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# Characterization of the Key Aroma Compounds in Soy Sauce Using Approaches of Molecular Sensory Science

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Application of aroma extract dilution analysis (AEDA) to the volatiles isolated from a commercial Japanese soy sauce revealed 30 odor-active compounds in the flavor dilution (FD) factor range of 8−4096, among which 2-phenylethanol showed the highest FD factor of 4096, followed by 3-(methylsulfanyl)propanal (methional), the tautomers 4-hydroxy-5-ethyl-2-methyl- and 4-hydroxy-2-ethyl-5-methyl-3(2*H*)-furanone (4-HEMF), 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (4-HDF), and 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone (sotolone), all showing FD factors of 1024. Thirteen odorants were quantified by stable isotope dilution assays, and their odor activity values (OAVs) were calculated as ratio of their concentrations and odor thresholds in water. Among them, 3-methylbutanal (malty), sotolone (seasoning-like), 4-HEMF (caramel-like), 2-methylbutanal (malty), methional (cooked potato), ethanol (alcoholic), and ethyl 2-methylpropanoate (fruity) showed the highest OAVs (>200). An aqueous model aroma mixture containing 13 odorants, which had been identified with the highest OAVs, in concentrations that occur in the soy sauce showed a good similarity with the overall aroma of the soy sauce itself. Heat treatment of the soy sauce resulted in a clear change of the overall aroma. Quantitation of selected odorants revealed a significant decrease in sotolone and, in particular, increases in 2-acetyl-1-pyrroline, 4-HDMF, and 4-HEMF induced by heating.

**KEYWORDS: Soy sauce; aroma extract dilution analysis; odor activity value; (*E*)-β-damascenone; ethyl 2-methylbutanoate**

## INTRODUCTION

Soy sauce is traditionally used as seasoning in eastern Asia, and its popularity in the Western part of the world is growing due to its intense umami taste accompanied by a very characteristic aroma. Japanese soy sauce (shoyu) is traditionally produced by fermentation of heated soybeans and wheat flour with *Aspergillus oryzae* or *Aspergillus sojae*. The resulting koji is fermented with *Pediococcus halophilus* and *Zygosaccharomyces rouxii* to yield moromi. Pressing of moromi then yields soy sauce, which is finally pasteurized and bottled. For the aroma development of Japanese soy sauce, koji culturing, moromi fermentation, and pasteurization are the most important steps. For Chinese soy sauce only soybeans, but no cereals, are used, whereas Korean soy sauce is produced from soybeans, barley meal, and various spices (*1*).

Many investigations on the volatile constituents of Japanese soy sauce have been performed in the past, and today nearly 300 compounds have been identified (*1*). Nunomura et al. (*2*−*6*) investigated the volatile compounds of Japanese soy sauce in numerous studies using a combination of vacuum distillation−solvent distillation and gas chromatography−mass spectrometry. They identified over 90 compounds in the acidic fraction and over 140 compounds in the neutral volatile fraction. The tautomers 4-hydroxy-5-ethyl-2-methyl-3(2*H*)-furanone and 4-hydroxy-2-ethyl-5-methyl-3(2*H*)-furanone (4-HEMF) were proposed to be of major importance for soy sauce aroma on the basis of sensory experiments (*7*). In the basic fraction of soy sauce various pyrazines were identified (*3*), and it was reported that their concentrations increased during pasteurization (*1*). Sensory experiments also revealed phenylacetaldehyde as an important flavor compound in Japanese soy sauce (*6*). However, the aroma impact of other single compounds was not systematically evaluated in these studies. The studies of Aishima (*8*) and Kihara (*9*−*12*) tried to find a correlation between the GC pattern of the volatile fraction and the sensory quality of Japanese soy sauce, and these authors confirmed, in particular, the crucial role of 4-HEMF for the overall soy sauce aroma.

A more comprehensive approach to evaluate the aroma relevance of individual volatile components was recently performed by Baek and Kim (*13*). They applied gas chromatography−olfactometry on a headspace extract obtained by solid-phase microextraction of a Japanese soy sauce. These authors suggested methional, 3-methylbutanoic acid, 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (4-HDF), 4-HEMF, 2-methoxyphenol, and benzaldehyde as important odorants. However, because the use of solid-phase microextraction (SPME) involves the risk of compound discrimination caused by, for example,

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the differences in volatilities of the compounds or the differences in the polarity of the fibers (*14*), respectively, exact quantitative data and a correlation with odor thresholds are needed to confirm results of screening procedures, such as GC-O. Futhermore, to separate the key odor-active compounds of foods from the bulk of odorless volatiles, it is necessary to combine analytical methods with human odor perception in each analytical step during odorant isolation and characterization, to finally obtain an aroma recombinate. This approach, which can be assigned as "molecular sensory science", leads to a blueprint of volatile constituents that interact with the human odorant receptors, thus causing the entire food aroma impression in the brain.

The aim of the present study was, therefore, first, to screen the key odorants of a commercial Japanese soy sauce by aroma extract dilution analysis, to quantify key aroma compounds using stable isotope dilution assays, to verify the results by flavor recombination, and, finally, to study changes caused by thermal processing.

