Shoyu (Soy Sauce) Flavor Components: Acidic Fractions and the Characteristic Flavor Component†

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From the volatile flavor concentrate of *shoyu*, acidic I and acidic II fractions were separated. *Shoyu* was also directly extracted with CH_2Cl_2 in order to cover the components that were not obtained by the above method, and the extract was separated into ten (A~J) fractions. C~I fractions were acidic ones. The above fractions were successively analyzed by gas chromatography and combined gas chromatography-mass spectrometry. Consequently, 93 components were identified, 36 of which have not been reported previously as constituents of *shoyu*. The identified compounds were 15 alcohols, 15 carbonyls, 9 esters, 4 lactones, 6 furans, 3 furanones, 5 pyrones, 7 phenols, 13 acids and 16 other compounds. Organoleptically, the caramel-like aroma compounds among them are important for the characteristic flavor of *shoyu*. 4-Hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2H)-furanone (HEMF) and 4-hydroxy-5-methyl-3(2H)-furanone seem to be important, and particularly, in view of the content and the organoleptic property, HEMF is concluded to be the main and most important constituent of the characteristic flavor of *shoyu*.

In the previous papers,^{1~4)} the authors reported that 80 volatile components were identified in *shoyu* flavor and that above all 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2H)-furanone (abbreviated to HEMF, tautomers ratio; ca. 3:2) which exhibited a weak acidic property was a main and important constituent of the characteristic *shoyu* flavor. Asao and Yokotsuka⁵⁾ pointed out that a weak acidic fraction of *shoyu* flavor imparted a main part of *shoyu* aroma. Japanese fermented *shoyu* is a weak acidic seasoning, pH of which is usually 4.8, so that there may be some other important acidic components in *shoyu* than HEMF.

This paper deals with identification of many components in acidic fractions of *shoyu*. Some important acidic compounds have been identified, but HEMF was found to be still most important in comparison with the other acidic components.

† Studies on Flavor Components in *Shoyu*. Part VI. For Part VI, see refs. 1~4 and *Nippon Nôgei-gakku Kaishi*, **51**, 709 (1977).

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MATERIALS AND METHODS

Shoyu. Commercially available genuine fermented *shoyu* (pH 4.8) was used for the following experiments.

Separation of a volatile flavor concentrate into acidic, basic and neutral fractions. Four fractions were obtained by the method shown in Fig. 1. A glass flash-vacuum-evaporator was utilized for distillation under reduced pressure and the distillate was condensed in an ice-NaCl trap and a dry ice-ethanol one. All operations were carried out at a temperature below 50°C.

Separation of a shoyu flavor concentrate (CH_2Cl_2 extract) into ten fractions. Ten fractions were obtained by the method shown in Fig. 2. Preparation of a *shoyu* flavor concentrate was carried out by the method reported in the previous paper.⁴⁾ The final extractions of the aqueous layers with dichloromethane were carried out by adjusting the pH two times in order to complete the extraction of flavor components. Each fraction was concentrated at a bath temperature below 50°C and atmospheric pressure. All operations were carried out at a temperature below 50°C.

Fractionation for minor components. The *shoyu* flavor concentrate and C, E, F and H fractions obtained above were further fractionated by means of gas chromatography. Minor components which gave small peaks on the gas chromatogram of each fraction were collected and the major components were removed.

Fig. 1. Separation Scheme of Volatile Flavor Concentrate into Basic, Acidic I, Acidic II and Neutral Fractions.

FIG. 2. Separation Scheme of *Shoyu* Flavor Concentrate (CH_2Cl_2 Extract) into Ten Fractions.

Fig. 3. Gas Chromatogram (Capillary Column) of *Shoyu* Flavor Concentrate (CH_2Cl_2 Extract).

Instrument, Hitachi Model 063-RMU-7M (GC-MS); column, FFAP, 0.28 mm i.d. $\times$ 30 m, glass, SCOT; oven temp., 50~170°C (3°C/min); carrier gas, He; ionization electron energy, 70 eV; ion source temp., 240°C; detector, total ion monitor, 10%.

