Apparatus Used for Small-Scale Volatile Extraction from Ethanol-Supplemented Low-Salt Miso and GC−MS Characterization of the Extracted Flavors

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An extraction apparatus was equipped with a nitrogen-flushing vessel to purge volatiles from a 10-g miso prepared solution at 40 °C, a reflux condenser to recover water, a coiled cold-trap to separate ethanol in advance, and a glass-lined stainless (GLS) trap filled with Tenax TA for flavor adsorption. Volatiles in the GLS tube were released with a thermal desorption device and condensed with a Micro-cryo trap prior to connection with GC and GC−MS for characterization. After analysis, a broad volatile profile comprising 9 categories of functional group and 97 identified compounds was achieved. As affected by ethanol supplementation for miso fermentation, most volatiles except alcohols and acetals in the low-salt products fermented with 5% NaCl and 7.5% ethanol were higher than those in the control products fermented with 9% NaCl and 0% ethanol and the high-ethanol supplemented products fermented with 5% NaCl and 15% ethanol. It reveals that supplementation of ethanol in miso at an appropriate level not only enabled a low-salt miso fermentation but also enhanced flavor formation.

Keywords: *Miso; low salt; flavor extraction; GC−MS; volatile compounds; esters*

INTRODUCTION

Because of health considerations, high salt levels in most indigenous and traditional foods are a concern to the public. Having low salt while maintaining the unique flavor characteristics of the indigenous and traditional foods is highly expected. In a previous report, a low-salt miso was fermented at 5% NaCl supplemented with 7.5% ethanol, and a similar or even better flavor rating in comparison with a commercial product was achieved (Chiou et al., 1999). In general, miso aroma is unique while mild, fragile, and not rich as that of soy sauce. Artificial flavors are eventually formed when flavor extraction and concentration are done at an elevated temperature (Iwabuchi et al., 1976, 1977). In addition, miso is a semisolid colloidal food, which makes it difficult to extract flavors.

In the literature, methods of direct solvent extraction, steam distillation/solvent extraction (SDE) or Likens−Nickerson (LN) extraction, vacuum distillation, porous polymer adsorption, and headspace volatile (HSV) analysis have been used to extract miso flavors (Sugawara et al., 1990; Mori et al., 1983, 1985; Honma 1987a,b). For most extractions, a large sample size is required, and a large quantity of solvent is needed. Heat is usually involved and may cause flavor changes. A series of cold traps are needed for vacuum distillation. Flavors are undetectable due to losses, being present in trace amounts, or being masked by ethanol (a normal product of miso fermentation) in HSV analysis. Thus, continued work in technique innovation for flavor extraction from miso products is urgent. In this study, an apparatus used for small-scale volatile extraction was designed and constructed for effective extraction of miso volatiles. The target was pursued with an attempt to minimize sample size and heat involvement and to condense ethanol in advance from masking other volatiles. Nitrogen flushing was monitored to facilitate volatile evaporation at a low temperature. The ethanol was condensed with a coiled cold-trap, and other volatiles were subsequently adsorbed onto a porous polymer matrix. The extracts were released with a thermal desorption device, condensed with a Micro-cryo device, and identified by GC−MS. Flavor formation in the miso products as affected by ethanol supplementation was thereby characterized.

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MATERIALS AND METHODS

Miso Preparation. Miso substrates containing 9% NaCl and 0% ethanol as a control, containing 5% NaCl and 7.5% ethanol as a low-salt product, and containing 5% NaCl and 15% ethanol as a high-ethanol supplemented product were respectively prepared following the procedure described by Chiou et al. (1999). The substrates in plastic bag were thoroughly blended by hand and deposited in a series of glass jars (500 mL) for fermentation. After 8 weeks of fermentation, the jars were stored in a refrigerator (4 °C) for flavor extraction and analyses.

Apparatus and Flavor Extraction. A schematic illustration of the apparatus for flavor extraction is shown in Figure 1. The apparatus comprises an extraction vessel (250 mL) in connection with a reflux condenser, a coiled cold-trap, and a glass-lined stainless steel (GLS) trap containing a porous adsorbent.

