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Changes in the Key Aroma Compounds of Raw Shiitake Mushrooms (*Lentinula edodes*) Induced by Pan-Frying as well as by Rehydration of Dry Mushrooms

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ABSTRACT:

Application of the aroma extract dilution analysis (AEDA) on an extract/distillate from raw shiitake mushrooms revealed 32 odorants among which 3-(methylthio)propanal (cooked potato), 1-octen-3-one and 1-octen-3-ol (both mushroom-like) showed the highest flavor dilution (FD) factors. An isotope enrichment experiment with raw shiitake tissue and either $^{13}C_{18}$-linoleic acid or $^{2}H_{4}$-1-octen-3-ol confirmed that both, 1-octen-3-ol and 1-octen-3-one are direct degradation products of the fatty acid, but it could be proven for the first time that the ketone is not formed by an oxidation of the alcohol. After pan-frying, 42 odor-active compounds appeared among which 3-hydroxy-4,5-dimethylfuran-2(5*H*)-one (savory), 1,2,4,5-tetrathiane (burnt, sulfury), 4-hydroxy-2,5-dimethylfuran-3(2*H*)-one (caramel-like), phenylacetic acid (honey-like), 3-(methylthio)-propanal and *trans*-4,5-epoxy-(*E*)-2-decenal (metallic) showed the highest FD factors. To get a deeper insight into their aroma contribution, 19 key odorants were quantitated in the raw shiitake and twenty-one in the pan-fried mushrooms by stable isotope dilution assays, and new methods for the quantitation of four sulfur compounds were developed. A calculation of odor activity values (OAV; ratio of concentration to odor threshold) showed that 1-octen-3-one was by far the most important odorant in raw shiitake. During pan-frying, in particular four aroma compounds were significantly increased, i.e., 4-hydroxy-2,5-dimethylfuran-3(2*H*)-one, dimethyl trisulfide, 1,2,4,5-tetrathiane and 1,2,3,5,6-pentathiepane. The overall aroma profile of pan-fried shiitake could very good be mimicked by an aroma recombinate consisting of 15 reference aroma compounds in the concentrations determined in the pan-fried mushrooms. Further results showed that the sulfur compounds were even higher in rehydrated dry shiitake as compared to the pan-fried mushrooms.

KEYWORDS: raw shiitake, pan-fried shiitake, dry,rehydrated shiitake, sensomics, odor activity value, [$^{2}H_{4}$]-1,2,3,5,6-pentathiepane, [$^{2}H_{4}$]-1,2,4,5-tetrathiane, [$^{2}H_{6}$]-1,2,4,6-tetrathiepane, stable isotope dilution analysis,

INTRODUCTION

The global market consumption of mushrooms (edible fungi) is about 12.7 million tons (2018) and button mushrooms (*Agaricus bisporus*) hold the highest market share followed by shiitake mushrooms (*Lentinula edodes*). The growing demand for foods produced without meat in particular in Western countries, the low content of fat and cholesterol in the mushroom tissue, the presence of a lot of micronutrients as well as the unique aroma are undoubtedly the main drivers for the increase in mushroom production/consumption in the past decades. Shiitake is mainly used for its flavor properties in the Asian cuisine, but is today also increasingly sought after in European countries. Several odorants have been suggested to be important for the shiitake aroma. Morita and Kobayashi[1] were among the first to identify 1,2,3,5,6-pentathiepane in shiitake suggesting that the sulfur compounds is an impact odorant due to its "shiitake-like" odor. Later, Chen and Ho[2] identified further 18 sulfur compounds, for example 1,2,4,5-tetrathiane, 1,2,3,5-tetrathiane and 1,2,4-trithiolane in raw shiitake. Hong et al.[3] isolated the volatile fraction from raw and dry shiitake by simultaneous distillation/extraction (SDE) and reported on carbon disulfide, dimethyl disulfide, dimethyl trisulfide and 1-(methylthio)-dimethyl disulfide besides 1-octen-3-ol, 1-octen-3-one, 3-octanol, 3-octanone, cis-2-octenol, cis-2-octenal and *n*-octanol as further volatile components. However, application of SDE to raw, enzyme containing foods is known to cause artifacts either during maceration of the tissue or by the thermal treatment during distillation.

It is meanwhile accepted in the scientific literature that not all volatiles occurring in a given food contribute to the overall aroma profile. One reference method to locate, identify and quantitate the key aroma compounds able to interact with the human odorant receptors is the Sensomics approach.[4] Mushrooms are scarcely used as such, but are mostly processed either by cooking or pan-frying, and thus, non-volatile precursors present in the raw tissue may be thermally converted into volatile compounds. To get an idea on such conversions, the Sensomics approach has previously[4] been used in a study on changes in the key aroma compounds of raw and pan-fried white mushroom (*Agaricus bisporus L.*), and 4-hydroxy-2,5-dimethylfuran-3(*2H*)-one (caramel-like) followed by 2-propionyl-1-pyrroline (popcorn-like) and 3-hydroxy-4,5-dimethylfuran-2(*5H*)-one (seasoning-like) could clearly be assigned as the key aroma compounds increasing during pan-frying.

However, systematic investigations on the influence of a thermal treatment on changes in the aroma compounds of shiitake mushrooms are rather scarce. Therefore, the aim of the present study was to (i) characterize the key aroma compounds in raw shiitake mushrooms by application of the AEDA and (ii) to quantitate the odor-active compounds by means of stable isotope dilution assays (SIDAs) followed by a calculation of odor activity values. The same approach should then be applied on pan-fried as well as dry, rehydrated shiitake to elucidate the compounds undergoing changes during the thermal treatment or the hydration process. To establish the analytical data, the aroma profile of pan-fried shiitake and the dry,rehydrated mushrooms should be mimicked by means of an aroma recombinate on the basis of the quantitative data measured in the mushroom samples. Finally, isotope labeling experiments should be undertaken to elucidate the formation pathway of 1-octen-3-one, the key aroma compound in raw shiitake also occurring in nearly all types of raw edible mushrooms.

