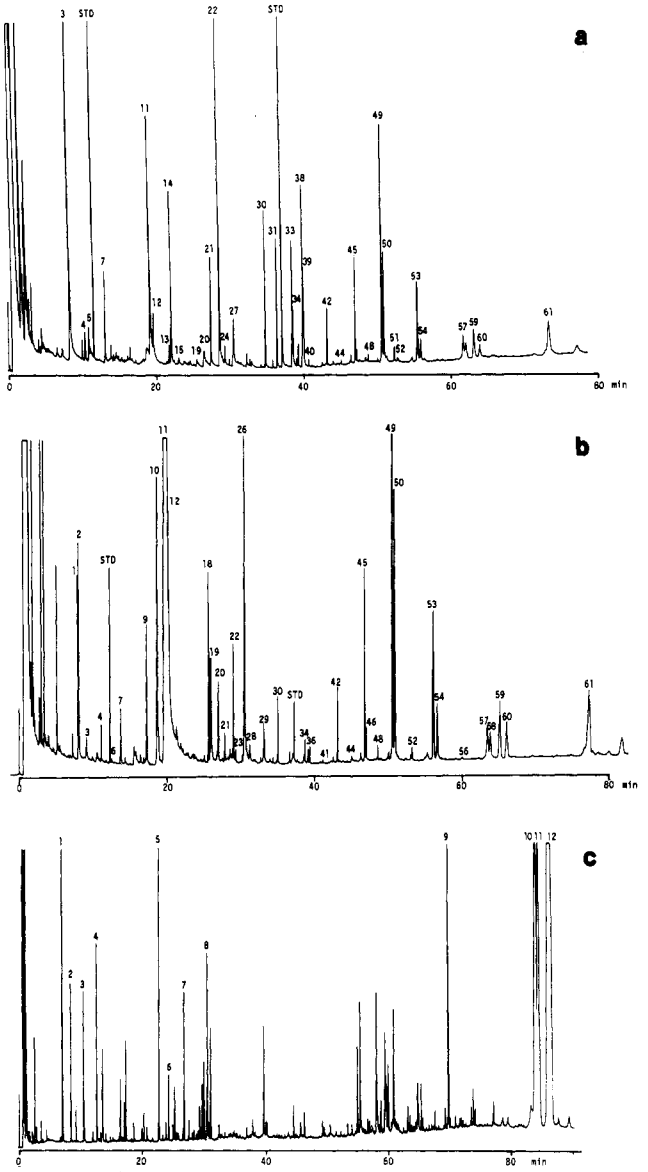

Figure 1. Representative chromatograms: (a) clover honey; (b) manuka and kanuka honeys; (c) chloroform extract of whole manuka flowers. For peak identification, see Table I. GC conditions are as stated in Materials and Methods except for (c) in which the detector and injector temperature was maintained at 270 °C and the column oven was temperature programmed from 35 °C (2-min initial hold) to 270 °C (30-min final hold) at 3 °C/min with carrier gas (H_2) linear velocity of 46 cm/s.

the absolute quantity varying from 2.5 μg/g (clover 1) to 66.9 μg/g (clover 2).

Other aromatic compounds detected were phenylacetic acid (peak 7), (*E*)-cinnamic acid (peak 12), and benzoic acid (peak 4). Their identities were also confirmed by direct comparison with authentic standards. Clover sample 8 revealed the presence of a large amount (20.6 μg/g) of 2-hydroxy-3-(4-methoxyphenyl)propionic acid (peak 20). This compound was not a major component in other clover honey samples (concentration in the range 0–0.2 μg/g). This suggests that another floral source contributed to clover sample 8.

A number of minor components could not be identified from the mass spectra. For example, peak 14 (see Figure 2) possessed a mass spectrum suggesting it to be a mixture of two components with molecular ions *m/e* 166 and 180. Loss of a methyl radical from these two M^+ ions would give rise to the ions at *m/e* 151 and 165, respectively.