## MATERIALS AND METHODS

**Soy Sauce.** A soy sauce originating from a Japanese manufacturer (Kikkoman), prepared from soybeans, wheat, water, and salt without further additives, was purchased at a local supermarket. The soy sauce was dark brown in color.

**Chemicals.** The following odorants were obtained from the commercial sources as follows: 2-acetyl-2-thiazoline, bis(2-methyl-3-furyl) disulfide, butanoic acid, 2,3-diethyl-5-methylpyrazine, 2-ethyl-3,5-dimethylpyrazine, ethyl 2-methylbutanoate, ethyl 2-methylpropanoate, 2-furylmethanethiol, 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone (sotolone), 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone, 4-hydroxy-3-methoxybenzaldehyde, 3-hydroxy-2-methyl-4-pyranone, 2-methoxyphenol, 2- and 3-methylbutanal, 2- and 3-methylbutanoic acid, 3-methylbutan-1-ol, methyl 2-methylpropanoate, 3-(methylsulfanyl)propanol, pentanoic acid, phenylacetaldehyde, 2-phenylacetic acid, 2-phenylethanol, 3-(methylsulfanyl)propanal (Sigma-Aldrich Chemie, Taufkirchen, Germany); 4-ethyl-2-methoxyphenol, 1-octen-3-one, 4-vinyl-2-methoxyphenol (Alfa Aesar, Johnson Matthey, Karlsruhe, Germany); acetic acid and ethanol (Merck, Darmstadt, Germany). 4-Hydroxy-5-ethyl-2-methyl-3(2*H*)-furanone was a gift from Givaudan, Dübendorf, Switzerland.

The following odorants were synthesized as reported in the literature: 2-acetyl-1-pyrroline (*15*), (*E*)-*β*-damascenone (*16*), *trans*-4,5-epoxy-(*E*)-2-decenal (*17*). Diethyl ether (Merck) was freshly distilled before use.

**Isotopically Labeled Compounds.** The following deuterium-labeled isotopologues were synthesized according to procedures previously published by our group: [$^2H_{2-5}$]-2-acetyl-1-pyrroline (*18*), [$^2H_3$]-ethyl 2-methylpropanoate (*19*), [$^{13}C_2$]-3-hydroxy-4,5-dimethyl-2(5*H*)-furanone (*20*), [$^{13}C_2$]-4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (*21*), [$^2H_3$]-4-hydroxy-5-ethyl-2-methyl-3(2*H*)-furanone (*22*), [$^2H_3$]-methional (*23*), [$^2H_3$]-3-methylbutanal (*24*), [$^2H_2$]-3-methylbutanoic acid, and [$^2H_2$]-3-methylbutan-1-ol (*25*) as well as [$^{13}C_2$]-phenylacetaldehyde and [$^{13}C_2$]-2-phenylethanol (*26*).

**Thermal Treatment of Soy Sauce.** Soy sauce (100 mL) was diluted with tap water (300 mL), filled in a glass cylinder, and reacted for 20 min at 145 °C (type II laboratory autoclave; Roth, Karlsruhe, Germany). After the final temperature was reached, the mixture was immediately cooled in ice−water.

**Isolation and Fractionation of Volatiles.** Soy sauce (100 mL) was diluted with tap water (300 mL) and extracted three times with diethyl ether (150 mL total volume). The combined extracts were dried over anhydrous sodium sulfate and concentrated to 100 mL using a Vigreux column (50 cm × 1 cm i.d.). The volatile fraction was separated from the nonvolatile material by SAFE distillation (*27*) at 40 °C. Acidic compounds were separated from the neutral and basic volatile fraction (NBF) by treatment of the distillate with aqueous sodium hydrogen carbonate (0.5 mol/L; three portions; 300 mL total volume). The alkaline aqueous phase was washed twice with diethyl ether (50 mL), acidified to pH 3.0 with hydrochloric acid (2 mol/L), and finally extracted with diethyl ether (3 × 50 mL) to yield the acidic volatiles fraction (AF). Both fractions were dried over anhydrous sodium sulfate and concentrated to 100 μL using a Vigreux column (50 cm × 1 cm i.d.) followed by a microdistillation apparatus (*28*).

**High-Resolution Gas Chromatography−Olfactometry (HRGC-O); Mass Spectrometry.** HRGC was performed by means of a type 8160 gas chromatograph (Fisons Instruments, Mainz, Germany) with helium serving as carrier gas at a pressure of 70 kPa. Samples were applied by cold on-column injection onto 30 m × 32 mm i.d., 0.25 μm, DB-5 or DB-FFAP capillary columns (J&W Scientific, Agilent Technologies, Waldbronn, Germany). The end of the capillary was connected to a deactivated Y-shaped glass splitter dividing the effluent of the column into two equal parts, which were then transferred via two deactivated but uncoated fused silica capillaries (50 cm × 0.25 mm) to a sniffing port and an FID, respectively. The sniffing port, which was mounted on a detector base of the GC, was heated to 180 °C, and the FID was operated at 240 °C. On-column injection of the samples (0.5 μL) was performed at an oven temperature of 40 °C. After 2 min, the temperature was raised by 6 °C/min to 240 °C. The final temperature was held for 10 min. During a GC run, the panelist placed his nose closely above the top of the sniffing port and evaluated the odor of the chromatographic effluent. Linear retention indices (RI) of the compounds were calculated from the retention times of *n*-alkanes.