MATERIALS AND METHODS

Mushrooms. Several batches of fresh shiitake mushrooms were purchased from a local supplier. The mushrooms contained 0.3 % lipids per 100 g fresh weight. The main fatty acid was linoleic acid with about 70 % after methanolysis of the total lipids. Linoleic acid was mainly bound in the phospholipid fraction (data not shown).
For pan-frying, fresh mushrooms were cut into slices, and fried in a hot pan (140 °C) for 5 min with several turnovers, but without addition of fat.
Dry shiitake mushrooms were purchased from Hawlik Vitalpilze (Straßlach; Germany). The drying temperature used was 40 °C (as indicated on the label).

Chemicals

Acetic acid, acetic anhydride, aluminium oxide 90 (neutral), anhydrous sodium sulfate, deuterated methylene chloride, methylene chloride, sulfur, silica 60, sodium iodide, sodium sulfide, sodium sulfite, and methylene dithiocyanate were obtained from Merck, (Darmstadt, Germany). Methylene chloride was freshly distilled before use. Liquid nitrogen was obtained from Linde (Munich, Germany).

Reference Odorants.

The following reference odorants were obtained from the commercial sources given in parentheses: acetic acid, 4-allyl-2-methoxyphenol, 2,3-butanedione, butanoic acid, *δ*-decalactone, *γ*-decalactone, 2,3-diethyl-5-methylpyrazine, (*E,E*)-2,4-decadienal, dimethyl trisulfide, (*Z*)-6-dodeceno-γ-lactone, 3,5-dimethyl-2-ethenylpyrazine, 3-ethylphenol, 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone, 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone, indole, 2-methoxy-5-methylphenol, 2-methoxyphenol, 3-methylbutanal, 2-methylbutanoic acid, 3-methylbutanoic acid, 3-(methylthio)propanal, (*E,E*)-2,4-nonadienal, *γ*-nonalactone, δ-nonalactone, (*E*)-2-nonenal, octanal, (*E*)-2-octenal, 1-octen-3-ol, pentanoic acid, phenylacetaldehyde, phenylacetic acid, 3-methylindole and *γ*-undecalactone (Sigma Aldrich Chemie, Taufkirchen, Germany); 1-octen-3-one (Alfa Aesar, Karlsruhe, Germany); 1,2,3,5,6-pentathiepane (Chemos Group, Regenstauf, Germany); vanillin (Merck); 4-*tert*-butylphenol, 2-isobutyl-3-methoxypyrazine, (Acros Organics BVBA, Geel, Belgium).

Synthesis of 1,2,4-Trithiolane and 1,2,4,6-Tetrathiepane

The compounds were synthesized following a procedure published earlier[5] with some modifications: Sodium sulfide nonahydrate (100 g; 0.42 mol) and sulfur (20g; 0.62 mol) were stirred in deionized water (330 mL) until an orange-red color appeared. The mixture was filtrated and after addition of dichloromethane (330 ml) stirred at room temperature (RT) for seven h. Then, the organic phase was washed with brine (total volume 240 mL) and dried over anhydrous sodium sulfate. The solvent was carefully removed in vacuo, and the residue was re-crystallized from dry dichloromethane. 1,2,4-Trithiolane (1.5 g) and 1,2,4,6-tetrathiepane (5.6 g) were characterized by mass spectrometry (see spectra in Figure 1A and Figure 2A).

Synthesis of 1,2,4,5-Tetrathiane

The odorant was synthesized following a method published previously[6] with several modifications: Methylene dithiocyanate (6.5 g) and sodium disulfate (11.4 g) were dissolved in demineralized water (50 mL) and refluxed for 15 min. Water was removed in vacuo and, after addition of potassium iodide (20 g), acetic acid (225 mL) followed by acetic acid anhydride (25 mL) refluxed for 1 h. After cooling, water (100 mL) was

added, the excess of iodine was removed by addition of ascorbic acid, and the mixture was extracted with dichloromethane (total volume 200 mL). The organic phase was washed with sodium bicarbonate (0.5 mol/l; 100 mL) and after drying over anhydrous sodium sulfate concentrated in vacuo to about 5 mL. The solution was filtered over neutral aluminium oxide using dichloromethane, and after concentration of the solution purified by column chromatography on silica gel flushed with n-pentane. The tetrathiane was obtained in a yield of 0.58 g and was characterized by mass spectrometry (Figure 3A).

The following reference compounds were synthesized according to literature cited: 2-acetyl-1-pyrroline,[7] trans-4,5-epoxy-(E)-2-decenal,[8] (*Z*)-1,5-octadien-3-one,[9] and 2-propionyl-1-pyrroline.[10]

Isotopically Labeled Reference Compounds.

The compounds, either labeled with deuterium or carbon-13, were synthesized according to the literature cited: [$^2H_{2-5}$]-2-acetyl-1-pyrroline,[7] [$^2H_3$]-2,3-diethyl-5-methylpyrazine, [$^2H_3$]-3,5-dimethyl-2-ethylpyrazine and [$^2H_3$]-2,5-dimethyl-3-ethylpyrazine,[11] [$^{13}C_2$]-3-hydroxy-4,5-dimethylfuran-2(5*H*)-one,[12] [$^{13}C_2$]-4-hydroxy-2,5-dimethylfuran-3(*2H*)-one,[13] [$^2H_3$]-3-(methylthio)propanal,[14] [$^2H_2$]-3-methylbutanal,[15] [$^2H_2$]-3-methylbutanoic acid,[16] [$^2H_4$]-1-octen-3-ol,[17] [$^2H_4$]-1-octen-3-one,[18] [$^{13}C_2$]-phenylacetaldehyde,[19] [$^2H_{2-4}$]-2-propionyl-1-pyrroline,[20] [$^2H_4$]-*trans*-4,5-epoxy-(*E*)-2-decenal;[21]

[$^2H_3$]-acetic acid and [$^{13}C_2$]-phenylacetic acid were obtained from Sigma-Aldrich (Taufkirchen, Germany); [$^2H_3$]-pentanoic acid was obtained from C/D/N isotopes (Quebec, Canada).