The fractionation was made on a JEOL Model 750 gas chromatograph with a thermal conductivity detector. The conditions were as follows: a 3 mm i.d. $\times$ 2 m stainless steel column containing 10% polyester FF on 60~80 mesh Diasolid L, a helium flow rate of 30 ml/min, and an injection temperature of 230°C. The oven temperature was programmed from 50 to 220°C at a rate of 4°C/min. The minor components were trapped in a glass tube cooled in a dry ice-ethanol mixture.

Combined gas chromatography-mass spectrometry (GC-MS). Combined GC-MS analyses were made on a Hitachi Model 063-RMU-7M gas chromatograph-mass spectrometer. The chromatographic conditions are presented in Fig. 3. The "acidic I" and "acidic II" fractions of Fig. 1, the *shoyu* flavor concentrate, C, D, E, F, G, H and I fractions of Fig. 2 and the five minor components fractions were analyzed. The gas chromatogram of the *shoyu* flavor concentrate is shown in Fig. 3.

Gas chromatography (GC). All samples obtained except the minor components fractions were quantitatively analyzed by use of GC method. The gas chromatogram of C fraction is shown in Fig. 4.

Isolation of 3-methoxy-2-methyl-4H-pyran-4-one.

Basic fraction concentrate was obtained from 125 liters of *shoyu* by the same way as that shown in the previous paper.³⁾ The concentrate was separated by means of preparative silica gel thin-layer chromatography. Two kinds of mixed solvent, *i.e.* benzene and acetone (3:1), and successively chloroform and tetrahydrofuran (9:1) were selected for development of the chromatogram. The ethanolic solution of Rhodamine B was used as the spray reagent with which one edge of the chromatogram was sprayed. The objective band was scraped and then eluted with acetone. Evaporation *in vacuo* gave oil of 42.6 mg. The oil was purified by GC method (10% FFAP column), and then the purified oil was subjected to spectrometrical investigation. The purified oil was colorless and transparent. UV $\lambda_{\text{max}}^{\text{EtOH}}$ nm: 211, 259. IR $\nu_{\text{max}}^{\text{film}}$ cm^{-1} : 1648, 1625, 1260, 1214, 1172. PMR $\delta_{\text{TMS}}^{\text{CDCl}_3}$: 2.31 (3 H, s, C-CH₃), 3.84 (3 H, s, O-CH₃), 6.35 (1 H, d, $J=5.9$ Hz, C-CH=), 7.58 (1 H, d, $J=5.9$ Hz, O-CH=). High-MS m/e (%): C₇H₈O₃, 141 (9), 140 (100), 139 (21), 125 (21), 122 (44), 111 (9), 110 (22), 109 (13), 97 (16), 95 (6), 82 (27), 81 (9), 71 (17), 70 (7), 69 (76), 67 (8), 66 (10), 55 (16), 54 (17), 53 (16), 52 (7), 43 (62), 42 (12), 41 (12), 39 (17), 29 (9), 27 (22), 26 (15), 15 (11).

FIG. 4. Gas Chromatogram of C Fraction Concentrate.

Instrument, Shimadzu 4BM-PF (FID); column, polyester FF 10%+terephthalic acid 3%, 3 mm i.d. $\times$ 2 m, glass; oven temp., 50~180°C (3°C/min); injector and detector temp., 240°C; carrier gas, N₂, 30 ml/min.

Preparation of 3-methoxy-2-methyl-4H-pyran-4-one.

This authentic compound was prepared by methylation⁶⁾ of 3-hydroxy-2-methyl-4H-pyran-4-one (maltol). Maltol (100 mg) in hot methanol (2 ml) was treated with Ag₂O (20 mg) and CH₃I (0.2 ml). When the vigorous reaction had subsided, a second portion of Ag₂O and CH₃I was added. The reaction was less vigorous and the mixture was heated in the steam bath for 15 min. A third addition of Ag₂O and CH₃I was then made and the mixture was refluxed for 20 min. The solution was filtered and the filtrate was concentrated *in vacuo*. The concentrate was purified by GC method, and the objective material (oil) was obtained.