Mass spectra were recorded using a 5890 series II gas chromatograph (Hewlett-Packard, Waldbronn, Germany) connected to a sector field MAT 95 S mass spectrometer (Finnigan, Bremen, Germany). Mass spectra in the electron ionization mode (MS/EI) were recorded at 70 eV ionization energy and mass spectra in the chemical ionization mode (MS/CI) at 115 eV using isobutane as the reactant gas.

**Aroma Extract Dilution Analysis (AEDA).** For AEDA, fractions NBF and AF were stepwise diluted 1:1 using diethyl ether as the solvent to obtain dilutions of 1:1, 1:2, 1:4, 1:8, 1:16, etc., of the original extracts (*28*). Sniffing of dilutions was continued until no odorant was detectable. Each odorant was thus assigned a flavor dilution factor (FD factor) representing the last dilution at which the odorant was detectable. The original extract was evaluated by three panelists to avoid that odorants may be overlooked, for example, by panelists who might be anosmic versus certain odorants. The entire AEDA was then performed by two panelists. The FD factors obtained by the two assessors were averaged, but differed by not more than two FD factors.

**Quantitation by Stable Isotope Dilution Assays in Combination with Two-Dimensional High-Resolution Gas Chromatography (TD-HRGC-SIDA).** The labeled internal standards (2−10 μg) dissolved in diethyl ether (0.5 mL) were added to aliquots of soy sauce containing the respective analytes in a similar concentration range, which was determined in preliminary experiments. After the addition of tap water and 1 h of stirring, the volatiles and the internal standards were isolated by extraction/distillation as described above.

TD-HRGC-MS was performed using a Mega 2 series GC (Fisons, Mainz, Germany) connected to a 5160 GC (Carlo Erba, Hofheim, Germany). In the first dimension, the separation of the extract was achieved on a DB-5 column (NBF) or on an FFAP column (AF), respectively. The elution range containing the selected odorant and the internal standard was transferred to a cold-trap (−100 °C) by means of a moving capillary stream switching device (Thermo, Dreieich, Germany). After complete trapping, the analyte and the internal standard were transferred onto the second 30 m × 0.32 mm i.d., 0.25 μm, fused silica capillary column (DB-FFAP for NBF and DB-1701 for AF) (Chrompack, Mühlheim, Germany) by heating the trap to 200 °C. For mass spectrometry, the second column was coupled in the open-split mode to an ITD 800 ion trap detector (Finnigan MAT, Bremen, Germany) running in the chemical ionization mode (CI) with methanol as reactant gas (ionization energy = 115 eV). The selected ions of the labeled standard and the aroma compound (**Table 1**) were monitored and their intensities calculated by means of a computer program. Concentrations were calculated and corrected using response factors obtained by measuring defined mixtures of the respective labeled and unlabeled compound.

**Quantitation of Acetic Acid and Ethanol.** Acetic acid and ethanol were determined enzymatically using commercial test combinations (R-Biopharm, Mannheim, Germany).

**Table 1.** Selected Ions (*m*/*z*) and Response Factors Used in the Stable Isotope Dilution Assays

| odorant | *m*/*z* | labeled internal standard | *m*/*z* | response factor |
|---|---|---|---|---|
| 2-acetyl-1-pyrroline | 112 | [$^2H_{2-6}$]-2-acetyl-1-pyrroline | 114–118[a] | 0.98 |
| ethyl 2-methylpropanoate | 117 | [$^2H_3$]-ethyl 2-methylpropanoate | 120 | 0.99 |
| 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone | 129 | [$^{13}C_2$]- 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone | 131 | 0.98 |
| 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone | 129 | [$^{13}C_2$]-4-hydroxy-2,5-dimethyl-3(2*H*)-furanone | 131 | 0.98 |
| 4-hydroxy-2-ethyl-5-methyl-3(2*H*)-furanone | 143 | [$^2H_3$]-4-hydroxy-2-ethyl-5-methyl-3(2*H*)-furanone | 146 | 0.98 |
| methional | 105 | [$^2H_3$]-methional | 108 | 0.98 |
| 2-methylbutanal | 69 | [$^2H_3$]-3-methylbutanal | 72 | 0.54 |
| 3-methylbutanal | 69 | [$^2H_3$]-3-methylbutanal | 72 | 0.94 |
| 3-methylbutanoic acid | 85 | [$^2H_2$]-3-methylbutanoic acid | 87 | 0.85 |
| phenylacetaldehyde | 121 | [$^{13}C_2$]-phenylacetaldehyde | 123 | 0.98 |
| 2-phenylethanol | 105 | [$^{13}C_2$]-2-phenylethanol | 107 | 0.98 |

[a] The sum of the isotopologues was used.