Synthesis of sulfur-containing isotopically labeled internal standards. [$^2H_4$]-1,2,3,5,6-pentathiepane, [$^2H_4$]-1,2,4,5-tetrathiane, [$^2H_6$]-1,2,4,6-tetrathiepane and [$^2H_4$]-1,2,4-trithiolane were synthesized following a procedure published for the unlabeled compounds[22] with some modifications: Sodium sulfide nonahydrate (7.5 g; 96 mmol) and sulfur (1.5 g; 57 mmol) were dissolved in distilled water (25 mL) and stirred for 1h at RT. The orange colored solution was filtered and [$^2H_2$]-dichloromethane (25 mL; 0.4 mmol) was added. After vigorous stirring for 7 h, the organic layer was washed with brine (25 mL) followed by distilled water (25 mL). The solution was

concentrated to ~200 µL at 52 °C using a Vigreux column. The compound mixture was purified by flash chromatography on silica with n-pentane. The mass spectra obtained after separation by gas chromatography are displayed in Figure 1B ([$^2H_4$]-1,2,4-trithiolane; Yield 89 mg, Figure 2B ([$^2H_6$]-1,2,4,6-tetrathiepane; yield 35,1 mg), Figure 3B ([$^2H_4$]-1,2,4,5-tetrathiane; yield 20,3 mg) and Figure 4B ([$^2H_4$]-1,2,3,5,6-pentathiepane; yield 3,9 mg).

The concentration of each compound was determined with methyl octanoate as the internal standard using a calibration curve determined with mixtures of methyl octanoate and the respective unlabeled compound.

Isolation of the Volatiles. Either fresh (cut into small slices), pan-fried or dry, rehydrated shiitake mushrooms (on the basis of 30 g fresh weight) were frozen in liquid nitrogen and ground by means of a commercial blender after addition of anhydrous sodium sulfate to bind water. For volatile isolation, the material was extracted with dichloromethane (total volume 300 mL) by vigorous stirring for 60 min. The mixture was filtered and the volatile fraction was isolated using the solvent assisted flavor evaporation (SAFE) technique.[23] The distillate obtained was dried over anhydrous sodium sulfate and finally concentrated at 45 °C to ~200 µL using a Vigreux column (50 cm × 1 cm i.d.) and a microdistillation apparatus.[24]

Fractionation of the Volatiles. For identification experiments, the mushroom material (300 g) was extracted and worked up as described above. To separate the acidic from the neutral and basic volatiles, the SAFE distillate was treated with an aqueous sodium bicarbonate solution (0.5 mol/L, total volume 300 mL) to isolate the neutral/basic volatile fraction in the solvent phase (NBF).[20] The combined aqueous layers were adjusted to pH 2 with hydrochloric acid, and the acidic volatiles (acidic fraction, AF) were extracted with methylene chloride (total volume 300 mL). Both fractions were dried over anhydrous sodium sulfate, and after filtration each fraction was concentrated to ~500 µL. The NBF was further fractionated by column chromatography using a water-cooled glass column (30 cm × 1.8 cm) filled with a slurry of 30 g of acid-washed silica (G60, 7% water) in n-pentane and separated into six fractions using n-pentane/diethyl ether mixtures of increasing polarity (fraction I, 100:0, 75 mL; fraction II, 95:5, 80 mL; fraction III, 90:10, 80 mL; fraction IV, 75:25, 80 mL; fraction V, 50:50, 80 mL;

fraction VI, 0:100, 110 mL)[20] plus a seventh fraction using methylene chloride (100 mL). Each fraction was dried over anhydrous sodium sulfate, concentrated to ~200 µL as described above, and analyzed by means of HRGC-O and HRGC-MS.

High-Resolution Gas Chromatography−Olfactometry (GC-O). GC-O analysis was performed using a Thermo Electron Trace Ultra gas chromatograph (Dreieich, Germany) with the following J&W Scientific fused silica capillary columns: DB-FFAP and DB-5 (both 30 m × 0.25 mm i.d.; 0.25 µm film thickness) (Folsom, CA, USA). The sample (1.0 µL) was injected cold on-column at 40 °C using helium as carrier gas at a flow rate of 1.2 mL/min. The oven temperature was programmed from 40 °C, held for 2 min, increased with 6 °C/min to 230 °C (for the DB-FFAP column or to 240 °C for the DB-5 column) and held for 5 min. The flow of the carrier gas was split at the end of the capillary column by a Chrompack Y-type quick-seal glass splitter (Frankfurt, Germany) and two deactivated fused-silica capillaries (50 cm × 0.32 mm i.d.). One part was directed to an FID (250 °C) and the other to a sniffing port (190 °C). A series of n-alkanes C6−C26 for the DB-FFAP and C6−C18 for the DB-5 was used to determine linear retention indices (RI).

Aroma Extract Dilution Analysis. For the determination of FD factors the concentrated SAFE distillate was subjected to GC-O on the FFAP column to detect and describe the odors of all aroma-active areas. To avoid overlooking of odor-active compounds, the original distillate was sensorially analyzed by at least three experienced panelists. Then, the distillate was diluted stepwise 1:1 (by vol) with methylene chloride, and each dilution was analyzed in 1.0 µL aliquots by HRGC-O.

High-Resolution Gas Chromatography−Mass Spectrometry (HRGC-MS). For compound identification, mass spectra were acquired using a Hewlett-Packard gas chromatograph 5890 series II (Waldbronn, Germany) coupled to a Finnigan MAT 95 S sector field mass spectrometer (Bremen, Germany). Mass spectra in the electron impact (MS-EI) mode were generated at 70 eV, and chemical ionization (MS-CI) was performed at 115 eV using isobutane as the reactant gas.

Quantitation of Odorants by Stable Isotope Dilution Assays (SIDA). Depending on the amounts of the odorants estimated in preliminary experiments, isotopically labeled internal standards (0.1−70 µg), dissolved in methylene chloride, were added to the mushroom material (1−50 g) suspended in methylene chloride. After 1 h of stirring for equilibration, the work-up procedure was performed as described above.

Quantitation of compounds present in higher concentrations was performed using a Varian CP 3800 gas chromatograph and a Varian Saturn 2000 ion trap mass spectrometer (Darmstadt, Germany) (system 1; Table 1). The sample was injected on the J&W Scientific DB-FFAP capillary column (60 m × 0.25 mm i.d.; 0.25 µm film thickness) by the cold on-column technique, and mass spectra of the separated compounds were recorded for the respective ions in the MS-CI mode using methanol as reagent gas (system 1; Table 1).