RESULTS AND DISCUSSION

Identification

From the volatile flavor concentrate, the basic, acidic I, acidic II and neutral fractions were separated. On the other hand, *shoyu* was also directly extracted with dichloromethane in order to cover the components that were not obtained by the above method, that is to say, in order to cover the flavor components that were not distilled or difficult to be distilled under reduced pressure, and then

the extract was separated into ten (A~J) fractions. The concentrates and fractions were subjected to GC and GC-MS analyses. The results described here concern the two volatile acidic fractions of Fig. 1, the direct dichloromethane extract and the C~I fractions of Fig. 2. The investigation on the volatile basic fraction has been reported already.³⁾

Ninety-three components were identified in the two acidic fractions of Fig. 1, the direct dichloromethane extract, the C~I fractions of Fig. 2 and the five minor components fractions, 36 of which have not been reported previously as constituents of *shoyu*. All the components were identified by the comparison of their mass spectra with those of authentic samples or those in references. Their GC retention times were also compared with those of authentic samples, when obtained. The confirmed compounds are listed in Table I.

2,3-Butanediol is a major component. This compound could be separated only by the direct extraction method with dichloromethane.

Detection of 2-methoxy-5-vinylphenol in

TABLE I. IDENTIFIED COMPONENTS IN *Shoyu*

Compounds ^a	Peak No. of Fig. 3	Fractions ^{a, b}															Methods for identi- fication	Ref. No.
		Vac. distilled volatiles		Direct extract							Minor components							
		Acidic I	Acidic II	Total	C	D	E	F	G	H	I	Total	C	E	F	H		
<i>Alcohols</i>																		
Methanol														+		+	MS,GC	
Ethanol			+									+	+	+	+	+	MS,GC	
1-Propanol					+	+			+	+		+	+				MS,GC	
2-Propanol																	MS,GC	
1-Butanol	11	+	+		+	+	+	+	+	+	+	+	+	+	+	+	MS,GC	
2-Methyl-1-propanol	9			+	+	+	+	+	+	+	+	+	+				MS,GC	
1-Pentanol	14			+							+						MS,GC	
2-Methyl-1-butanol			+						+		+	+	+	+	+	+	MS,GC	
3-Methyl-1-butanol	13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	MS,GC	
3-Pentanol*												(+)					MS,GC	
1-Penten-3-ol*												(+)					MS,GC	
Benzylalcohol		+	+									+	+				MS,GC	
2-Phenylethanol	50	+		+	+	+				+		+	+			+	MS,GC	
L-2,3-Butanediol*	30			+		+	+			+	+						MS,GC	
meso-2,3-Butanediol*	32			+		+	+			+							MS,GC	
<i>Carbonyls</i>																		
Acetaldehyde		+										+	+			+	MS,GC	
Propanal		+									(+)		+	+		+	MS,GC	
2-Methylpropanal					+	+			+	+		+					MS,GC	
3-Methylbutanal	5			+	+	+			+	+	(+)		+	+	(+)		MS,GC	
Hexanal																	MS,GC	
Benzaldehyde			+		+	+			+	+	+	+	+	+	+	+	MS,GC	
Phenylacetaldehyde					+	+	+	+	+	+	+	+	+	+	+	+	MS,GC	
Acetone	3																MS,GC	
1-Hydroxy-2-propanone* (acetol)	18			+	+	+	+	+	+	+	+	+	+	+	+	+	MS,GC	
3-Hydroxy-2-butanone (acetoin)	17	+		+	+	+	+	+	+	+	+	+	+	+	+	+	MS,GC	
2,3-Butanedione											(+)						MS,GC	
2,3-Pentanedione											(+)					+	MS,GC	
2-Hexanone					+	+											MS,GC	
2,3-Hexanedione		+	+						+							+	MS,GC	