For each extraction, 10 g of miso, 20 g of NaCl, and 100 mL of deionized water were deposited in the vessel. The vessel was immersed in a water bath at 40 ± 5 °C and stirred with a magnetic stirrer. A nitrogen flushing tube was inserted into the miso solution and regulated at 15 mL/min of flow rate. Total flushing nitrogen volume was 60 L. On the basis of an extensive preliminary experiment focused on temperature adjustment, a reflux condenser was controlled at −6 ± 0.5 °C by circulation with a coolant to recover water. A coiled cold-

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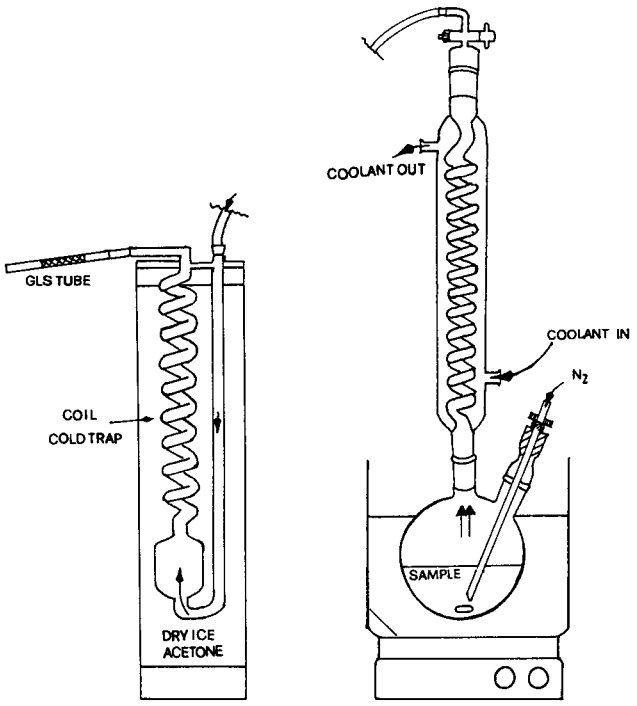

Figure 1. Schematic illustration of the apparatus used for miso flavor extraction.

trap for ethanol recovery was immersed in a dry ice-cooled acetone bath. Then, a GLS steel tube (0.4 cm i.d., 10 cm length) (SIS Co., USA) containing 0.15 g of Tenax TA (Ohio Valley Specialty Chemical, OH) was used as a porous absorbent. The GLS tube containing Tenax TA was conditioned 2 h at 300° under N_2 prior to use. For each sample, the extraction time was 400 min. The volatile compounds were stepwise collected in the coiled cold-trap and GLS tube.

After extraction, the collected volume and weight in the coiled cold-trap were measured, and the volatile concentration was calculated by dividing the weight of extract by weight of miso used for extraction. Then, the extract was stored in a 2-mL glass vial at −20 °C for further analysis. The adsorbed volatiles in the GLS tube were subjected to a thermal desorption device and GC−MS analysis.

Volatile Analysis. A gas chromatography (GC) (HP 5890 II, Hewlett-Packard Co., CA) equipped with a flame ionization detector (FID) and a HP-Innowax column (30 m length, 0.25 mm i.d., and 0.5 μm film thickness) was used for quantitative analysis of the volatiles. For identification and quantitation of the volatile compounds in the GLS tube, a short path thermal desorption devise (model TD-4, SIS Co., USA) was used in connection with the inlet of GC column. The GLS tube after incorporation with 1 μL of internal standard solution (containing 10 μg of tetradecanol in 1 μL of methanol) was flushed with N_2 for 7 min at room temperature to clean up methanol and oxygen residues. Then, the temperature was heated to 200 °C and increased to 220 °C at 20 °C/min and held 5 min. The released volatiles were re-trapped and condensed with a Micro-cryo trap (SIS Co., USA) at -100 ± 2 °C in conjunction with the inlet of GC column. Then, a GC program was initiated with column temperature set at 30 °C for 5 min, increased to 40 °C at 5 °C/min, held for 5 min, further increased to 220 °C at 5 °C/min, and held for an additional 10 min. Helium as a carrier gas was monitored at 1.0 mL/min of flow rate.

Identification of the volatile compounds was performed with a HP 5890 II GC coupled with a HP 5972 MSD (mass selective detector). The temperature and ionizing voltage of the MC−MS transfer line were 265 °C and 70 eV, respectively. Data were collected using a HP G1030A VL24/66 ChemStation controller interfaced with HP 1034C MS ChemStation software. The mass spectrometer was initiated automatically immediately after sample injection. The mass fragments between 40 and 400 amu were detected and integrated in a total ion chromatogram. Unknown peaks were identified based on the retention index and compared with the reference spectra from HP G1035A MS ChemStation Libraries. For quantitative analysis, the GC conditions were identical to those for identification analysis described above except that the MSD detector was substituted with a FID and the detector temperature was 270 °C. The concentration of volatiles was calculated from the integrated area of each individual peak to that of internal standard, and the response factor was assumed as 1.