For the separation of co-eluting compounds, a two dimensional-GC-MS system (TD-GC-MS) consisting of a Thermo Scientific Trace GC 2000 series (Dreieich, Germany) coupled to a Varian CP 3800 gas chromatograph and a Varian Saturn 2000 ion trap mass spectrometer was used. After cold on-column injection, separation of the distillate in the first dimension was achieved on the J&W Scientific DB-FFAP capillary column (30 m × 0.32 mm i.d.; 0.25 µm film thickness). The elution range containing the selected odorant and the internal standard was then transferred into a cold trap by a moving capillary stream switching system (MCSS). After complete trapping, the analyte and the standard were transferred onto the second column, a J&W Scientific DB-1701 (30 m × 0.25 mm i.d.; 0.25 µm film thickness) by heating the trap to 200 °C. Mass spectra were recorded in the MS- CI mode using methanol as reagent gas (system 2; Table 1).

For compounds present in low concentrations comprehensive two-dimensional gas chromatography (GC x GC) was performed using an Agilent 6890N gas chromatograph (Böblingen, Deutschland) equipped with a Gerstel PTV 4 injector (Mühlheim an der Ruhr, Deutschland) and a CTC-analytics PAL autosampler (Zwingen, Schweiz) (system 3; Table1). After cold on-column injection (-20 °C) and immediate increase of the temperature to 240 °C, separation of the distillate in the first dimension was achieved on the J&W Scientific DB-FFAP capillary column (30 m × 0.32 mm i.d.; 0.25 µm film thickness) with helium as carrier gas at a flow rate of 1.2 mL/min. The effluent was transferred onto the second column, a J&W Scientific DB-5 (1 m × 0.25 mm i.d.; 0.25 µm film thickness). Mass spectra were recorded using a Leco

Pegasus 4 time-of-flight mass spectrometer (St. Joseph, USA). Ionization energy was set to -70 eV, detector voltage to 1700 V. Mass spectra were recorded with a frequency of 100 scans/s. Mod. temperature offset was set to +50 °C. Data were recorded with the LECO ChromaTOF software (v. 4.50.8.0, LECO Corporation, Michigan, USA). The autosampler software was Gerstel Maestro 1 (v. 1.4.12.14) (Mülheim an der Ruhr). Data were treated using GC-Image (v. 2.2b4 GCxGC) (Lincoln, NE, USA). Response curves were prepared from defined mixtures of the labeled and the unlabeled compound as detailed for the four sulfur compounds below.

Quantitation of *trans*-4,5-epoxy-(*E*)-2-decenal was performed using an Agilent 7890B gas chromatograph and an Agilent ion trap 240 mass spectrometer (Waldbronn, Germany). The sample was injected on the J&W Scientific DB-FFAP capillary column (60 m × 0.25 mm i.d.; 0.25 µm film thickness) by the cold on-column technique, and mass spectra of the separated compounds were recorded in the negative MS-CI mode using methanol as reagent gas (system 4;Table 1).

For the quantitation of 2- and 3-methylbutanoic acid, the sum of both acids coeluting on the respective gc stationary phase was determined by SIDA. Then, the ratio of 2- and 3-methylbutanoic acid in the samples was determined by HRGC-MS by calculating the intensities of the fragments *m/z* 60 (3-methylbutanoic acid) and *m/z* 74 (2-methylbutanoic acid). For method standardization, defined mixtures of 2- and 3-methylbutanoic acid were analyzed under the same conditions, and a calibration line was drawn plotting the intensity ratio of the ions against the percentage of 3-methylbutanoic acid in the mixture.

Response curves for the quantitation of [$^2H_4$]-1,2,3,5,6-pentathiepane, [$^2H_4$]-1,2,4,5-tetrathiane, [$^2H_6$]-1,2,4,6-tetrathiepane and [$^2H_4$]-1,2,4-trithiolane

Mixtures of the respective labeled and unlabeled compound were prepared in five different mass ratios (1:5, 1:3, 1:1, 3:1, and 5:1) and analyzed by HRGC-MS to calculate the response factor (RF) from the intensities of the mass fragments. The response curves obtained are shown in the supplementary information (Figures S1 to S4).

Determination of Orthonasal Odor Thresholds. Orthonasal odor thresholds were determined using the triangular test with decreasing concentrations of aqueous

odorant solutions against odorless water as the control. Glass vessels either filled with the odorless water (Volvic; 20 mL) or with the respective aqueous odorant solution (20 mL) were presented to a panel of 15−20 trained assessors, who were asked to identify the different sample in each row and describe the odor quality. Calculation of odor thresholds was performed as described before.[25]

Aroma Profile Analysis. Aroma profiles were determined by a trained panel consisting of 20-25 panelists, who participated in weekly sensory sessions to train their ability to recognize and describe different aroma qualities. The following reference compounds were used to define the aroma attributes: 2-acetyl-1-pyrroline (popcorn-like, roasty), (*E,E*)-2,4-decadienal (fatty; fried), 2,3-diethyl-5-methylpyrazine (earthy), *trans*-4,5-epoxy-(*E*)-2-decenal (metallic), 3-hydroxy-4,5-dimethylfuran-2(*5H*)-one (seasoning-like), 1-octen-3-one (mushroom-like), 3- (methylthio)propanal (cooked potato-like), phenylacetaldehyde (flowery), and 1,2,4,5-tetrathiane (sulfury, burnt). The intensities of the respective aroma qualities were ranked on a seven-point scale from 0 (not perceivable) over 0.5, 1.0, 1.5, ..., to 3.0 (strongly perceivable). The judgements of the panelists were averaged. Samples (20 g) were presented in glass vessels at room temperature (RT).

Aroma Recombination Experiments. An aqueous aroma model was prepared using all quantitated reference aroma compounds with OAVs >1 in their actual concentrations determined in the pan-fried mushrooms. The recombinate and the pan-fried mushrooms were each placed in closed glass vessels (20 g each) and presented to the sensory panel at RT. The overall aroma was evaluated on the basis of the same scale as used for aroma profile analysis.

