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Components in Commercial Douchi－a Chinese Fermented Black Bean Product by Supercritical Fluid Extraction

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Abstract

Douchi, a popular seasoning agent in Chinese dishes prepared by the *Aspergillus oryzae* fermentation of black beans, was subjected to supercritical fluid extraction (SFE) and gas chromatography-mass spectrometry analysis for its volatile components. A total of 73 components were identified in two commercial brands, which were positively confirmed and quantified. Among the common components in the two brands were 18 acids, 12 alcohols, 11 aldehydes, 9 esters, 1 furan, 11 other oxygen-containing compounds, 4 pyrazines, 2 pyridines and 5 miscellaneous compounds. The most abundant components found were acetic acid, benzoic acid, 2,6-dimethylpyrazine, 2-piperidinone, 3-methylbutanoic acid, maltol, 4-ethylphenol, 2-methylpropanoic acid, butanoic acid, 2-pyrrolidinone, all fatty acids and some esters.

Key words: douchi, black bean, fermentation, SFE, volatile

INTRODUCTION

Douchi is a popular condiment in Chinese dishes. It consists of fermented and salted whole black beans. Douchi is thought to be one of the oldest fermented bean products in China. In historical records (the Shih-chi), written in the second century B.C., the product is mentioned as a popular food seasoning and preservative, second to salt (1). Douchi-making spread to Japan, where it is called hamanatto, and to the Philippines and Singapore (tou-si) (2). To make douchi, black beans are washed, soaked, boiled to make them soft, cooled down, covered with straw and inoculated. Though *Aspergillus oryzae* is traditionally used for inoculation, other microorganisms are also used, such as *Mucor* spp. and *Bacillus* spp. The mixture is incubated at room temperature for several days until the beans are covered with a white mold. The result is called *koji*, which is used as a starting culture for several other fermented soy products such as soy sauce and miso (1). The koji is then washed to completely remove all mycelia and spores from the beans. If the beans are not properly washed, this can result in a bitter taste. The beans are salted with brine, transferred to earthenware jars and left to age. The final product is sun-dried (1). Japanese hamanatto has a similar production process, but wheat is added to the beans before inoculation.

A supercritical fluid is a form of matter in which the liquid and gaseous states are indistinguishable. Fluids or gases turn supercritical at temperature-time combinations above the critical temperature (T_c) and critical pressure (P_c). In this supercritical region, no liquefaction will take place on raising the pressure and no gas will be formed on increasing the temperature. Supercritical fluids have, like gases, low viscosities, high diffusion rates and very low surface tension, while sharing high densities and excellent solvent powers with liquids. These properties make supercritical fluids very suitable for extraction purposes (3). Since most flavor components are fat soluble, supercritical fluid extraction (SFE) is used to extract flavor components from douchi. Though the flavor profile of other fermented soy products, such as soy sauce and miso (fermented soybean paste), has been extensively studied, not much information is available on douchi. The objective of this study was to analyze the volatile components present in the douchi using SFE as the extraction method.

MATERIALS AND METHODS

Two samples (brands B and D) of douchi, Chinese-type fermented black bean, were bought at a local market in Hong Kong. Douchi of brand B was produced in Hong Kong, brand D originated from Guangdong, China. Fermented beans were ground using a mortar and pestle before extraction to increase their contact surface.

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Supercritical fluid extraction (SFE)

Three grams of the ground douchi was transferred to a 10 mL polymer cartridge (Isco Inc., Lincoln, Nebraska, USA). The cartridge was filled with inert WetSupport™ (Isco Inc., Lincoln, Nebraska, USA) to reduce the void space. Finally, 1 mL of 0.458 μg/mL 2,4,6-trimethylpyridine was added as an internal standard. Douchi of both brands was extracted in triplicate. Supercritical fluid extraction was performed on an Isco SFX™ 3560 automated supercritical fluid extraction system (Isco Inc., Lincoln, Nebraska, USA). Supercritical carbon dioxide was used as the extraction fluid. Extraction was performed at 60°C, 7000 psi operating pressure and 2.0 mL/min restriction flow rate. The extraction method consisted of two steps, a static and dynamic extraction step, set at 5.0 min and 30.0 min, respectively. Extract was collected in glass collection tubes containing 20 mL of dichloromethane. Initially extractions were performed to minimize solvent and volatile loss during extraction by varying the collection temperature, collection pressure and solvent replenishment condition. Finally, extract was collected under pressure at -10°C and slowly reheated to room temperature after finishing the extraction. Solvent (0.5 mL) was automatically added to the collection tube every 10 min for replenishment. Extracts were dehydrated by pouring through a 2.5 g of anhydrous sodium sulfate column and then drying to a volume of approximately 0.1 mL using prepurified nitrogen gas to prepare for GC-MS separation and analysis.