**Table 2.** Most Odor-Active (FD ≥ 8) Acidic Volatile Constituents in Soy Sauce

| | | | RI[a] on | | | |
|---|---|---|---|---|---|---|
| no. | odorant[b] | odor quality[c] | FFAP | DB-5 | FD factor | previously reported as volatile constituent in soy sauce |
| **1** | acetic acid | sour | 1436 | 645 | 64 | *2* |
| **2** | unknown | caramel-like | 1614 | nd | 8 | |
| **3** | butanoic acid | Parmesan cheese-like | 1620 | 1602 | 16 | *2* |
| **4** | 2- and 3-methylbutanoic acid | sweaty, cheese-like | 1648 | 873 | 256 | *2* |
| **5** | pentanoic acid | sweaty | 1723 | nd | 8 | *5* |
| **6** | 2-methoxyphenol | burnt | 1846 | 1090 | 128 | *2* |
| **7** | 3-hydroxy-2-methyl-4-pyranone | caramel-like | 1967 | nd | 16 | *30* |
| **8** | 4-ethyl-2-methoxyphenol | burnt | 2002 | 1282 | 8 | *30* |
| **9** | 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone | caramel-like | 2030 | 1071 | 1024 | *5* |
| **10** | unknown | caramel-like | 2060 | nd | 8 | |
| **11** | 4-hydroxy-5-ethyl-2-methyl-3(2*H*)-furanone[d] | caramel-like | 2088 | 1135/1140 | 1024 | *7* |
| **12** | 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone | seasoning-like | 2190 | 1082 | 1024 | *31* |
| **13** | 4-vinyl-2-methoxyphenol | spicy | 2200 | 1317 | 64 | *32* |
| **14** | 2-phenylacetic acid | hot chocolate, sweet | 2540 | 1262 | 256 | *5* |
| **15** | 4-hydroxy-3-methoxybenzaldehyde | vanilla-like | 2560 | 1406 | 128 | *30* |

[a] Retention index; nd, not determined. [b] The odorant was identified by comparing it with the reference compound on the basis of the following criteria: (i) retention indices on the GC stationary phases detailed in the table; (ii) mass spectra obtained by MS-EI and MS-CI; and (iii) odor quality and odor threshold determined by GC-O. [c] Odor quality perceived at the sniffing port. [d] The compound coelutes with its tautomer, 4-hydroxy-2-ethyl-5-methyl-3(2*H*)-furanone.

**Sensory Experiments. Descriptive Profile Fest.** Assessors were recruited from the German Research Center for Food Chemistry and were trained to describe and recognize the odor qualities of about 80 odorous chemicals. The assessors were subjected to a ranking test with a series of seven suprathreshold aqueous solutions (25 mL in Teflon vessels) of 3-methylbutanal (malty), 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (caramel-like), methional (cooked potato), 2-phenylethanol (flowery), ethanol (alcoholic), acetic acid (sour), and 2-acetyl-1-pyrroline (roasty, popcorn-like) and were asked to score the odor intensities on a seven-point linear scale from 0 to 3. Ten panelists were then selected for the evaluation of soy sauce and the aroma model. Sensory analyses were performed in a sensory panel room at 21 ± 1 °C at three different sessions.

The evaluation of the odor (orthonasal) of the soy sauce was performed in the following way: The panelists were asked to evaluate the intensities of the eight odor qualities represented by the chemicals given above using a seven-point linear scale. The results obtained at three different sessions were averaged for each odor note and plotted in a spider web diagram. The values judged by the single assessors differed by not more than 20%.

An aroma model mixture consisting of the 13 soy sauce odorants in the concentrations given in **Table 4** was prepared in tap water. The overall aroma profile was determined in the same way as described above for the soy sauce.

In a separate session, the overall similarities of the soy sauce aroma and the model aroma recombinate (with addition of brown color) were compared. The similarity was estimated using a seven-point scale from 0 to 3.

## RESULTS AND DISCUSSION

**Identification of Odor-Active Compounds.** The soy sauce exhibited an overall malty, caramel-like, and seasoning-like aroma, and a small portion of the distillate, isolated by solvent extraction and SAFE distillation, clearly represented the typical aroma of the soy sauce when sniffed on a strip of filter paper.

To avoid interferences during GC-O, the distillate was fractionated into the NBF containing neutral and basic volatiles and the AF containing acidic volatiles. Then AEDA was applied to both fractions. Because, in particular, the AF elicited an intense caramel- and seasoning-like aroma, the identification experiments were first focused on this fraction. Application of GC−olfactometry (GC-O) revealed 15 odor-active areas (**Table 2**) among which compounds **9**, **11**, and **12** showed very intense caramel- and seasoning-like odor qualities. By sniffing of serial dilutions of the distillate, these three compounds turned out to be the most odor-active ones based on the highest FD factor of 1024. The determination of their retention indices on two columns of different polarities as well as their odor quality and odor activity suggested compound **9** as 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone, compound **11** as 4-hydroxy-5-ethyl-2-methyl-3(2*H*)-furanone [or 4-hydroxy-2-ethyl-5-methyl-3(2*H*)-furanone], and compound **12** as 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone (**Figure 1**) on the basis of a comparison of the retention indices and odor qualities and intensities with data available in