Isotopologue Enrichment Study with Carbon-13 Labeled Linoleic Acid and Deuterium labeled 1-Octen-3-ol

Either [$^{13}C_{18}$]-linoleic acid or [$^{2}H_{4}$]-1-octen-3-ol were dissolved in phosphate buffer (18 mL; pH 6.5, 0.1M, according to *Sørensen*) with the aid of Tween 80 (0.1%). In separate experiments, the amount of each labeled compound was used in an equimolar concentration compared to the respective unlabeled compound measured in the mushroom homogenate. Mushrooms were added and homogenized with an ultraturrax homogenizer for 30 s. To stop enzymatic reactions, glass vials containing

hydrochloric acid (1 mL; 2mol/l) were prepared. In the experiment with labeled linoleic acid, 1 mL (n=3) of the homogenate was transferred after 1, 2, 4, 8, 15 and 30 min into these vials followed by the internal standards 2-nonanone, [$^2H_4$]-1-octen-3-ol, [$^2H_4$]-1-octen-3-one and [$^2H_2$]-(E)-2-nonenal. The formation of 1-octen-3-ol, 1-octen-3-one, 3-octanone and (E)-2-octenal as well as the four eight-fold carbon-13 labeled isotopologues (formed from [$^{13}C_{18}$]-linoleic acid) was measured by solid phase micro extraction (SPME) after 15 min equilibration. The experiment with [$^2H_4$]-1-octen-3-ol followed the same approach, but only 2-nonanone was added as the internal standard.

RESULTS AND DISCUSSION

Identification of Odor-Active Compounds in Raw Shiitake.

Freshly macerated raw shiitake mushrooms showed an intense mushroom-like, metallic aroma, and the volatile fraction isolated by extraction and SAFE distillation elicited the same overall aroma profile when evaluated on a strip of filter paper. Application of the AEDA resulted in 34 odorants in the FD factor range of 4 to 4096 (Figure 5), among which **13** exhibiting a potato-like odor showed the highest FD factor of 4096, followed by **5** and **12,** both with a mushroom-like odor attribute. With somewhat lower FD factors, **30** with a metallic odor quality and **52** exhibiting a vanilla-like odor were detected. The odor qualities, retention indices and mass spectra (MS-EI and MS-CI) were first compared with data available in an in-house database established by the analysis of more than 1000 odor-active compounds as recently reported for a group of sulfur containing odorants.[26] By GC/O and GC/MS analysis of the respective reference compound, the following aroma compounds were subsequently identified: **13** (3-(methylthio)propanal), **5** (1-octen-3-one), **12** (1-octen-3-ol), **30** (*trans*-4,5-epoxy-(*E*)-2-decenal) and **52** (vanillin)(Figure 6). Besides these five odorants, 3-methylbutanal (**1**, malty), (*E*)-2-octenal (**10**, fatty, nutty), phenylacetaldehyde (**19**, flowery), 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (**32**, caramel-like), and 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone (**40**, seasoning-like) also reached quite high FD factors among the twenty-eight odor-active compounds identified (Table 2). Only four out of the compounds identified in this study (**5,12,41,53**) have previously been described as volatiles in shiitake. Among them, the sulfury and burnt smelling compounds 1,2,3,5,6-pentathiepane (**41**) and 1,2,4,5-tetrathiane (**53**)

have earlier been identified in a steam distillate of fresh or dried shiitake mushroom[1,2,27-30] and were reported to be enzymatically formed in raw shiitake by a degradation of the precursor lentinic acid.[31-33]

Development of stable isotope dilution assays for the quantitation of 1,2,3,5,6-pentathiepane, 1,2,4,5-tetrathiane, 1,2,4,6-tetrathiepane and 1,2,4-trithiolane

During GC/O the entire amount of a single component present in an extract is vaporized and is, thus, available for the odorant receptors during GC/O.[34] However, in a food only the relative proportion present in the headspace above a food can be used to evaluate the aroma contribution of an odorant. To approach this challenge, a quantitation followed by the calculation of odor activity values (OAV; ratio of concentration to odor threshold) is an appropriate method, because the influence of the food matrix on the volatility of a given aroma compound is taken into account.

However, while the isotopically labeled internal standards for 15 out of the 19 odorants selected or quantitation were available in our group (see experimental part), except for the unlabeled pentathiepane, neither the other three sulfur containing unlabeled compounds nor the four deuterium labeled isotopologues were commercially available. Following the synthetic route described above, the three unlabeled sulfur compounds could successfully be synthesized as confirmed by the mass spectra displayed in Figures 1A to 3A. The deuterium labeled isotopologues [$^2H_4$]-1,2,3,5,6-pentathiepane, [$^2H_4$]-1,2,4,5-tetrathiane, [$^2H_6$]-1,2,4,6-tetrathiepane and [$^2H_4$]-1,2,4-trithiolane were synthesized in a one pot reaction (Figure 7) using [$^2H_2$]-dichloromethane to introduce the labeling into the four target compounds. Their mass spectra are contrasted in Figures 1B to 4B to the spectra of the unlabeled analytes. The successful introduction of either four or six deuterium atoms is confirmed by a comparison of the respective molecular ions. The response curves obtained by monitoring the molecular ions in defined mixtures of the respective unlabeled and labeled compound by mass chromatography are shown in Figures S1 to S4 in the supporting information. To verify that no deuterium/hydrogen exchange occurred during the work-up procedure, a 1:1 mixture of all eight compounds was added to a raw mushroom homogenate, and the volatile fraction was isolated as described for the samples.