Kanuka and Manuka Honeys. Kanuka and manuka

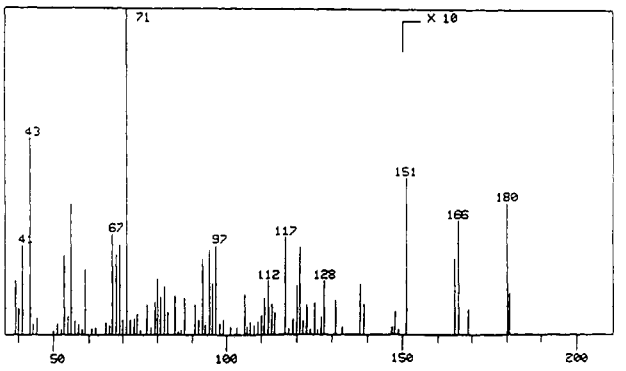

Figure 2. Mass spectrum of an unidentified compound that occurs as a significant component in clover honey extracts.

are of the same family and genus. Consequently it can be anticipated that the two species may give rise to similar types of honeys, and this indeed was observed. Accordingly, they are discussed together under the same heading.

Two kanuka and six manuka honeys were analyzed (sample numbers 9–16); a representative GC trace is shown in Figure 1b. As in the clover honey, the GC analyses were complicated by a series of ethyl ester peaks. Table IV lists the components and their concentrations found in the two kanuka and six manuka honey samples studied. It is evident from Table IV that the manuka honeys possessed much higher concentrations of aromatic acids than the clover honeys. Hydrocarbon and higher fatty acid compositions and concentrations were similar to clover honey and are not reported.

Aromatic acids were invariably dominated by 2-hydroxy-3-phenylpropionic acid (peak 11). Lesser amounts of 2-methoxybenzoic acid (peak 10), 2′-methoxyacetophenone (peak 9), 2-decenedioic acid (peak 22), and 4-hydroxy-3,5-dimethoxybenzoic acid (peak 26) were also detected. The latter compound was consistently detected in all the samples studied. It was also one of the components detected by Russell (1983) in her studies of antibacterial activity in honey. This was one of the few acids found to be in the honey mainly in the methyl ester form.

Manuka Flower Extractives. Because of the high levels of phenolic and acidic compounds recovered from manuka honey, an attempt was made to correlate the extractives from manuka flowers with the components found in the honey.

Figure 1c depicts the GC profile of the methylated extractive. Little similarity exists between this GC trace (Figure 1c) and the representative manuka honey profile shown in Figure 1b.

The principal compounds recovered from manuka flower were demonstrated to be triterpenoids (peaks 10–12). PLC on silica gel was used to isolate fractions for ^1H and ^{13}C NMR. The molecular weights of peaks 10–12, as determined by probe MS, were in each case 470. From a combination of the MS and ^1H and ^{13}C NMR data, peaks 10–12 were shown to be triterpene acid methyl esters: methyl ursolate, methyl oleanolate, and methyl betulinate.

The principal classes of the minor components were monoterpenes and sesquiterpenes. Chief amongst the monoterpenes were α-pinene (peak 1), β-pinene (peak 2), cineole (peak 3), and linalool (peak 4), while the principal sesquiterpenes appeared to be cadinene (peak 6), caryophyllene (peak 7), and unidentified peaks 5 and 8. A collection of other substances having molecular weights in the range 220–262 were also detected. These appeared to be oxygenated sesquiterpenes. Since none of these substances were found in the honey, their structures were not investigated further. Some hydrocarbons were also