3-Octanone*	(+) MS,GC]				
Esters					
Ethyl acetate	+			+	MS,GC
Ethyl propionate				+	MS,GC
2-Oxopropyl acetate* (acetol acetate)		+		+	MS
19	+	+	+	+	14
Ethyl 2-hydroxypropionate (ethyl lactate)	+	+	+	+	MS,GC
1-Methylpropyl acetate*	+			(+)	MS,GC
2-Ethoxyethyl acetate*				+	MS,GC
Diethyl succinate					MS,GC
37	+	(+)			MS,GC
Ethyl 4-hydroxy-3-methoxybenzoate (ethyl vanillate)			(+)		+
4-Acetoxy-3-methoxybenzaldehyde* (vanillin acetate)					14
Lactones					
4-Butanolide*	+		(+)	+	MS,GC
4-Pentanolide			+		MS,GC
2-Methyl-4-butanolide*	+		+		MS,GC
5-Methyl-2(5 H)-furanone* (β-angelicalactone)			+		MS
13,14					
Furans					
Furfuryl alcohol	+	+	+	+	MS,GC
27	+	+	+	+	MS,GC
5-Methyl-2-furfural*		+	+	(+)	MS,GC
Allyl 2-furoate*					MS
2-Acetylfuran	+				MS,GC
2-Acetyl-3-hydroxyfuran* (isomaltol)		+	(+)	+	MS
13					
Furanones					
4-Hydroxy-5-methyl-3(2 H)-furanone (HMF)	+	+	(+)	+	MS,GC
57					
4-Hydroxy-2,5-dimethyl-3(2 H)-furanone* (HDMF)	(+)	(+)	+	+	MS,GC
55					
4-Hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2 H)-furanone (HEMF)	+	+	+	+	MS,GC
56					

TABLE I. (Continued)

Compounds ^a	Peak No. of Fig. 3	Fractions ^{a, b}															Methods for identi- fication	Ref. No.
		Vac. distilled volatiles		Direct extract								Minor components						
		Acidic I	Acidic II	Total	C	D	E	F	G	H	I	Total	C	E	F	H		
<i>Pyrones</i>																		
3-Hydroxy-2-methyl-4H-pyran-4-one (maltol)	52	+		+	+	+	+	+	+	+	+	+	+	(+)	+	+	MS,GC	
3-Methoxy-2-methyl-4H-pyran-4-one* (isolated)	51	+		+	+					+	+	+	+	+	+	+	MS,GC	
5-Hydroxy-2-methyl-4H-pyran-4-one*				(+)						(+)		+	(+)	+			MS	14
3,5-Dihydroxy-2-methyl-4H-pyran-4-one*						+							(+)				MS	19
3,5-Dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one*				+	+	+	+					+	+				MS	18
<i>Phenols</i>																		
Phenol*																	MS,GC	
2-Methoxyphenol (guaiacol)	48		+	+			+			+	+	+	+	+	+	+	MS,GC	
2-Methoxy-4-ethylphenol (4-ethylguaiacol)	54	+	+	+			+			+	+	+	+	+	+	+	MS,GC	
2-Methoxy-5-vinylphenol*	59	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	MS	7
4-Ethylphenol		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	MS,GC	
2,6-Dimethoxyphenol	61	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	MS,GC	
Catechol						+											MS,GC	
<i>Acids</i>																		
Acetic acid	26			+	+	+	+	+	+	+	+	+	+	+	+	+	MS,GC	
Propionic acid		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	MS,GC	
Butanoic acid			+		+	+	+	+	+	+	+	+	+	+	+	+	MS,GC	
2-Methylpropionic acid		+															MS,GC	
trans-2-Butenoic acid* (Crotonic acid)										+	(+)	+	+	(+)	+	+	MS,GC	
Pentanoic acid																	MS,GC	
2-Methylbutanoic acid*						+											MS,GC	
3-Methylbutanoic acid		+		+	+	+	+					+	+	+	+	+	MS,GC

Fig. 5. Caramel-like Aroma Compounds Found in *Shoyu*.

natural products has been described before only as a constituent of the aroma of cloudberries by Honkanen and Pyysalo.⁷⁾

5-Hydroxy-2-methyl-4H-pyran-4-one and 3,5-dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one are said to be odorless, and yet the latter compound is important as a intermediate of maltol formation. The former has been isolated as a component of cigarette smoke.⁸⁾