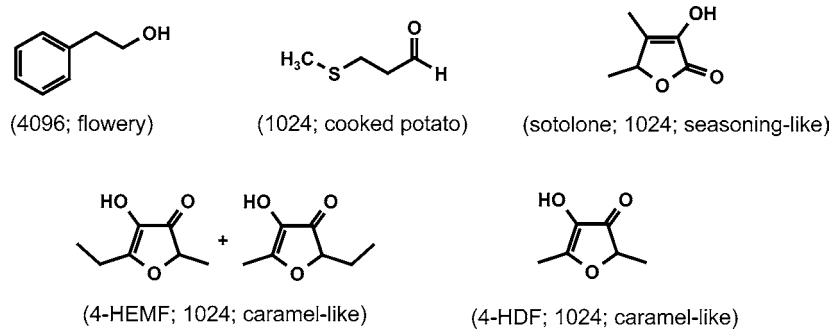


**Figure 1.** Structures of the most odor-active compounds (FD ≥ 1024) identified in soy sauce. Flavor dilution factor and odor quality are given in parentheses.

**Table 3.** Most Odor-Active (FD ≥ 8) Neutral-Basic Volatile Constituents in Soy Sauce

| | | | RI[a] on | | | |
|---|---|---|---|---|---|---|
| no. | odorant[b] | odor quality[c] | FFAP | DB-5 | FD factor | previously reported as volatile constituent in soy sauce |
| **16** | methyl 2-methylpropanoate | fruity | 900 | 684 | 16 | *33* |
| **17** | 2- and 3-methylbutanal | malty | 930 | 652/663 | 8 | *2*, *34* |
| **18** | ethyl 2-methylpropanoate | fruity | 959 | 747 | 256 | |
| **19** | ethyl 2-methylbutanoate | fruity | 1038 | 847 | 32 | *34* |
| **20** | 3-methylbutan-1-ol | malty | 1210 | 732 | 64 | *2* |
| **21** | 1-octen-3-one | mushroom-like | 1300 | 980 | 32 | |
| **22** | 2-acetyl-1-pyrroline | roasty, popcorn-like | 1319 | 926 | 8 | |
| **23** | methional | cooked potato | 1453 | 906 | 1024 | *2* |
| **24** | 2,3-diethyl-5-methylpyrazine | roasted potato | 1484 | 1158 | 16 | |
| **25** | phenylacetaldehyde | honey-like | 1647 | 1047 | 8 | *2* |
| **26** | 3-(methylsulfanyl)propanol (methionol) | cooked potato | 1715 | 980 | 16 | *2* |
| **27** | (*E*)-*β*-damascenone | cooked apple | 1806 | 1389 | 128 | |
| **28** | 2-phenylethanol | flowery | 1900 | 1117 | 4096 | *35* |
| **29** | *trans*-4,5-epoxy-(*E*)-2-decenal | metallic | 1989 | 1382 | 16 | |
| **30** | bis(2-methyl-3-furyl) disulfide | meat-like | 2127 | 1530 | 32 | |

[a] Retention index. [b] The odorant was identified by comparing it with the reference compound on the basis of the following criteria: (i) retention indices on the gc stationary phases detailed in the table; (ii) mass spectra obtained by MS-EI and MS-CI; and (iii) odor quality and odor threshold determined by GC-O. [c] Odor quality perceived at the sniffing port.

an in-house database. The compound structures were finally confirmed by comparing the mass spectra (MS-EI; MS-CI), the retention indices, and the sensory attributes of the three odorants with those of the respective reference compounds (**Figure 1**). The two tautomers of 4-hydroxy-5-ethyl-2-methyl- and 4-hydroxy-2-ethyl-5-methyl-3(2*H*)-furanone coeluted on the stationary phase used and, thus, are further assigned as 4-hydroxy-5-ethyl-2-methyl-3(2*H*)-furanone (4-HEMF). On the basis of the same approach, compounds **4** and **14**, showing somewhat lower FD factors, were identified as 2- and 3-methylbutanoic acid and 2-phenylacetic acid, respectively. As indicated in the last column of **Table 2**, the results confirmed previous data on the presence of these compounds in soy sauce; however, our data report for the first time a ranking of the volatiles on the basis of their odor activity in air (GC-O).

Application of AEDA on the NBF revealed another 15 odor-active compounds in the FD factor range of 8−4096 (**Table 3**). The highest FD factors were shown by compounds **28** (flowery), **23** (cooked potato), **18** (fruity), and **27** (cooked apple-like). The results of the identification experiments, performed as described above, revealed 2-phenylethanol, methional, ethyl 2-methylpropanoate, and (*E*)-*β*-damascenone as the most odor-active constituents in this fraction (**Table 3**). The last two mentioned odorants are reported for the first time as constituents of soy sauce.

**Quantitation of Odor-Active Compounds.** Dilution to odor threshold techniques, such as AEDA, are valuable tools for the screening of odor-active compounds in a given food. However, AEDA does not provide information on the aroma contribution of single compounds, because, for example, matrix effects on volatile release are neglected. However, the volatility of an odorant and its concentration in the headspace above the food, which is available for the odorant receptors in the nose, is significantly influenced by the matrix (*36*). For this reason, the odor activity value concept was applied to the odorants of the soy sauce. For this purpose, the five odorants that showed the highest FD factors (>1024) were quantified by means of stable isotope dilution assays, and their odor activity values (ratio of concentration to odor threshold) were calculated. Because high amounts of free amino acids are formed during soy fermentation, additionally, all compounds known as metabolites of amino acids, such as 2- and 3-methylbutanal, phenylacetaldehyde, 2-acetyl-1-pyrroline, 3-methylbutanol, and 2-phenylethanol, and, also, ethanol and acetic acid, which are both known as major soy sauce volatiles (*1*), were quantified.