392 **Quantitation of Selected Odorants in Raw Shiitake Mushrooms and**
393 **Calculation of Odor Activity Values.**

394 Quantitation of 19 odorants in the raw shiitake revealed by far the highest
395 concentrations for 1-octen-3-ol, followed by phenylacetaldehyde, phenylacetic acid
396 and 1-octen-3-one (Table 3). The sulfur compounds 1,2,3,5,6-pentathiepane, 1,2,4,5-
397 tetrathiane and 1,2,4,6-tetrathiepane were only present in quite low amounts
398 For OAV calculations, the odor thresholds of trans-4,5-epoxy-(E)-2-decenal, 2,3-
399 diethyl-5-methylpyrazine and of the four sulfur compounds were newly determined in
400 this study (Table 4). The results showed that only 14 of the 19 odorants quantitated
401 exceeded their odor threshold in water. By far the highest OAV of 2913 was calculated
402 for 1-octen-3-one, followed by 3-(methylthio)propanal, 3-methylbutanal and 1-octen-3-
403 ol with OAVs >75, while 1,2,3,5,6-pentathiepane, 1,2,4,5-tetrathiane and 1,2,4,6-
404 tetrathiepane showed only low OAVs between 9 and 12. The importance of 1-octen-3-
405 one for the typical mushroom-like odor is well in line with data on the high OAV in raw
406 white mushrooms.[4] However, aldehydes known as degradation products of amino
407 acids, i.e. phenylacetaldehyde, 3-(methylthio)propanal and 3-methylbutanal (Table 4)
408 were previously not reported as constituents of raw shiitake, but the same observation
409 was reported for aroma compounds in white mushrooms.[4] However, while these
410 aldehydes are well-known to be formed in a thermal degradation reaction of amino
411 acids with alpha dicarbonyls (Strecker reaction), obviously these can also be formed
412 at lower temperatures, probably catalyzed by pyridoxamine, a well-known catalyst in
413 the enzymatically induced amino acid metabolism.
414
415 **Isotopologue enrichment study on the formation of C-8 aroma compounds**
416 **in raw shiitake**

417 Most mushrooms contain linoleic acid as the main fatty acid, and it has been
418 reported for *Boletus edulis* many years ago[35] that the fatty acid is enzymatically
419 converted via the 10-hydroperoxide into the mushroom-like smelling 1-octen-3-ol.
420 Furthermore, several other C-8 compounds, such as 1-octen-3-one, 3-octanone and
421 (E)-2-octenal were found in shiitake[36] and could also be formed from linoleic acid as
422 precursor. In particular the most odor-active mushroom aroma compound 1-octen-3-
423 one is assumed to be formed by an oxidation of the alcohol. However, this reaction
424 step has not yet been proven by a systematic experiment. An isotope enrichment

approach is the most reliable way to identify a non-volatile precursor of an aroma compound directly in the food itself. This idea is explained in the following: The food, here the mushrooms, are administered with the same amount of an isotopically labeled precursor (^{13}C or ^{2}H labeled) compared to the assumed, unlabeled precursor quantitated in the food itself. In this case, the isotope ratio in the precursor for either carbon-12 to carbon-13 or deuterium to hydrogen is 1:1. Thus, any reaction product only formed from this assumed precursor during thermal treatment should consequently appear in the same isotope ratio. But, if additional precursors would be present, the ratio should be shifted to a higher number for ^{12}C or ^{1}H, respectively.

The experiments were first done with $^{13}C_{18}$-linoleic acid, which was added to a shiitake homogenate, and the formation of four C-8 degradation products was monitored in a time span between 20 s and 30 min. The data indicated that the unlabeled 1-octen-3-ol showed by far the highest concentration.as compared to 1-octen-3-one, 3-octanone and (E)-2-octenal, respectively (Table 5). For all four compounds the maximum concentration was reached after 8 min. The enzymatic conversion of the $^{13}C_{18}$ linoleic acid was a bit slower compared to the natural linoleic acid, but a nearly 1:1 ratio for 1-octen-3-ol and [$^{2}H_4$]-1-octen-3-ol was reached after about 8 to 15 min. This result clearly corroborated that this odorant was exclusively formed from linoleic acid. A similar trend was observed for 1-octen-3-one and (E)-2-octenal showing a delayed formation from the labeled linoleic acid, but reaching a ratio close to 1:1 ratio in the unlabeled to labeled compound after about 15 min. The quite late formation of the $^{13}C_8$ 3-octanone suggests that this compound is probably not directly formed from linoleic acid, but obviously by an enzymatic hydrogenation of 1-octen-3-one.

In the next experiment, the role of 1-octen-3-ol in the formation of 1-octen-3-one was studied (Table 6). The results showed that the amount of [$^{2}H_4$] -1-octen-3-ol added to the mushroom homogenate remained nearly unchanged within an incubation time of 60 min, while the unlabeled alcohol was generated from linoleic acid. However, the data clearly showed that no [$^{2}H_4$]-1-octen-3-one was formed by conversion of the labeled alcohol (Table 6), while labeled 3-octanone as well as labeled (E)-2-octenal were formed, however, in very small amounts. These results suggest that a direct formation pathway of 1-octen-3-one from linoleic acid does exist, but not involving 1-octen-3-ol as the intermediate.

Identification of Odor-Active Compounds in Pan-fried Shiitake Mushrooms. To get an insight into changes induced by a thermal treatment, the same batch of shiitake was pan-fried, and the volatile fraction was isolated by extraction/SAFE distillation. In the FD factor region of 8 to 8192 fifty-three odor-active areas were detected by GC/O (Figure 8) among which **40** exhibiting a seasoning-like odor showed the highest FD factor of 8192, followed by **41** and **51** with a sulfury, burnt and a honey-like odor quality, respectively, as well as **32** with a caramel-like aroma. The identification experiments in combination with the FD factors revealed **40** as 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone, **41** as 1,2,4,5-tetrathiane, **51** as phenylacetic acid and **32**. 4-hydroxy-2,5-dimethylfuran-3(*2H*)-one (Table 7). Among the further forty-eight odorants identified in the pan-fried mushrooms, 3-(methylthio)propanal (**13**) and trans-4,5-epoxy-(E)-2-decenal (**30**) also showed high FD factors. However, although a few compounds, in particular the sulfur containing odorants and 1-octen-3-one, have previously been reported in heat treated shiitake, sensory studies on their impact on the aroma have been performed for the first time in the present study. In addition, 33 compounds were newly reported as odorants of shiitake (Table 7).