Gas chromatography–Mass spectrometry (GC-MS)

One microliter of dehydrated and dried extract was injected at splitless mode into a Hewlett-Packard 6890 GC (Hewlett-Packard Co., Palo Alto, CA, USA). Separation of components was performed on a Supelcowax 10 polar capillary column (60 m × 0.25 mm i.d. × 0.25 μm film thickness; Supelco Inc., Bellefonte, PA, USA), using helium as a carrier gas at 30 cm/s flow rate. Injection temperature was 220°C. The GC oven temperature was kept at 35°C for 5 min, increased to 195°C at 6°C/min and held at 195° until all components were eluted out. The GC instrument was coupled with a Hewlett-Packard 5973 mass selective detector (Hewlett- Packard Co., Palo Alto, CA, USA). MS interface temperature was set to 250°C. Ion source and MS quadruples temperatures were 230 and 106°C, respectively. Ionization voltage was 70 eV. Scans were made in the mass range of 33～450 amu, at a scan rate of 2.94 scans/s. The electron multiplier voltage was 1494 V.

Component identification and quantification

Components were identified by comparing retention indices (RI) and mass spectra with those of authentic standards under identical analytical conditions (4). Tentative identifications were based on matching mass spectra of unknown components with those in the *Wiley Library of Mass Spectral Database* (7Nth edition, Wiley, New York, NY, USA). Components were quantified by comparing the peak area of a specified fragment of a component to the peak area of the fragment with m/z 121 of the internal standard (5). Positively identified components were quantified using three-point calibration curves of corresponding authentic standards. The relative abundance of tentatively identified components was estimated by the area ratios of these components to the area of the internal standard.

RESULTS AND DISCUSSION

In the two brands of douchi, a total of 73 components were positively identified (Table 1). These common components were categorized as 18 acids, 12 alcohols, 11 aldehydes, 9 esters, 1 furan, 11 other oxygen-containing compounds, 4 pyrazines, 2 pyridines and 5 miscellaneous compounds.

Many fatty acids such as hexadecanoic acid, (*Z*)-9-octadecenoic acid and (*E,E*)-9,12-octadecanoic acid were present in the extracts in very high quantities. There were no significant differences in the concentrations of these components between the extracts of the two brands. The fatty acids are likely to be produced by the action of fungal lipases on the lipids of the beans (6). The smaller acid components such as acetic acid were highly abundant in the extracts. Acetic acid was found in both brands in very high quantities and had a strong pungent sour odor (7). The penetrating, sour tasting 3-methylbutanoic acid (8) was highly abundant in brand B, but only present in small amounts in brand D ($p>0.05$). The same could be said for 2-methylpropanoic acid. The concentration difference was 51% in the latter and 70% in the former. The former was also reported to have a rancid, cheese-like, sweaty, fecal and putrid flavor (7) and the odor of the latter was described as penetrating, rancid butter (7). Both components are thought to be produced during fermentation.