The data revealed in particular ethanol (21700 mg/L) and acetic acid (1050 mg/L) with the highest concentrations, followed by 4-HEMF (14.5 mg/L), 3-methylbutanoic acid (23.9 mg/L), 2-phenylethanol (5.88 mg/L), 3-methylbutan-1-ol (4.27 mg/L), and 3-methylbutanal (2.3 mg/L) (**Table 4**). On the other hand, 2-acetyl-1-pyrroline [1-(3,4-dihydro-2*H*-pyrrol-5-yl)ethanone] was present in the lowest concentration (1.3 *μ*g/L). According to the odor activity value concept, odorants should contribute to the overall aroma if they exceed their odor

**Table 4.** Concentrations, Orthonasal Odor Thresholds (OOT), and Odor Activity Values (OAVs) of 13 Key Aroma Compounds in Soy Sauce

| compound | concn[a] (μg/L) | OOT (μg/L) | OAV[b] |
|---|---|---|---|
| 3-methylbutanal | 2300 | 1.2[c] | 1920 |
| 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone (sotolone) | 1080 | 1.1[c] | 982 |
| 4-hydroxy-5-ethyl-2-methyl-3(2*H*)-furanone (4-HEMF) | 14500 | 20[d] | 725 |
| 2-methylbutanal | 2150 | 4.4[c] | 489 |
| methional | 343 | 1.4[c] | 245 |
| ethanol | 21700000 | 100000[e] | 217 |
| ethyl 2-methylpropanoate | 20 | 0.1[e] | 200 |
| 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (4-HDF) | 1980 | 25[e] | 79 |
| phenylacetaldehyde | 201 | 4[e] | 50 |
| acetic acid | 1050000 | 22000[e] | 48 |
| 3-methylbutanoic acid | 23900 | 1200[c] | 20 |
| 2-phenylethanol | 5880 | 390[c] | 15 |
| 2-acetyl-1-pyrroline | 1.3 | 0.1[c] | 11 |

[a] Data are mean values of triplicates differing not more than ±0%. [b] Calculated by dividing the concentration by the odor threshold in water. [c] Czerny et al., 2007 (in preparation). [d] Odor threshold taken from ref *37*. [e] Odor threshold taken from ref *38*.

threshold in a given matrix (*28*). Because water is the major constituent of soy sauce, the odor thresholds of the selected aroma compounds in water (*37*, *38*) were used to calculate their odor activity values (OAVs).

On the basis of the results (**Table 4**), all 13 odorants quantified should contribute to the aroma of the soy sauce, because their concentrations clearly exceeded their odor thresholds. The highest odor activity value of 1920 was calculated for the malty-smelling 3-methylbutanal, followed by the seasoning-like-smelling sotolone, which was second in rank. Due to its high volatility, 3-methylbutanal had shown only a low FD factor in the AEDA, but a high OAV on the basis of the quantitative results. In addition, 4-HEMF, 2-methylbutanal, methional, ethanol, and ethyl 2-methylpropanoate showed OAVs > 200 and are, therefore, also suggested to contribute significantly to the overall aroma of soy sauce. Somewhat lower OAVs (11−79) were calculated for 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone, phenylacetaldehyde, acetic acid, 3-methylbutanoic acid, 2-phenylethanol, and 2-acetyl-1-pyrroline.

**Aroma Recombination.** The calculation of OAVs is a useful tool to predict a possible contribution of single odorants to the overall aroma. However, by this approach no masking or additive effects caused by other aroma compounds can be evaluated. Therefore, a model aroma mixture of the soy sauce was prepared in tap water of the same pH as measured in the soy sauce containing the 13 odorants in the same concentrations as present in the soy sauce (see data in **Table 4**). Odor attributes of the original soy sauce and the aroma model, respectively, were then evaluated singly in a descriptive profile test by 10 trained panelists. Additionally, the similarity of the overall odor of the soy sauce and the aroma model was evaluated on a seven-point scale from 0 (no similarity) to 3 (very good similarity).

The intensities of the main odor qualities of the original soy sauce were judged as follows: malty (2.4), seasoning-like (2.4), caramel-like (1.7), cooked potato (1.1), flowery (1.6), ethanolic (1.3), sour (1.3), and roasty, popcorn-like (1.2). The evaluation of the aroma profile of the model aroma mixture matched all of these odor qualities present in the soy sauce; however, the flowery and the malty odor qualities, in particular, were rated lower in the reconstituted aroma, whereas the cooked potato odor was a bit more pronounced (**Figure 2**). The reason for these differences might be the presence of matrix compounds, such as peptides and amino acids, which could change the overall aroma by odorant binding mechanisms. Nevertheless, the similarity between the overall odors of the two samples was judged to be 2.4 points of a maximum of 3.0.