For analysis of the volatile compounds collected in the coiled cold-trap in which ethanol was predominant, the extract was directly subjected to GC-FID analysis following the procedures described above with a different temperature program. The sample injection volume was 1 μL and injected with a splitless mode at 250 °C of injector temperature. The column temperature was initiated at 30 °C for 15 min to elute ethanol and programmed to increase column temperature to 220 °C at 5 °C/min and hold for an additional 10 min. The ethanol concentration expressed as percentage was calculated according to the peak area of ethanol to the total area of detected peaks and multiplying by 100.

Table 1. Volatiles in the Coiled Cold-Trap Extracted from Control Miso Products Fermented with 9% NaCl and 0% Ethanol, Low-Salt Products Fermented with 5% NaCl and 7.5% Ethanol, and High-Ethanol Supplemented Products Fermented with 5% NaCl and 15% Ethanol

	ethanol supplementation, %		
items of determination	0	7.5	15
proportional volatile concn, mg/g of miso	12.15	15.01	25.07
ethanol concn[a], %	99.3	98.5	99.8

[a] Percentage of the peak area of ethanol in proportion to the total detected peak area.

RESULTS AND DISCUSSION

Volatile compounds collected in the coiled cold-trap are shown in Table 1. On the basis of the total GC peak areas, the extracts were predominantly comprised of ethanol (higher than 98.5%). The total collected volatiles in proportion to the miso weight used for flavor extraction increased with an increase of ethanol from 0 to 15% supplemented for miso fermentation. Since ethanol is a normal metabolite of holophilic yeasts during miso fermentation (Abiose et al., 1982; Ebine, 1989), it was also predominant in the extract of control miso in which ethanol was not supplemented. The collected volatiles were 12.15, 15.01, and 25.07 mg/g of miso and approximately equivalent to 1.2, 1.5 and 2.5% of ethanol on weight basis, respectively. In comparison, the obtained ethanol concentrations were lower than the original ethanol levels supplemented for miso fermentation. It is likely that some ethanol might have undergone chemical reactions during fermentation, some might not be evaporated by nitrogen flushing at 40 °C with NaCl supplemented in the miso solution, and small amounts of ethanol might go through this trap. Nevertheless, it is apparent that the coiled cold-trap was effective in ethanol separation in advance from masking other volatiles.

The volatile compounds passing through the coiled cold-trap and adsorbed in the GLS tube are shown in Table 2. Categories of the compounds were grouped into alcohols, phenols, esters, aldehydes, acetals, ketones, acids, hydrocarbons, and miscellaneous based on their functional groups. In total, 97 compounds were identified, and some compounds were unable to be identified.

Table 2. Volatiles in GLS Trap Extracted from Control Miso Products Fermented with 9% NaCl and 0% Ethanol, Low-Salt Products Fermented with 5% NaCl and 7.5% Ethanol, and High-Ethanol Supplemented Products Fermented with 5% NaCl and 15% Ethanol