Of the common components in the alcohol class, 4-ethylphenol was the most abundant, though it was as much as six times more abundant in brand B than in D. The component was also found in extracts of the plain fermented soybean curd (5) and is reported to have a pleasant strong, sweet honey-like odor and a bittersweet taste (8). Other alcohols also found in plain soybean curd include 2-heptanol, benzenemethanol, phenol and 2-me-

Table 1. Volatile components in two commercial brands of douchi (B and D)

Compound	CAS No.[1]	m/z[2]	RI[3]	Brand B		Brand D		p t-test[4]	sig[5]
				conc. (μg/mg)	SD	conc. (μg/mg)	SD		
Acids (18)									
acetic acid	64-19-7	43	1401	14163.10	2726.98	12153.98	2184.53	0.378	no
2-methylpropanoic acid	79-31-2	43	1515	27.59	4.42	13.57	0.68	0.029	yes
butanoic acid	107-92-6	60	1555	22.25	1.63	25.68	0.72	0.051	no
3-methylbutanoic acid	503-74-2	60	1581	83.91	19.18	25.35	4.29	0.029	yes
pentanoic acid	109-52-4	60	1666	0.94	0.69	0.88	0.23	0.905	no
hexanoic acid	142-62-1	60	1789	6.58	2.89	6.71	1.44	0.946	no
2-ethyl-hexanoic acid	149-57-5	88	1898	3.75	1.99	1.31	2.27	0.234	no
heptanoic acid	111-14-8	60	1901	1.01	0.06	0.63	0.64	0.422	no
octanoic acid	124-07-2	60	2008	3.03	0.82	2.53	0.36	0.404	no
nonanoic acid	112-05-0	60	2114	0.89	0.25	0.69	0.08	0.284	no
benzoic acid	65-85-0	105	2414	529.10	203.69	–	–	n.c.	n.c.
tetradecanoic acid	544-63-8	73	2636	52.06	16.12	21.42	18.80	0.100	no
pentadecanoic acid	1002-84-2	242	2737	14.91	8.78	7.28	1.12	0.270	no
hexadecanoic acid	57-10-3	256	2887	272.53	148.25	203.85	20.77	0.507	no
heptadecanoic acid	506-12-7	73	3073	66.38	63.72	44.65	45.09	0.657	no
octadecanoic acid	57-11-4	284	3349	213.30	92.00	2097.66	2936.39	0.382	no
(*Z*)-9-octadecenoic acid	112-80-1	264	3445	9295.90	4635.92	289836.85	462518.75	0.404	no
(*Z*,*Z*)-9,12-octadecadienoic acid	60-33-3	280	3663	16610.20	8228.65	15868.49	13749.11	0.941	no
Alcohols (12)									
2-heptanol	543-49-7	45	1248	0.08	0.07	0.21	0.04	0.073	no
2-cyclohexen-1-ol	822-67-3	70	1433	0.15	0.14	0.31	0.01	0.195	no
2-ethyl-1-hexanol	104-76-7	57	1454	0.52	0.46	0.85	0.07	0.347	no
2-furanmethanol	98-00-0	98	1574	0.51	0.33	0.49	0.02	0.923	no
2-chlorocyclohexanol	1561-86-0	80	1586	1.63	1.46	3.18	0.11	0.205	no
2-undecanol	1653-30-1	45	1725	0.25	0.01	0.15	0.04	0.045	yes
2-methoxy-phenol	90-05-1	109	1817	2.80	0.79	5.78	1.57	0.062	no
benzenemethanol	100-51-6	108	1832	0.23	0.22	0.05	0.09	0.298	no
benzeneethanol	60-12-8	91	1876	0.88	0.81	1.58	0.20	0.269	no
phenol	108-95-2	94	1959	1.99	0.45	1.43	0.39	0.180	no
4-ethylphenol	123-07-9	107	2125	38.69	10.13	6.75	2.51	0.026	yes
2-methoxy-4-vinylphenol	7786-61-0	150	2151	0.26	0.12	0.35	0.07	0.329	no
Aldehydes (11)									
n-hexanal	66-25-1	56	1059	2.81	0.64	2.92	0.54	0.838	no
n-nonanal	124-19-6	57	1367	1.94	0.93	3.08	0.20	0.162	no
2-furancarboxaldehyde	98-01-1	95	1428	0.06	0.01	0.06	0.01	0.564	no
(*E*,*E*)-2,4-heptadienal	4313-03-5	81	1468	0.14	0.05	0.09	0.01	0.261	no
benzaldehyde	100-52-7	105	1500	2.62	0.77	2.51	0.16	0.832	no
phenylethanal	122-78-1	91	1578	1.13	0.26	1.27	0.27	0.539	no
(*E*,*E*)-2,4-decadienal	25152-84-5	81	1780	0.88	0.36	0.72	0.14	0.531	no
2-phenyl-2-butenal	4411-89-6	146	1906	1.03	0.23	0.55	0.03	0.065	no
1*H*-pyrrole-2-carboxaldehyde	1003-29-8	95	1988	0.55	0.15	0.56	0.04	0.949	no
5-methyl-2-phenyl-2-hexenal	21834-92-4	117	2044	4.09	1.42	2.48	0.20	0.187	no
2-formyl-1-methylpyrrole	1192-58-1	109	2059	0.41	0.21	0.15	0.02	0.162	no
Esters (9)									
butyl acetate	123-86-4	56	1049	0.45	0.03	0.30	0.20	0.313	no
methyl benzoate	93-58-3	105	1565	0.99	0.31	0.05	0.01	0.034	yes
ethyl benzoate	93-89-0	105	1593	2.61	0.77	0.06	0.01	0.029	yes
methyl benzeneacetate	101-41-7	35	1721	–	–	0.01	0.02	n.c.	n.c.
methyl hexadecanoate	112-39-0	74	2184	73.93	85.14	29.27	25.41	0.464	no
ethyl hexadecanoate	628-97-7	88	2223	98.85	54.96	49.33	5.89	0.258	no
ethyl octadecanoate	111-61-5	88	2432	0.79	0.56	0.47	0.08	0.435	no
ethyl (*Z*)-9-octadecenoate	111-62-6	310	2449	0.28	0.19	0.22	0.06	0.628	no
ethyl 9,12,15-octadecatrienoate	1191-41-9	306	2551	8.58	5.81	14.91	19.14	0.631	no