Table IV. Concentration (μg/g) of Methylated Components in Kanuka and Manuka Honeys

peak	compound	sample number 9	10	11	12	13	14	15	16	mean
1	unknown	6.5	3.2	71.2	1.3	5.4	9.8	0.8	1.5	12.5
2	unknown	2.5	2.0	84.0	1.7	2.5	5.1	1.0	1.6	12.6
3	dimethyl succinate	7.5	23.4	21.1	7.0	19.1	59.2	65.0	27.4	28.7
4	methyl benzoate	2.7	1.0	9.0	2.5	2.1	1.8	3.1	1.5	3.0
6	methyl caprylate (8:0)	0.4	0.3	0.4	0.3	0.2	0.6	1.1	0.2	0.4
7	methyl phenylacetate	2.2	1.8	12.4	2.5	15.3	4.3	34.4	2.6	9.4
9	2′-methoxyacetophenone	15.9	5.0	52.1	32.9	17.8	33.4	21.3	8.1	23.3
10	methyl 2-methoxybenzoate	103	21.5	143	20.5	36.6	19.0	12.0	14.7	46.3
11	methyl 2-hydroxy-3-phenylpropionate	733	816	4474	1440	1668	1466	2348	1209	1769
12	methyl (*E*)-cinnamate	1.6	327	200	8.0	5.1	115	95.7	92.7	106
13	dimethyl octanedioate	0.4	0.8	1.5	0.5	0.2	0.6		1.2	0.7
16	methyl laurate (12:0)	0.2	1.2	0.5	0.2	0.1	0.3		0.3	0.4
17	dimethyl nonanedioate	0.7	1.4	2.6	1.1	0.2	1.4	1.3	1.2	1.2
18	methyl 3,5-dimethoxybenzoate	22.9	0.5	52.2	6.6	8.9	20.4	3.3	3.6	14.8
19	methyl 3,4-dimethoxybenzoate	7.2	2.5	42.0	7.3	6.3	6.9	2.1	10.0	10.5
20	methyl 2-hydroxy-3-(4-methoxyphenyl)propionate	22.4	44.3	29.5	6.2	14.4	6.5	508	12.4	80.5
21	dimethyl decanedioate	5.4	3.4	10.5	3.4	0.3	6.0	8.6	11.5	6.1
22	dimethyl 2-decenedioate	19.6	39.2	42.5	13.1	3.5	16.3	59.5	55.1	31.1
23	methyl 3,4,5-trimethoxybenzoate	21.4	3.7	7.3	28.6	2.5	16.3	8.5	4.8	11.6
25	methyl myristate (14:0)	0.4	0.5	2.3	0.3	0.2	0.3	1.6	0.7	0.8
26	methyl 4-hydroxy-3,5-dimethoxybenzoate	470	26	355	329	306	372	312	105	277
28	unknown	3.3	4.2	5.9	0.5	20.6	26.4	4.6	3.9	8.7
29	unknown	6.0	9.5	10.6	5.4	13.0	18.3		6.4	9.9
30	methyl palmitate (16:0)	3.0	1.5	23.0	2.4	0.5	9.1	13.2	6.4	7.4
32	methyl linoleate (18:2)	1.1[a]	0.4	3.8[a]	0.3	0.1	0.3	7.0	2.3[a]	4.8[a]
33	methyl α-linolenate (18:3)		0.5		1.0	0.5	0.6	20.5		
34	methyl oleate (18:1)	2.3	1.3	9.6	2.4	0.5	2.2	9.4	4.3	4.0

[a] Fatty acids 18:2 and 18:3 unresolved.

characterized, nonacosane (peak 9) being dominant.

DISCUSSION

The results of GC/MS studies have shown that honey extracts are intricate mixtures containing various classes of components, the concentrations of which vary from 0.1 to 4000 μg/g. The principal constituents recovered were hydrocarbons (C_{21}–C_{33}), fatty acids (C_8–C_{28}), phenolic acids, and diacids. The majority of the substances listed in Table I have been reported previously in honey apart from the diacids.

The presence of high molecular weight hydrocarbons in honey extracts were not unexpected. Undoubtedly they arise from beeswax, which has not been completely separated during harvest and processing. Further characterization of this fraction was not attempted as it was not likely to give significant biological activity. A detailed compositional analysis for chestnut honey has been reported (Bonaga et al., 1986), including structural elucidation of unsaturates. They showed that the abundance order of hydrocarbons was C_{27} alkane and then C_{29} alkane followed by C_{23} alkane and C_{25} alkane. The concentrations of odd-numbered *n*-alkanes were much higher than the even-numbered *n*-alkanes. This is similar to the results found in the present study except that the level of C_{25} alkane is always higher than C_{23} alkane. In fact the relative amounts of alkanes found in this study are more closely related to those reported by Graddon et al. (1979) and Tulloch and Hoffman (1972).