3-Methoxy-2-methyl-4H-pyran-4-one, which is one of the major components, was isolated from *shoyu* and its UV, IR, PMR and MS spectra were obtained. The representative values of the spectra agreed with those in references.^{9~12)} The authors were interested in its odor, and synthesized this compound. However, the purified synthetic compound was odorless. Its MS spectrum was reported only by Elmenhorst⁹⁾ and McGillivray and Poulton¹⁰⁾ till now. Isolation of this compound from a natural product was described here for the first time. By the way, the retention times of maltol and this compound were very near and the two compounds were difficult to be separated by gas chromatographic method. However, it was found that this compound was contained in all fractions, the con-

tent of which was largest in neutral fraction, and that maltol was contained only in acidic fractions. Therefore, isolation of this compound was carried out from the basic fraction which was first separated.

Three lactones were also newly found in *shoyu*. 5-Methyl-2(5H)-furanone has been reported as a product of acid-catalyzed degradation of D-fructose.¹³⁾

Caramel-like aroma compounds

The caramel-like aroma compounds found in *shoyu* are presented in Fig. 5. Recently, these compounds are generally considered to be very important as flavor components of foods, and especially maltol is well known as a flavor enhancer.

4-Hydroxy-2,5-dimethyl-3(2H)-furanone (abbreviated to HDMF) has been first isolated from pineapple.²⁰⁾ The quantity of HDMF is very small in *shoyu*. Isolation of 4-hydroxy-5-methyl-3(2H)-furanone (abbreviated to HMF) from *shoyu* was described in detail in the previous paper,⁴⁾ and the importance of its odor was discussed in it. Both furanones are probably formed from sugars in heating processes such as heat treatment of raw materials

TABLE II. FLAVOR QUALITIES, AND HEMF AND HMF CONTENTS OF ACIDIC FRACTIONS

Fractions	Flavor qualities	GC peak area (%) ^a		
		HEMF	HMF	Others ^b
C	Caramel-like aroma similar to <i>shoyu</i> aroma	38.0	14.2	—
D	Caramel-like aroma with somewhat heavy note similar to the odor of C fraction	5.3	33.6	2-Methyl-1-propanol (13.3)
E	Rancid and somewhat sweaty odor	trace	1.3	Methionol (19.1), 2,6-Dimethoxyphenol (17.0)
F	Intense caramel-like aroma similar to <i>shoyu</i> aroma	77.6	0	—
G	Fermented fish sauce-like odor	6.6	0.8	2-Methyl-1-propanol (24.3) 1-Butanol (14.8) 3-Methyl-1-butanol (12.2)
H	Steamed soybean-like and somewhat roasted sweet potato-like odor	3.2	0	2-Methyl-1-propanol (16.0) 4-Ethylguaiacol (14.3)
I	Fermented fish sauce-like odor	0.1	0	2-Methyl-1-propanol (21.3) 1-Butanol (12.9) 3-Methyl-1-butanol (10.3)

^a The percentage of the peak area to the sum of all peak areas except solvent peak.

^b The compound the content of which is over 10 %.

and pasteurization of the liquid part of mash.

2-Hydroxy-3-methyl-2-cyclopenten-1-one (cyclotene), 2-acetyl-3-hydroxyfuran (isomaltol) and maltol have been found in various foods, and they are known to be formed in the Maillard reaction.

3,5-Dihydroxy-2-methyl-4H-pyran-4-one has been isolated from roast barley²¹⁾ and has a weak maltol-like odor. This compound has been also isolated from degradation of D-glucose with acetic acid and methylamine.¹⁹⁾

All compounds in Fig. 5 contain hydroxy group of enol in their structures; therefore they are considered to exhibit weak acidic properties and to be water-soluble.

Flavor quality and HEMF content of each acidic fraction

Table II shows flavor qualities of the acidic fractions. The percentage of the HEMF peak area to the sum of all peak areas except solvent peak in each fraction was calculated. The HMF content was also obtained in the same way. The gas chromatogram in Fig. 4, for example, was used for the calculation in C fraction.