volatile compd	volatile concn (ng/g of miso) at % ethanol supplementation			volatile compd	volatile concn (ng/g of miso) at % ethanol supplementation		
	0	7.5	15		0	7.5	15
Alcohols							
ethanol	96.9	262.0	294.1	1-heptanol		17.7	
butanol	5.2	5.2		2-ethyl-1-hexanol	26.2	38.5	7.5
isoamyl alcohol		16.2	3.8	2-octen-1-ol		7.8	
1-hexanol	53.9	121.9	68.6	nonanol		25.7	3.1
3-hexen-1-ol	7.4	12.4	2.7	furfuryl alcohol		6.1	
3-octanol	10.9	56.8	20.2	diethylene glycol	6.9	1.9	
1-octen-3-ol	188.4	746.8	160.5	subtotal	395.8	1319.0	560.5
Phenols							
2-methoxyphenol (guaiacol)		11.5		2,4-di-*tert*-butylphenol		35.2	
phenol	6.9	4.1		subtotal	6.9	50.8	
Esters							
ethyl acetate	132.2	63.8		ethyl octanoate		30.8	7.6
ethyl propanoate		20.4		ethyl nonanoate		10.2	
ethyl isobutyrate	13.1	28.6		ethyl decanoate		7.3	
ethyl butyrate		40.8		ethyl dodecanoate		9.2	
ethyl 2-methylbutyrate	19.3			ethyl palmitate		4.5	1.7
ethyl 3-methylbutyrate	15.0	28.2	4.1	ethyl stearate		11.8	
ethyl pentanoate	8.9	23.6		ethyl lactate		7.2	
ethyl 2-butenoate		9.7		ethyl benzoate	57.0	147.1	1.7
ethyl hexanoate	72.6	209.5	34.5	ethyl phenylacetate		15.9	
ethyl 3-hexenoate		16.4		isobutyl acetate		9.5	
ethyl heptanoate	8.6	20.4	3.7	subtotal	326.7	714.9	53.3
Aldehydes							
2-methylbutanal	75.1	124.5		2-methylundecanal		10.9	
3-methylbutanal		217.5		2-furfural	21.4	81.2	13.2
hexanal	49.2	228.5	31.5	5-methyl-2-furfural		10.0	
heptanal		24.8		benzaldehyde	41.2	172.9	17.2
2-pentenal		21.0		benzeneacetaldehyde		38.3	
octanal	8.9	29.8	4.5	2-phenyl-2-butenal		9.5	
nonanal	20.9	67.7	6.5	methional		11.6	
2-octenal	4.5	14.2		subtotal	229.9	1092.3	78.7
decanal	8.7	29.9	5.8				
Acetals							
acetal		73.2	189.2	3-methylbutanal diethyl acetal		99.3	86.7
cyclic 1,2-dimethylethylene acetal		14.8	12.6	subtotal		261.5	297.7
isobutanal diethyl acetal		74.2	9.2				
Ketones							
acetone	55.4	3.6	18.2	6-methyl-5-hepten-2-one	8.1	20.4	
4-methyl-2-pentanone	4.0	18.3		4-hydroxyl-4-methyl-2-pentanone			3.8
3-octanone	25.7	33.6	8.0	acetophenone		23.3	
2-octanone		3.5		geranyl acetone		11.6	
1-octen-3-one	9.5	19.3		subtotal	102.7	133.5	29.9
Acids							
acetic acid	17.3	5.2		subtotal	17.3	17.6	
palmtic acid		12.4					
Hydrocarbons							
hexane		9.6	15.8	*m*-xylene	8.9	73.1	50.8
isooctane + octane		21.8	27.3	*o*-xylene	2.8	52.0	16.1
1-octene		3.7		propylbenzene		2.9	
4-methyloctane		6.6		ethenylbenzene	31.7	195.9	82.9
3-methyloctane		3.5		1-methyl-4-ethylbenzene		9.8	10.5
decane		21.1		1-methyl-3-ethylbenzene		18.2	
undecane	1.8	10.7		1-methyl-2-ethylbenzene	7.3	35.7	7.7
limonene	12.7	21.3	10.8	1-ethyl-2,3-dimethylbenzene		4.9	
tetradecane		11.4		2-ethtyl-1,4-dimethylbenzene		5.7	0.7
pentadecane		22.9		3-ethyl-1,5-dimethylbenzene		2.6	
heptadecane		6.2		dichlorobenzene	20.5	14.0	4.3
toluene	27.0	340.7	118.0	naphthalene	5.9	29.2	4.3
ethylbenzene	5.1	49.2		subtotal	127.0	1004.2	375.5
p-xylene	3.3	31.5	26.3				
Miscellaneous							
2-pentylfuran	27.7	33.7	4.4	benzothiazole		4.4	
pyridine		15.4		subtotal	27.7	53.5	4.4
total volatile concn, ng/g miso[a]					1861.8	5303.3	1558.0
identified peaks/total peaks					43/79	95/164	41/55

[a] Calculated from all peaks including unidentified ones.

On the basis of the fact that all compounds were originated from 10-g samples of miso, the apparatus (Figure 1) and applied procedure for flavor extraction were efficient. Sine the compounds were carried by nitrogen flushing from a vessel containing a miso solution at 40 °C and flown through a water condenser, a coiled cold-trap to recover ethanol, and finally adsorbed by GLS trap, the absorbed compounds must be very volatile either with comparatively low molecular weights or with low hydrophilic properties. This clue was supported by the consequent identification of the ester compounds shown in Table 2. In comparison to vacuum distillation, ethyl esters of less than 8-carbon acids and esters of acetic acid were not detected (Sugawara et al., 1990).