Quantitation of Selected Odorants in Pan-Fried Shiitake and Calculation of Odor Activity Values. Twenty-one aroma compounds detected with high FD factors in the pan-fried shiitake were quantitated by stable isotope dilution assays. The highest concentrations within these odorants were measured for 1-octen-3-ol, 4-hydroxy-2,5-dimethyl-3(2H)-furanone, 2,3-butanedione, 1,2,3,5,6-pentathiepane, 2-methylbutanoic acid and 1,2,4,5-tetrathiane (Table 8). However, a calculation of odor activity values revealed that only 14 aroma compounds were present above their odor threshold (Table 9). The highest OAVs of 981 and 891 were calculated for 1,2,3,5,6-pentathiepane and 1,2,4,5-tetrathiane. High OAVs were also found for 2,3-butanedione, 3-(methylthio)propanal, (*E,E*)-2,4-decadienal, 3-methylbutanal, 1-octen-3-one and dimethyl trisulfide.

Aroma Simulation. To study the aroma interactions among the key odorants in a mixture, an aroma recombinate was prepared in an aqueous solution containing all 15 aroma compounds odorants with OAVs ≥1 in their actual concentration in the pan-fried shiitake mushrooms (Table 8). The aroma profile was evaluated by a descriptive

aroma profile analysis and also compared to the overall odor elicited by a powder made from freshly prepared pan-fried mushrooms. Eight aroma descriptors agreed in preliminary sessions of the panel were used: mushroom-like, sulfury/burnt, fatty/fried, seasoning-like, metallic, earthy, popcorn-like/roasty, and cooked potato-like. The sensory panel agreed that the recombinate elicited the typical aroma of pan-fried shiitake mushrooms with small differences in the attributes mushroom-like and metallic (Figure 9). The similarity of the aqueous aroma recombinate was judged to be 2.6 of 3.0, thus corroborating the quantitative data obtained.

Changes induced by the thermal treatment

Besides a remarkable overall difference in the aroma profiles of the raw and pan-fried shiitake (data not shown), also clear changes in nearly all important odorants were observed, and either a formation or a degradation occurred (Table 10). In particular, 4-hydroxy-2,5-dimethyl-3(2*H*) furanone (4-HDF) was increased by a factor of over 150 in the pan-fried mushrooms followed by dimethyl sulfide, which was increased by a factor of over 50. The caramel-like smelling 4-HDF is known to be formed by a degradation of carbohydrates in processed foods, and hexose phosphates as well acetylformoin were previously confirmed as important precursors.[37]

Also 1,2,3,5,6-pentathiepane and 1,2,4,5-tetrathiane were much higher in the pan-fried material, obviously due to a thermal degradation of the precursor lentinic acid. Although both sulfur compounds have previously been reported as constituents of a steam distillate of shiitake,[30] this is the first sensory study confirming the impact of these two compounds in the aroma of heat-treated shiitake.

Nine aroma compounds were lost/degraded during pan-frying, and in particular the mushroom like smelling 1-octen-3-one and 1-octen-3-ol were lower by a factor of 100 or 10, respectively, compared to the amounts in the raw mushroom (Table 3). As it was recently shown for the homologue of 1-octen-3-one, the 1-penten-3-one, such alpha, beta unsaturated ketones with a double bond at the end may easily undergo aldol-type reactions during thermal treatment of foods and can also bind to thiols.[38]

Changes induced by rehydration of dry shiitake

To increase their shelf-life and to maintain the availability over the year, mushrooms are often dried. When such mushrooms are rehydrated for use in the

preparation of dishes, it is common knowledge that an intense aroma is quickly released, which is, however, quite different from the aroma of the fresh mushroom. To get a first insight into changes in key aroma compounds, dry mushrooms from the trade were rehydrated and the concentrations of 28 odorants detected with high FD factors in the dry/rehydrated mushrooms (Table S1; supporting information) were quantitated in the dry as well as in the dry/rehydrated mushrooms to see the influence of water addition on aroma compound generation (Table S2; supporting information). In Table 11 the amounts of ten aroma compounds showing an increase during rehydration by at least a factor of 5 are contrasted. In particular the sulfur compound 1,2,4-trithiolane showed the highest increase during rehydration, while 1,2,4,5-tetrathiane and 1,2,3,5,6-pentathiepane showed the highest concentrations among the sulfur compounds formed by rehydration. The increase in 1,2,3,5,6-pentathiepane corroborates data previously published by Hirade et al.[38] However, the quantitative results confirm for the first time that the latter two compounds are more effectively formed by rehydration of dry shiitake compared to thermal processing and both sulfur compounds reached odor activity values of 11800 and 7500, respectively in the rehydrated mushroom tissue (Table S2; supporting information). Aroma profile analyses (data not shown) of the dry/rehydrated mushrooms as well as an aroma recombinate prepared on the basis of the quantitative data in Table S2 (supporting information) confirmed that the overall aroma of the dry, rehydrated shiitake was mainly influenced by the sulfury, burnt odor attributes of both key sulfur aroma compounds. On the other hand, the mushroom-like odor note was rated lower by the sensory panel as compared to the pan-fried shiitake.

In conclusion, the data showed for the first time that 1-octen-3-one, a long-known key odorant of many edible mushrooms, is also the key odorant in raw shiitake. As confirmed by isotope labeling experiments with U-^{13}C-linoleic acid, the ketone as well as the corresponding alcohol 1-octen-3-ol are rapidly formed during storage of a shiitake homogenate at room temperature. However, a second labeling experiment clearly showed that, against the literature view[35], the ketone is not formed by an enzymatic oxidation of the alcohol suggesting a yet unknown intermediate in its generation from linoleic acid.

A thermal treatment by heating in a pan led to a significant change in the concentrations of aroma compounds leading to over 30 odorants newly characterized

559 as aroma compounds of pan-fried shiitake. While in particular the caramel-like smelling
560 4-HDF, a carbohydrate degradation product, was significantly increased during pan-
561 frying, the typical mushroom-like smelling compounds 1-octen-3-one and 1-octen-3-ol
562 were drastically decreased. As it was recently shown for the homolog of 1-octen-3-
563 one, the 1-penten-3-one, such alpha, beta unsaturated ketones with a double bond at
564 the end may easily undergo aldol-type reactions during thermal treatment of foods and
565 can also bind to thiols.[39]

566 Also 1,2,3,5,6-pentathiepane and 1,2,4,5-tetrathiane were generated during pan-
567 frying of shiitake confirming previous data on their formation during steam distillation.[30]
568 However, simply the addition of water to dry shiitake, led to a much more effective
569 generation of both sulfur compounds. In general, these results can be used to develop
570 special shiitake products for use in food flavoring.