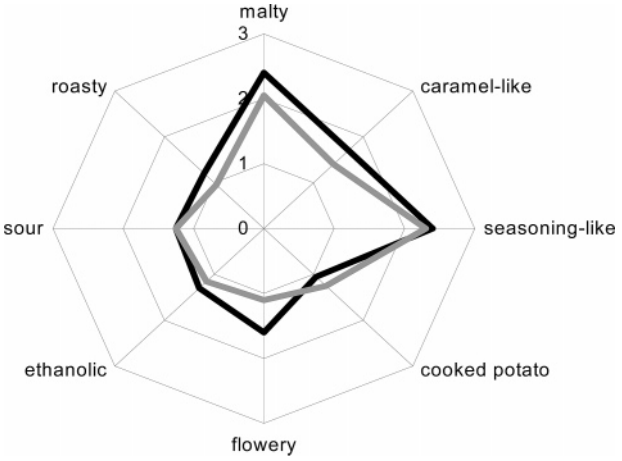


**Figure 2.** Aroma profile analysis of the soy sauce (black) and the model aroma mixture (gray).

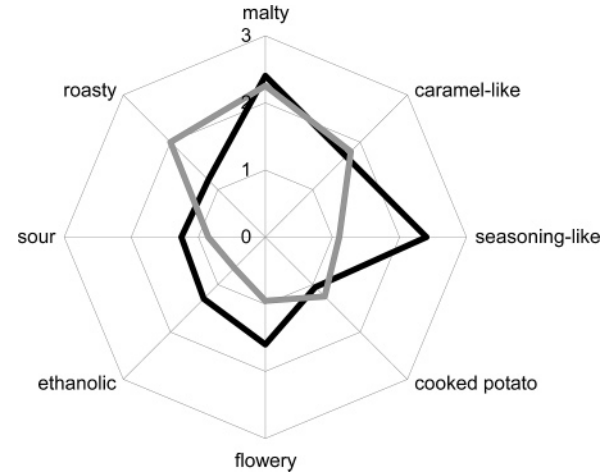


**Figure 3.** Aroma profile analysis of the soy sauce before (black) and after heat treatment (gray).

**Changes during Heat Treatment.** Soy sauce is used as a seasoning during cooking, so that aroma compounds can either be newly formed or degraded. To investigate the influence of a heat treatment on its major odorants, soy sauce was heated in a laboratory autoclave. The heated soy sauce exhibited an overall malty, bread-like aroma. Evaluation of the aroma by trained panelists resulted in the following scaling of odor qualities: malty (2.3), caramel-like (1.8), seasoning-like (1.1), cooked potato (1.3), flowery (1.0), ethanolic (0.7), sour (0.9), and roasty, popcorn-like (2.0). Compared to the untreated soy sauce, in particular, the seasoning-like odor note was significantly decreased, whereas the roasty note was markedly higher in the heated sample (**Figure 3**).

By means of a comparative AEDA (*28*), the FD factors of odorants present in the heated soy sauce were determined and compared to the data of the nonheated soy sauce. **Table 5** lists those compounds showing clear differences in their FD factors between both samples.

The results revealed three additional compounds, which had not been found in the nonheated soy sauce: 2-furylmethanethiol (roasty, coffee-like), 2-acetyl-2-thiazoline [1-(4,5-dihydro-1,3-

**Table 5.** Odorants Showing Clear Differences in Their FD Factors before and after Thermal Treatment of Soy Sauce

| odorant | odor quality | RI on FFAP | RI on DB-5 | FD factor before | FD factor after |
|---|---|---|---|---|---|
| 2- and 3-methylbutanal | malty | 930 | 652/663 | 8 | 64 |
| 2-acetyl-1-pyrroline | roasty, popcorn-like | 1319 | 926 | 8 | 256 |
| 2-furylmethanethiol | roasty, coffee-like | 1417 | 911 | <1 | 64 |
| 2-ethyl-3,5-dimethylpyrazine | potato-like | 1435 | 1079 | <1 | 16 |
| butanoic acid | Parmesan cheese-like | 1620 | 1602 | 16 | 256 |
| phenylacetaldehyde | honey-like | 1647 | 1047 | 8 | 512 |
| 2- and 3-methylbutanoic acid | sweaty, cheese-like | 1648 | 873 | 256 | 1024 |
| 2-acetyl-2-thiazoline | roasty | 1748 | 1106 | <1 | 64 |
| pentanoic acid | sweaty | 1723 | nd[a] | 8 | 256 |
| 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone (sotolone) | seasoning-like | 2190 | 1082 | 1024 | 256 |

[a] nd, not determine.

**Table 6.** Concentrations of Eight Selected Aroma Compounds in Soy Sauce before and after Thermal Treatment

| compound | concn[a] ($\mu$g/L) before | concn[a] ($\mu$g/L) after |
|---|---|---|
| 3-methylbutanal | 2300 | 1560 |
| 2-methylbutanal | 2150 | 580 |
| 2-acetyl-1-pyrroline | 1.3 | 15 |
| phenylacetaldehyde | 201 | 1110 |
| 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (4-HDF) | 1980 | 3560 |
| 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone (sotolone) | 1080 | 121 |
| 4-hydroxy-5-ethyl-2-methyl-3(2*H*)-furanone (4-HEMF) | 14500 | 17200 |
| 3-methylbutanoic acid | 23900 | 18900 |

[a] Data are mean values of triplicates differing not more than ±10%.

thiazol-2-yl)ethanone; roasty], and 2-ethyl-3,5-dimethylpyrazine (potato-like). These odorants were detected in the heated sauce with FD factors of 64, 64, and 16, respectively (**Table 5**).