As affected by miso product, total volatile concentrations were much higher in the low-salt miso products containing 5% NaCl and 7.5% ethanol than in the control products containing 9% NaCl and 0% ethanol or in the high-ethanol supplemented products containing 5% NaCl and 15% ethanol. On the basis of the categorized volatiles, except alcohols and acetals being highest in the high-ethanol supplemented products, most categories of volatiles were higher in the low-salt miso products than in the other two products.

Among the detected alcohols, 1-octen-3-ol was much higher in the low-salt miso products than in other products. 1-Octen-3-ol is an enzymatic product of linoleic acid (Tressl et al., 1982; Wurzenberger and Grosch, 1986) and the key compound of mushroom flavors with sweet and butter characteristic notes (Cornin and Ward, 1971). It may enhance flavor performance of miso. Linoleic acid is rich in soybean, a major miso substrate, and may form 1-octen-3-ol formation through koji enzymes during fermentation. In addition, considerable amounts of 1-hexanol and 2-ethyl-1-hexanol were observed in the low-salt miso products. These two compounds are green soybean, coconut-like, pungent (Simpson, 1979; Kato et al., 1981; del Rosario et al., 1984), and cooked rice flavors (Honma, 1987b), respectively. In particular of interest, furfuryl alcohol was also detected in the low-salt miso products. This was in agreement with the observations of Sugawara et al. (1990, 1991a,b) who reported that furfuryl alcohols can be collected by the porous polymer (Porapak Q) but not by SDE or vacuum distillation.

In comparison of the ester compounds, total contents varied significantly as affected by ethanol supplementation. The proportional concentrations were highest in the low-salt miso products and followed in order in the control products and the high-ethanol supplemented products. They were 714.9, 326.7, and 53.3 ng/g of miso, respectively. In general, most esters are pleasant in sensory perception and enhance miso flavor quality. In particular, ethyl esters of C6, C8, C10, and ethyl phenyl acetate contribute strongly to the aroma of white wines (Simpson, 1977; Pyysalo et al., 1977; Marais and Pool, 1980; Shinohara and Watanabe, 1981). Among the 21 identified esters, 20 ester compounds were detected in the low-salt miso products containing 5% salt and 7.5% ethanol. Most esters were higher in the low-salt miso products than in the other products except that ethyl acetate was higher in the control products than in the other products.

Total aldehyde concentrations were also highest in the low-salt miso products. The content was 5 times higher than that in the control products and 14 times higher than that in the high-ethanol supplemented products. In addition, considerable amounts of hexanal, 2-methylbutanal, benzaldehyde, 2-methylbutanal, and 2-furfural were also present in the low-salt miso products. Both furfuryl alcohol and 2-furfural were simultaneously collected in the GLS trap. This was not in agreement with the observation of Sugawara et al. (1990, 1991a,b), who used a porous polymer to detect furfuryl alcohol and used SDE or vacuum distillation to detect 2-furfural.

In addition, acetals, ketones, acids, and hydrocarbons were present in the extracts of miso products. Diethyl acetal was highest in the high-ethanol supplemented products. Since microorganisms were not detected in the products supplemented with 15% ethanol (Chiou et al., 1999), the formation of acetals was mostly due to chemical reactions rather than microorganism involvement. On the contrary, acids were detected in the control and low-salt miso products in which microorganisms were involved in varied extents. Hydrocarbons were also higher in the low-salt products than the other products.

In conclusion, through the apparatus and procedure developed in our laboratory, 10-g samples of miso were sufficient for flavor extraction and subjection to instrumental analysis. A broad volatile profile comprising 97 identified compounds was observed in the extracts of miso products. Since the extractions were conducted at temperatures lower than 40 °C, the possible formation of artificial flavors was minimized. As affected by ethanol supplementation, most volatiles except alcohols and acetals were higher in the low-salt miso products fermented with 5% NaCl and 7.5% ethanol than in the control products fermented with 9% NaCl and 0% ethanol and the high-ethanol supplemented products fermented with 5% NaCl and 15% ethanol. This was supportive to the previous observation that the sensory flavor rating of miso products fermented at 5% NaCl and 7.5% ethanol was higher than the ratings of other products fermented at lower or higher ethanol levels (Chiou et al., 1999). It also reveals that appropriate supplementation of miso with ethanol for fermentation not only enabled a low-salt fermentation but enhanced flavor formation.