While the absolute amounts of the individual fatty acids varied from sample to sample, the relative amounts (see Table III) did not vary greatly. The major components were palmitic acid (16:0) (peak 30) and lignoceric acid (24:0) (peak 50), followed by oleic acid (18:1) (peak 34) and α-linolenic acid (18:3) (peak 33).

The composition of fatty acids in cotton honey has previously been reported by Smith (1963, 1966) and in beeswax by Tulloch and Hoffman (1972). Smith reported that the highest fatty acid level in cotton honey was oleic acid (18:1). On the other hand, Tulloch's report on the studies of 80 Canadian beeswax samples showed the highest level of fatty acid to be lignoceric acid (24:0). Unsaturated fatty acids of the same chain length were not assessed separately in his study. [Components of the lipid fraction of beeswax were described in an earlier paper (Tulloch, 1971).] In addition, Tulloch's report also includes a range of much higher carbon fatty acids up to C_{52}. These fatty acids were not detected in the present study because the GC conditions used only eluted methyl esters up to C_{30}.

It is interesting to note that, as early as 1911, Ehrlich and Jacobsen (1911) demonstrated that 2-hydroxy-3-phenylpropionic acid could be produced by the action of microorganisms on phenylalanine. Its presence in honey was first reported by Hodges and White (1966), who isolated one optically active isomer, namely the (+)-form from a New Zealand honey.

The concentrations of trace organics in manuka/kanuka honey were 1000 times greater than those in white clover honey (Tables II and IV). Even modest contamination of the presumed unifloral source clover honey samples by (for example) manuka honey would dramatically increase the level of 2-hydroxy-3-phenylpropionic acid detected.

It is accepted that a truly unifloral honey is impossible to obtain. Strong flavored and dark colored honeys such as manuka may also be derived from a significant percentage of clover nectar. Although pollen analysis can give some guide, the proportions of pollen may differ from the proportions of nectars contributing to a honey.

New Zealand white clover honeys were characterized by low overall recoverable trace organics. Only three constituents of the clover honeys were generally present in concentrations greater than 5 μg/g, these being the unknown component (peak 14), succinic acid (peak 3), and 2-decenedioic acid (peak 22). Those honeys with 2-hydroxy-3-phenylpropionic acid as a major component are considered to contain contributions from other floral sources.

Manuka and kanuka honeys are characterized by the presence of 4-hydroxy-3,5-dimethoxybenzoic acid (peak 26), 2′-methoxyacetophenone (peak 9), and 2-methoxybenzoic acid (peak 10), with 2-hydroxy-3-phenylpropionic acid (peak 11) dominating the GC trace. These components serve to differentiate manuka or kanuka honey from clover honey.

The presence of triterpenoids in manuka flowers was not surprising since Cambie (1976) reported the presence of betulic acid, oleanolic acid, and ursolic acid acetate in the bark of manuka. Despite the dominance of these triterpenoids in manuka flower, no traces were found in the honey studied. Similarly, monoterpenes and sesquiterpenes were not detected in manuka honey. The absence of 2-hydroxy-3-phenylpropionic acid, 4-hydroxy-3,5-dimethoxybenzoic acid, 2′-methoxyacetophenone, and 2-methoxybenzoic acid in manuka flowers suggests that these substances may be derived from honeydew.

Manuka plants are generally attacked by an Australian scale insect (*Eriococcus orariensis*), resulting in what is commonly known as manuka blight (Hoy, 1961). This insect lies on the bark and drains the sap, copiously excreting honeydew. Sooty mold fungi grow on the honeydew, giving rise to a blackened appearance of the bark and leaves. Honeydew is known to be collected by foraging bees. Phenylalanine in honeydew could be metabolized to 2-hydroxy-3-phenylpropionic acid by bacteria or fungi in the honeydew or by the bee during honey production.