Moreover, after heat treatment several compounds showed clearly higher FD factors as compared to the nonheated soy sauce; for example, phenylacetaldehyde increased by a factor of 64 and 2-acetyl-1-pyrroline by a factor of 32. Additionally, pentanoic acid, butanoic acid, and 2- and 3-methylbutanoic acid showed higher FD factors after heat treatment. In contrast, sotolone appeared with a 3 times lower FD factor.

Quantitation of selected odorants subsequently confirmed the significant changes in the concentration of the key aroma compounds of soy sauce caused by the heat treatment. In particular, the concentration of sotolone was significantly lower, and also the concentrations of 2-methylbutanal, 3-methylbutan-1-ol, and 3-methylbutanal decreased by factors of 1.5−4 (**Table 6**). In contrast, the amount of 2-acetyl-1-pyrroline increased by a factor of 11. Clear increases were also observed for phenylacetaldehyde, methional, 4-HDMF, and 4-HEMF. A comparison of the quantitative data (**Table 6**) with the odor profile analysis (**Figure 3**) suggested that, in particular, the increase in 2-acetyl-1-pyrroline (popcorn-like, roasty) and the decrease in sotolone (seasoning-like) might be the main reasons for the different profiles of the unheated and the heated soy sauces.

In summary, this study revealed 3-methylbutanal, sotolone, 4-hydroxy-5-ethyl-2-methyl-3(2*H*)-furanone, 2-methylbutanal, methional, ethanol, ethyl 2-methylpropanoate, 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone, phenylacetaldehyde, acetic acid, 3-methylbutanoic acid, and 2-phenylethanol as the 12 most important odorants in Japanese soy sauce. 3-Methylbutanal, 4-HEMF, methional, 4-HDMF, phenylacetaldehyde, acetic acid, and 3-methylbutanoic acid had already been identified by Nunomura and co-workers (*2*, *5*, *7*), and 2-methylbutanal was reported by Yokotsuka et al. (*35*) as a volatile component in Japanese soy sauce. As early as after 1952, ethanol was identified as a soy sauce component in fractional distillation (*40*). 2-Phenylethanol was already detected by means of thin-layer chromatography as a soy sauce component in 1967 (*36*). However, the aroma contribution of all compounds had not been evaluated in these studies.

The seasoning-like-smelling compound 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone (sotolone) had already been suggested as a key aroma constituent in soy sauce by Blank et al. (*31*), who showed that sotolone rather than 5-ethyl-3-hydroxy-4-methyl-2(5*H*)-furanone (abhexone) is responsible for the "hydrolyzed vegetable protein-like" odor note. A compound so far unknown in soy sauce was ethyl 2-methylpropanoate.

Although Baek and Kim (*13*) applied GC-O and sample dilution techniques to a soy sauce extract, they did not detect 2- and 3-methylbutanal, sotolone, ethyl 2-methylpropanoate, and phenylacetaldehyde, which have been revealed as major odorants of soy sauce in this study. The use of solid-phase microextraction, which had been used as a sample preparation method by these authors, can, however, be suggested as a reason for the differences. SPME may discriminate volatiles during extraction, for example, on the basis of the polarity of the fiber used or on the basis of the total binding capacity of the fiber, respectively.

Heat treatment of the soy sauce resulted in a clear change in the aroma, which was correlated with some increases and decreases in the concentrations of important aroma compounds. The increase of the two roasty-smelling odorants, 2-furylmethanethiol and 2-acetyl-1-pyrroline, during heat treatment of soy sauce can readily be explained by the high concentrations of their precursor amino acids cysteine and ornithine, respectively, in the soy sauce (data not shown). The role of cysteine in the formation of 2-furylmethanethiol during Maillard-type reactions has been established in previous studies (*40*), whereas ornithine was earlier shown to be an effective precursor of 2-acetyl-1-pyrroline (*41*).

The formation of 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (4-HDMF) when fructose-1,6-bisphosphate is heated was established previously (*42*), and a reaction pathway via 2,4-dihydroxy-2,5-dimethyl-3-furanone (acetylformoine) as intermediate was established (*42*, *43*). Sasaki et al. (*44*) proved that 4-hydroxy-5-ethyl-2-methyl-3(2*H*)-furanone is produced in soy sauce by a biosynthetic pathway involving yeasts, suggesting the pentose−phosphate cycle as a key step. However, 4-HEMF was also shown to be formed through Maillard reaction of pentoses during heating (*45*).

The quantitative data combined with the sensory experiments show that the major impact of heat treatment on the aroma of

soy sauce is the loss of the dominating seasoning-like aroma note due to a significant decrease of sotolone. This is well in line with the findings of Dagan et al. (*46*), who reported a rapid decomposition of sotolone at temperatures above 80 °C.

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