Table 1. Continuded

Compound	CAS No.[1]	m/z[2]	RI[3]	Brand B		Brand D		p t-test[4]	sig[5]
				conc. (μg/mg)	SD	conc. (μg/mg)	SD		
Furans (1)									
2-pentylfuran	3777-69-3	81	1204	2.81	0.59	3.66	0.40	0.117	no
Other oxygen-containing compounds (11)									
2-cyclohexen-1-one	930-68-7	68	1415	0.53	0.07	0.67	0.02	0.068	no
4-cyclopentene-1,3-dione	930-60-9	96	1539	1.26	0.43	0.65	0.09	0.129	no
dihydro-5-methyl-2(3*H*)-furanone	108-29-2	85	1559	1.83	0.26	1.18	0.06	0.043	yes
tetrahydro-2*H*-pyran-2-one	542-28-9	42	1562	0.16	0.01	0.09	0.01	0.000	yes
dihydro-2(3*H*)-furanone	96-48-0	86	1570	2.25	1.00	1.96	0.19	0.660	no
1-methyl-2-pyrrolidinone	872-50-4	99	1597	0.37	0.13	0.20	0.11	0.162	no
maltol	118-71-8	126	1935	46.58	15.68	25.37	4.42	0.135	no
2,5-dimethyl-4-hydroxy-3(2*H*)-furanone	3658-77-3	128	1982	0.43	0.41	1.74	0.52	0.029	yes
2-pyrrolidinone	616-45-5	85	2003	24.70	7.77	10.24	7.96	0.088	no
2-piperidinone	675-20-7	99	2095	437.74	152.61	150.79	19.87	0.080	no
2,5-pyrrolidinedione	123-56-8	99	2411	16.64	14.47	14.28	4.14	0.808	no
Pyrazines (4)									
methylpyrazine	109-08-0	94	1232	0.62	0.16	0.62	0.09	0.958	no
2,5-dimethylpyrazine	123-32-0	108	1283	2.91	0.55	2.75	1.53	0.875	no
2,6-di-methyl-pyrazine	108-50-9	108	1287	946.86	178.45	1054.63	219.56	0.547	no
2,3,5-trimethylpyrazine	14667-55-1	122	1374	0.13	0.22	0.43	0.08	0.130	no
Pyridines (2)									
2,4,6-trimethylpyridine**	108-75-8	121	1332	149.69	0.85	151.01	1.25	0.215	no
2-phenyl-pyridine	1008-89-5	155	2216	0.43	0.15	0.36	0.02	0.531	no
Miscellaneous (5)									
octane	111-65-9	43	800	0.22	0.10	0.20	0.10	0.875	no
toluene	108-88-3	91	1020	0.18	0.01	0.31	0.20	0.381	no
1-methanol-3-cyclohexene	10482-56-1	136	1622	0.21	0.06	0.24	0.16	0.783	no
naphthalene	91-20-3	128	1724	0.23	0.01	0.10	0.07	0.080	no
benzeneacetonitrile	140-29-4	90	1901	−	−	0.10	0.01	n.c.	n.c.