Behavioral Substances. Of the compounds identified, the diacids are of special interest. Octanedioic, nonanedioic, decanedioic, and 2-decenedioic acids are reported in honey for the first time, despite the fact that they have long been recognized as part of the pheromone system of the honey bee *Apis mellifera*. Octanedioic acid has been reported in the extracts of worker larvae (Gochnauer and Shearer, 1981) while nonanedioic, decanedioic, and 2-decenedioic have been reported in the extracts of queen larvae or royal jelly (Collow et al., 1964; Lercker et al., 1981, 1982).

2-Decenedioic acid was the most prominent diacid in the honeys. Its concentration in clover, kanuka, and manuka honeys ranged from 3.5 to 181.2 μg/g, with a mean level of 32.1 μg/g. The presence of these compounds in honey poses interesting questions as to their possible significance to the bee pheromone system, particularly in relation to behavioral control of worker bees or sexual development of bee larvae.

CONCLUSIONS

Liquid/liquid extraction provides a mild and efficient means of extracting organic compounds from honey. Patterns of extractives, mostly acidic, are produced that are characteristic of the source of the honey. Manuka/kanuka honeys contain much higher concentrations of aromatic acids than those derived from white clover, which serves to differentiate the two types. Their origin does not appear to be directly related to extractives in manuka flowers. Diacids are prominent extractives in both clover and manuka/kanuka honeys. Investigations are continuing to determine the contribution of honeydew to manuka honey, the origin of the diacids, and the general utility of organic extractive profiles for charactering floral sources of honeys.

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Registry No. 8:0, 124-07-2; 12:0, 143-07-7; 14:0, 544-63-8; 16:0, 57-10-3; 16:0 ethyl ester, 628-97-7; 18:0, 57-11-4; 18:0 ethyl ester, 111-61-5; 18:1, 112-80-1; 18:1 ethyl ester, 111-62-6; 18:2, 60-33-3; 18:2 ethyl ester, 544-35-4; 18:3, 463-40-1; 18:3 ethyl ester, 1191-41-9; 20:0, 506-30-9; 22:0, 112-85-6; 24:0, 557-59-5; 26:0, 506-46-7; 28:0, 506-48-9; C_{21}, 629-94-7; C_{22}, 629-97-0; C_{23}, 638-67-5; C_{24}, 646-31-1; C_{25}, 629-99-2; C_{26}, 630-01-3; C_{27}, 593-49-7; C_{28}, 630-02-4; C_{29}, 630-03-5; C_{30}, 36731-14-3; C_{31}, 630-04-6; $C_{31:1}$, 77046-64-1; $C_{33:1}$, 85792-06-9; succinate, 110-15-6; benzoate, 65-85-0; phenylacetate, 103-82-2; 2′-methoxyacetophenone, 579-74-8; 2-methoxybenzoate, 579-75-9; 2-hydroxy-3-phenylpropionate, 156-05-8; (*E*)-cinnamate, 140-10-3; octanedioate, 505-48-6; nonanedioate, 123-99-9; 3,5-dimethoxybenzoate, 1132-21-4; 3,4-dimethoxybenzoate, 93-07-2; 2-hydroxy-3-(4-methoxyphenyl)propionate, 28030-15-1; decanedioate, 111-20-6; 2-decenedioate, 6048-93-7; 3,4,5-trimethoxybenzoate, 118-41-2; 4-hydroxy-3,5-dimethoxybenzoate, 530-57-4; ethyl methyl succinate, 627-73-6; ethyl phenylacetate, 101-97-3; ethyl 2-hydroxy-3-phenylpropionate, 15399-05-0; dimethyl decanedioate, 106-79-6; dimethyl 2-decenedioate, 28598-91-6; ethyl methyl decanedioate, 692-88-6; ethyl methyl 2-decenedioate, 111495-84-2; 2,6-di-*tert*-butyl-4-methylphenol, 128-37-0.