It was the odor of C fraction that was most similar to *shoyu* aroma. The second was that of F fraction. The aroma of D fraction was somewhat less caramel-like and heavier than that of C fraction. E, G, H and I fractions exhibited bad odors. Both the fermented fish sauce-like odor and the roasted sweet potato-like odor seem to be undesirable to the overall *shoyu* aroma.

The results shown in Table II indicate that C and F fractions which have good aroma contain a large quantity of HEMF, and that D fraction contains plenty of HMF in place of HEMF. On the other hand, E, G, H and I fractions which possess bad odors contain little of either HEMF or HMF. Namely, the results imply that the fractions which have good aroma contain a great amount of HEMF or HMF. The bad odors of E, G, H and I fractions are probably due to the low content of HEMF and HMF. The alcohols in G and I fractions proved to contribute little to good aroma.

The characteristic flavor component of *shoyu*

The volume ratios of the fractions to the

total amount in case of direct extraction of *shoyu* with dichloromethane were as follows: basic fractions (A and B) 27%, acidic fractions (C~I) 57%, and neutral fraction (J) 16%. The acidic fractions gave the highest value and amounted to over a half.

Then, acidic I fraction of Fig. 1 and C and F fractions of Fig. 2 organoleptically imparted the characteristic flavor of *shoyu*, and these fractions also had an intense caramel-like aroma as described above. Acidic I, C and F fractions were separated with 5% NaHCO_3 , saturated NaHCO_3 and 5% Na_2CO_3 , respectively, but the extraction with dichloromethane was carried out at pH of 5.3 or 7.2. Therefore, much of acids was considered not to be extracted. Many acids were actually detected in E and G fractions obtained at pH of 2.0. So that, it is considered that water-soluble acidic compounds, which were not strong acidic, were contained in acidic I, C and F fractions. Considering in this point and the organoleptic point, some of the caramel-like aroma compounds in Fig. 5 seem to contribute greatly to the aroma of the three acidic fractions.

Each content of HEMF, HMF and maltol in *shoyu* is large among the compounds in Fig. 5. Especially, HEMF is by far abundant in quantity (50~100 ppm). Then, the odor of HEMF is known to be more intense than that of HDMF,²²⁾ and the odor threshold value of HDMF is 0.04 ppb (in water).²³⁾ So that, the odor value of HEMF is calculated at more than 1,250,000. Consequently, among the above-described caramel-like aroma compounds, HEMF makes the greatest contribution to the good aroma of the acidic fractions.

On the other hand, in the previous paper,²⁾ the authors reported that the aroma of the fraction containing the higher-boiling compounds of the volatile flavor concentrate constituted the base of *shoyu* flavor, and that HEMF was one major and principal component of the fraction.

HEMF itself has an intense sweet and fruity aroma which reminds of the best part of fermented *shoyu* flavor.

From the results presented above, the authors conclude that HEMF is the main and most important constituent of the characteristic good flavor of *shoyu*.

Asao and Yokotsuka⁵⁾ pointed out that a weak acidic fraction of *shoyu* had an intense and very good aroma and was very important from a viewpoint of flavor, and they isolated the crystalline material, the molecular formula of which was $\text{C}_7\text{H}_{12}\text{O}_8$, from the weak acidic fraction as the result of seeking a good aroma compound. Raw *shoyu* was extracted with chloroform, and the extract was treated with 5% Na_2CO_3 . The aqueous layer was extracted with diethyl ether at pH of 7.2, and then the weak acidic fraction was obtained. The material of $\text{C}_7\text{H}_{12}\text{O}_8$ was isolated from the fraction by the method of aluminum column chromatography. After that, the authors²⁴⁾ proved that the material of $\text{C}_7\text{H}_{12}\text{O}_8$ was obtained by oxidation of HEMF during the experimental treatment, and then determined the structure. The purified material of $\text{C}_7\text{H}_{12}\text{O}_8$ was odorless, as Asao and Yokotsuka reported that the odor activity of the isolated compound was low. Accordingly, the good aroma of the weak acidic fraction obtained by Asao and Yokotsuka was also probably due to HEMF.

In addition, the content of HEMF was found to be positively correlative to the quality of *shoyu* in another experiment of the authors which was founded on accurate and reproducible statistical data. The data will be presented in detail later.

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