**Internal standard.
[1]Chemical Abstract Service Registry Number.
[2]Mass/charge value of specified fragment used for concentration calculation.
[3]Retention indices calculated on the basis of van den Dool and Kratz (4).
[4]Probability that the concentrations of the component in the two brands is identical (two-tailed Student t-test, n=3). n.c= not calculated.
[5]Significant difference in concentration of the component in the two brands according to a two-tailed Student t-test (α=0.05), yes or no.

thoxy-4-vinylphenol. The last compound has a spicy, clove-like flavor and is thought to be the thermal degradation products of the lignin-related phenolic carboxylic acids (9). But the compound is considered to be an off-flavor in orange juice (10). 2-Ethyl-1-hexanol, a component with a mild, oily, sweet, slight rosy aroma, was also found in red fermented soybean curd by Chung (11). 2-Undecanol was found in low concentrations in both brands, but the concentration was significantly higher in brand B ($p>0.05$). Alcohols have fatty odors with a fruity note and taste (8).

The abundance of aldehydes in the douchi extracts was relatively low. Among the more abundant aldehydes were *n*-hexanal, 5-methyl-2-phenyl-2-hexanal, benzaldehyde, nonanal and phenylethanal. *n*-Hexanal contributes to the green aroma of soybean products and is, together with benzaldehyde, thought to be produced by lipid oxidation and degradation (5,12). The last compound has an aromatic taste similar to almond and is thought to contribute to the aroma of roasted, cooked fermented soybean cotyledons just as nonanal, which has a strong fatty odor and a citrus flavor (8,11). 5-Methyl-2-phenyl-2-hexenal was also found in extracts of red fermented soybean curds by Chung (11). It was described as having a prune-like flavor. Phenylethanal, which did not exceed the threshold value of 4.0 μg/kg, is described as having a rosy flavor in red fermented soybean curds (11). The component is known to be a Strecker degrada-

tion product of the amino acid phenylalanine (10). Other components present both in douchi and in white fermented soybean curds were (*E*,*E*)-2,4-heptadienal and (*E*,*E*)-2,4-decadienal.

Esters found in high quantities in the extracts were all methyl or ethyl esters of the highly abundant fatty acids. Ethyl hexadecanoate has a faint waxy odor, ethyl (*Z*)-9-octadecenoate has a floral note and ethyl 9,12,15-octadecatrienoate has been described as sweet and mild creamy (5,8,11). The concentrations of these components in douchi however did not exceed the threshold values of 2.5×10^7, 1.3×10^5 and 3.4×10^6 μg/kg, respectively and are not likely to have great influence on the douchi flavor. The high abundance of methyl- and ethyl esters of the fatty acids suggests the presence of methanol and ethanol during fermentation (5,13). Concentrations of smaller esters were considerably lower. Methyl and ethyl benzoate were more abundant in brand B than in brand D. Their flavors are described as fruity, where the taste of the methyl ester is similar to cananga and the taste of the ethyl ester resembles apricot. Butyl acetate is present in small amounts in both brands and has a strong fruity odor and a burning, sweet pineapple flavor (8).