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Lipid Content and Fatty Acid Composition of Brown Rice of Cultivars of the United States

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The lipid content and fatty acid composition of brown rice of U.S. cultivars were investigated on a nonwaxy group of 14 long-, 10 medium-, and 2 short-grain types and a waxy group of 2 short-grain types. No significant difference among 3 nonwaxy grain types was observed in lipid content and fatty acid composition except arachidic and behenic acids between long- and medium-grain types. The significant difference between nonwaxy and waxy groups was shown in palmitic acid content. As for the relationship between fatty acid contents, there was the highest and negative correlation between oleic and linoleic acids in the nonwaxy group. The scatter diagram between both fatty acids could not be divided into grain types in nonwaxy groups but might be divided into nonwaxy and waxy groups.

Rice cultivars in the United States are divided by grain size and shape into long-, medium-, and short-grain types. In the southern rice-growing area, long- and medium-grain types are grown. In California, medium- and short-grain types are produced. However, the short-grain type is also produced on a small acreage in the southern rice-growing area, and the first commercial-scale long-grain type was produced in California in 1982.

Rice cultivars can be classified into Indica and Japonica types. Most of the rice grown in California belongs to the Japonica type, while most of that in the southern rice-growing area belongs to the Indica type or is a hybrid derivative of two types (Leonard and Martin, 1967). As for the classification of rice cultivars, the Javanica type was proposed as intermediate between Indica and Japonica types on the basis of phylogenetic remoteness by Morinaga (1954) and Chang (1976). Recently, Sinica was further separated from Indica on the basis of results of isozymic and phylogenetic analysis of rice plant (Nakagahra, 1978, 1986). According to the classification, the cultivars called Indica in the United States belong to the Javanica type.

In previous work, it was shown that the fatty acid composition of nonstarch lipid of brown rice differed between Indica and Japonica types (Taira and Chang, 1986), among Indica, Sinica, Javanica, and Japonica types (Taira et al., 1988), and between Japonica and Indica–Japonica hybrid types (Taira and Lee, 1988). Therefore, investigations were undertaken to study the lipid content and fatty acid composition of brown rice of three grain types of U.S. cultivars.

National Food Research Institute, Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Ibaraki 305, Japan (H.T.), and Hiroshima Agricultural College, Saijyo, Higashihiroshima, Hiroshima 724, Japan (T.I.).

MATERIALS AND METHODS

Mature grains of U.S. cultivars were collected from a field experiment conducted by Hiroshima Agricultural College, Japan, in 1986. Cultivars tested: (a) California cultivar, 2 long-grain types (California Belle, L 202), 5 medium-grain types (M 101, M 201, M 202, M 302, M 401), 1 short-grain type (S 201), and 2 short-grain waxy types (Calmochi 101, Calmochi 202); (b) Southern cultivars, 12 long-grain types (Lemont, Lebonnet, Labelle, Starbonnet, Newrex, Bond, Tebonnet, Skybonnet, Newbonnet, Bonnet 73, Toro 2, Bellemont), 5 medium-grain types (Mars, Saturn, Brazos, Pecos, Nato), and 1 short-grain type (Nortai). The seeding and transplanting times were May 14 and June 21, respectively. The heading dates are shown in Table I. Amounts of fertilizer per hectare were as follows. Basal dressing: N, 56 kg; P_2O_5, 68 kg; K_2O, 52 kg. Top dressing: N, 15 kg; P_2O_5, 2 kg; K_2O, 15 kg.

The analytical methods for lipid and fatty acids were identical with those used in the previous paper (Taira and Chang, 1986).

RESULTS AND DISCUSSION

The lipid content and fatty acid composition of long-, medium-, and short-grain types of brown rice are shown as mean values of duplicated data in Table I.

The difference test for lipid and fatty acid contents has been carried out among three nonwaxy grain types by analysis of variance for one-way layout. There was no significant difference among three grain types in lipid and fatty acid contents except for significant differences between long- and medium-grain types in arachidic acid content at the 5% level and behenic acid content at the 1% level on F value. In the previous paper (Taira et al., 1988), significant differences were shown among Indica, Sinica, Javanica, and Japonica types in the fatty acid

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