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LITERATURE CITED

Abiose, S. H.; Allen, M. C.; Wood, B. J. Microbiology and biochemistry of miso (soypaste) fermentation. *Adv. Appl. Microbiol.* **1982**, *28*, 239−244.

Chiou, R. Y.-Y.; Ferng, S.; Beuchat, L. R. Fermentation of low-salt miso as affected by supplementation with ethanol. *Int. J. Food Microbiol.* **1999**, *48*, 11−20.

Cornin, D. A.; Ward, M. K. The characterization of some mushroom volatiles. *J. Sci. Food Chem*. **1971**, *22*, 477−479.

del Rosario, R.; de Lumen, B. O.; Habu, T.; Flath, R. A.; Mon, T. R.; Teranishi, R. Comparison of headspace volatiles from winged beans and soybean. *J. Agric. Food Chem*. **1884**, *32*, 1011−1015.

Ebine, H. Industrialization of Japanese miso fermentation. In *Industrialization of Indigenous Fermented Foods*; Steinkraus, K. H., Ed.; Marcel Dekker: New York, 1989; pp 89−126.

Honma, N. Miso flavor and its composition (part 1). *J. Brew. Soc. Jpn*. **1987a**, *82*, 480−488.

Honma, N. Miso flavor and its composition (part 2). *J. Brew. Soc. Jpn.* **1987b**, *82*, 547−553.

Iwabuchi, S.; Sato, M.; Shibasaki, K. Studies on the aroma of miso [food pastes]. III. The carbonyl compounds produced during storage and heating. *J. Food Sci. Technol. Tokyo* **1976**, *23*, 191−195.

Iwabuchi, S.; Sato, M.; Shibasaki, K. Studies on the aroma of miso [food pastes]. IV. Role of miso components in volatile carbonyl production by heating. *J. Food Sci. Technol. Tokyo* **1977**, *24*, 65−71.

Kato, H.; Doi, Y.; Tsugita, T.; Kosai, K.; Kamiya, T.; Kurata, T. Changes in volatile flavor components of soybeans during roasting. *Food Chem.* **1981**, *7*, 87−94.

Marais, J.; Pool, H. J. Effect of storage time and temperature on the volatile composition and quality of dry white table wines. *Vitis* **1980**, 151−164.

Mori, Y.; Kiuchi, K. A method for determination of basic flavor components of miso. *J. Brew. Soc. Jpn.* **1985**, *80*, 274−277.

Mori, Y.; Kiuchi, K.; Tabei, H. Flavor components of miso: basic fraction. *Agric. Biol. Chem.* **1983**, *47*, 1493−1499.

Pyysalo, T.; Suihko, M.; Honkanen, E. Odour thresholds of the major volatiles identified in Cloudberry (*Rubus chamaemorus* L.) and arctic bramble (*Rubus arcticus* L.). *Lebensm. Wiss. Technol.* **1977**, *10*, 36−39.

Shinohara, T.; Watanabe, M. Gas chromatographic analysis of volatile esters in wine. *Agric. Biol. Chem.* **1981**, *45*, 2903−2905.

Simpson, R. F. Some important aroma components of white wine. *Food Technol. Aust.* **1979**, *31*, 516−522.

Sugawara, E. Search for aroma components of miso. *J. Brew. Soc. Jpn.* **1991a**, *86*, 411−416.

Sugawara, E. Changes in aroma components of miso with aging. *Nippon Shokuhin Kogyo Gakkaishi* **1991b**, *38*, 1093−1097.

Sugawara, E.; Ito, T.; Odagiri, S.; Kubota, K.; Kogayashi, A. Comparison of miso aroma components in preparations by different methods. *Nippon Nogei Kagaku Kaishi* **1990**, *64*, 171−176.

Tressl, R.; Bahri, D.; Engel, K.-H. Formation of eight-carbon and ten-carbon components in mushroom. *J. Agric. Food Chem.* **1882**, *30*, 89−93.

Wurzenberger, M.; Grosh, W. Enzymic oxidation of linolenic acid to 1,*Z*-5-octadien-3-ol, *Z*-2,*Z*-5-octadiene-1-ol and 10-oxo-*E*-8-decenoic acid by a protein fraction from mushroom. *Lipid* **1986**, *21*, 261−266.

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