Only one furan was found in douchi. 2-Pentylfuran was present in small amounts. It has a fruity odor and a metallic, green bean aroma (8,12) and was reported in concentrations exceeding its odor threshold value in roasted, cooked, fermented soybean cotyledons. As much as 11 other different common oxygen-containing compounds were found in the two brands of douchi, of which maltol, was present in very high quantities in both brands. Maltol (3-hydroxy-2-methyl-4H-pyran-4-one) has a warm sweet, fruity odor (8). 2,5-Dimethyl-4-hydroxy-3(2*H*)-furanone, also known as furaneol (10), was present in small amounts, but in significantly higher concentrations in brand D than in brand B. It has a strawberry-like note at low concentrations and serves as character impact component in pineapple, strawberry and muscadine grape juice (8,10). Wu and Cadwallader (10) could detect the component in considerable concentrations in a meat-like process flavoring made from enzyme-hydrolyzed soybean protein. Chung (11) reported its presence in plain fermented soybean curd, whereas dihydro-2(3*H*)-furanone, a component with a faint, sweet, caramel flavor was only detected in red fermented soybean curd (11). According to Chung (5) most heterocyclic ketones do not naturally occur in soybeans, but are thermally generated in soybean products during heating. 2-Piperidinone, 2-pyrrolidinone and 2,5-pyrrolidinedione were present in very high quantities in both brands.

2,6-Dimethylpyrazine was predominant in the class of pyrazines. All pyrazines found are also found in fermented soybean curds and have a nutty aroma (5). Because of their abundance, pyrazines are often suggested to be important contributors to the characteristic odor of fermented soybean products such as douchi, hamanatto, natto, sufu and fermented soybean curd (5,13,14). Owens et al. (12) reported the degradation of 2,6-dimethylpyrazine during fermentation of roasted, cooked soybean cotyledons. No significant difference could be found in concentrations of pyrazines present in brands B and D. Besides 2,4,6-trimethylpyridine (IS), which was added as an internal standard, only 2-phenylpyridine was found in douchi in small concentrations.

All of the components in the miscellaneous class were present in small quantities. Naphthalene was described to have a mothball-like flavor and was also found in fermented soybean curd by Chung (5). The component was responsible for a strong unpleasant odor and was suggested to be a contaminant from the environment.

While most of the 73 different components found in SFE extracts of douchi were present in similar amounts in both brands B and D, brand B seemed to have a sig-

Table 2. Flavor components with large difference in concentration between brands B and D

Compound class	Components	Difference (%)[1)]	Reported flavor[3)]
Acid	benzoic acid	>>[2)]	sweet and sour, acrid
	3-methylbutanoic acid	70	penetrating, sour, like stinky rubber
	3-methylpropanoic acid	51	bitter, like stinky feet
Alcohol	4-ethylphenol	81	strong sweet, honey-like odor, bitter taste
Ester	methylbenzoate	95	fruity, cananga
	ethylbenzoate	98	fruity, apricot
Other oxygen-containing compound	2,5-dimethyl-4-hydroxy-3(*2H*)-furanone	-77	strawberry

[1)]Difference calculated as percentage of the more abundant component, a positive value means the component is more abundant in brand B.
[2)]>> meaning present in high quantity (>100 μg/mg) in brand B, but absent in D.
[3)]Flavor references: Aldrich (7); Fenaroli (8).

nificantly higher concentration of important flavor components. Major differences in concentrations of flavor components between the two brands are summarized in Table 2. The data suggests that the aroma of douchi of brand B is both more bitter and sour. However, since sensory analysis was not done, this could not be confirmed. The high abundance of ethyl esters of fatty acids suggests the presence of ethanol. Since ethanol was absent in SFE extracts, it is expected that ethanol was originally present in douchi, but evaporated from the extract with the solvent during extraction. Generally, the extracted amount of each component was between 10^{-4}～10^{-3} μg/g, while abundant components were in range of 0.1～1.0 μg/g. Acetic acid was one of the most abundant components at a concentration of 14 μg/g.

Seventy-three components were found in SFE extracts of douchi and were divided into difference classes including 18 acids, 12 alcohols, 11 aldehydes, 9 esters, 1 furan, 11 other oxygen-containing compounds, 4 pyrazines, 2 pyridines and 5 miscellaneous compounds. Though many of these components were found in both brands in similar amounts, some of the more abundant components were significantly more abundant in brand B. These results suggest that brand B might have more bitter and sour aromas than the other sample.

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