571

572 **Supporting information**

573 **Table S1**: Most Odor-Active Compounds (FD ≥ 8) Identified in dry, rehydrated
574 shiitake

575 **Figures S1 to S4:** Response curves for the quantitation of [$^2H_4$]-1,2,3,5,6-
576 pentathiepane, [$^2H_4$]-1,2,4,5-tetrathiane, [$^2H_6$]-1,2,4,6-tetrathiepane, [$^2H_4$]-1,2,4-
577 trithiolane by stable isotope dilution assays

578

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Figure captions

Figure 1. Mass spectra of 1,2,4-trithiolane (Figure 1 A) and [$^2H_4$]-1,2,4-trithiolane (Figure 1B

Figure 2 Mass spectra of 1,2,4,6 tetrathiepane (Figure 2 A) and [$^2H_6$]- tetrathiepane (Figure 2B).

Figure 3 Mass spectra of 1,2,4,5-tetrathiane (Figure 3 A) and [$^2H_4$]- 1,2,4,5-tetrathiane (Figure 3B)

Figure 4 Mass spectra of 1,2,3,5,6-pentathiepane (Figure 4 A) and [$^2H_4$]- 1,2,3,5,6-pentathiepane (Figure 4B)

Figure 5. Flavor dilution (FD) chromatogram of the volatile fraction isolated from raw shiitake mushrooms.

Figure 6. Structures of five key odorants in raw shiitake mushrooms showing the highest FD factors (numbering refers to Table 2).

Figure 7. Synthetic route used in the preparation of deuterium labeled [$^2H_4$]-1,2,4-trithiolane, [$^2H_6$]- tetrathiepane, [$^2H_4$]- 1,2,4,5-tetrathiane and [$^2H_4$]- 1,2,4,5-tetrathiane

Figure 8. Flavor dilution (FD) chromatogram of the volatile fraction isolated from pan-fried shiitake mushrooms.

Figure 9. Aroma profile analysis of pan-fried shiitake (grey line) and the aroma recombinate (black line)

Table 1. Isotopically Labeled Standards, Selected Ions, and Accuracy of Calibration Lines Used in the Quantitation of 32 Shiitake Aroma Compounds by Stable Isotope Dilution Assays.

odorant	isotope label	ion (*m/z*) [a]		R^2 [b]	System [c]
		analyte	internal standard		
acetic acid	$^{2}H_3$	61	64	0.9999	1
2-acetyl-1-pyrroline	$^{2}H_{2\text{-}5}$	112	114-117[d]	1	3
2,3-butanedione	$^{13}C_4$	87	91	1	2
butanoic acid	$^{2}H_2$	89	91	1	1
(*E*,*E*)-2,4-decadienal	$^{2}H_{3\text{-}5}$	153	156-158[d]	0.9985	2
δ-decalactone	$^{2}H_2$	170	172	1	2
2,3-diethyl-5-methylpyrazine	$^{2}H_3$	151	154	0.9998	2
2,6-dimethoxyphenol	$^{2}H_{5\text{-}8}$	154	159-162[d]	0.9999	3
3,5-dimethyl-2-ethenylpyrazine	$^{2}H_2$	134	136	0.9999	3
dimethyl trisulfide	$^{2}H_6$	127	133	1	2
trans-4,5-epoxy-(*E*)-2-decenal	$^{2}H_4$	167	171	0.9998	4
3-ethylphenol	$^{2}H_2$	122	124	0.998	3
4-hydroxy-2,5-dimethyl-3(2*H*)-furanone	$^{13}C_2$	128	130	0.998	3
3-hydroxy-4,5-dimethyl-2(5*H*)-furanone	$^{13}C_2$	128	130	0.9996	3
2-methoxy-4-(prop-1-en-1-yl)phenol	$^{2}H_3$	165	168	0.9997	2
3-methylbutanal	$^{2}H_2$	87	89	1	2
2- and 3-methylbutanoic acid	$^{2}H_3$	103	105	0.998	1
3-(methylthio)propanal	$^{2}H_3$	105	108	0.997	2
2-methoxyphenol	$^{2}H_3$	124	127	1	2
2-methoxy-4-propylphenol	$^{2}H_{2\text{-}4}$	167	169-171[d]	1	2
(E,E)-2,4-nonadienal	$^{2}H_2$	139	141	0.9991	2
(*E*)-2-octenal	$^{2}H_2$	127	129	0.999	2
1-octen-3-ol	$^{2}H_{2\text{-}4}$	111	113-116[d]	0.995	1
1-octen-3-one	$^{2}H_2$	127	131	0.996	2
pentanoic acid	$^{2}H_3$	103	106	0.997	1
1,2,3,5,6-pentathiepane	$^{2}H_4$	189	193	0.9999	1
phenylacetaldehyde	$^{13}C_2$	120	122	0.996	3
phenylacetic acid	$^{13}C_2$	136	138	0.9993	3
1,2,4-trithiolane	$^{2}H_4$	125	129	0.9999	2
1,2,4,5-tetrathiane	$^{2}H_4$	157	161	0.9997	1
1,2,4,6-tetrathiepane	$^{2}H_6$	171	176	1	1
vanillin	$^{2}H_3$	152	155	1	3

[a] Ions used for quantitation by either MS-CI or MS-EI.

[b] Coefficient describing the accuracy of the calibration line; number of calibration points: n = 5.

[c] The different equipments used in the quantitation.
GC-MS (1)
GCxGC-MS; (2)
Comprehensive GCxGC-TOF-MS (3);
(4) GC-MS (MS-CI neg.)

[d] Internal standard was used as a mixture of isotopologues.

Table 2. Most Odor-Active Compounds (FD ≥ 8) Identified in Raw Shiitake Mushrooms.

no.	odorant [a]	odor quality [b]	RI on FFAP	RI on DB-5	FD [c]	previously identified [d]
1	3-methylbutanal	malty	927	640	128	-
2	2,3-butanedione	buttery	983	<600	16	-
3	unknown	garlic-like	1256	n.b.	16	-
4	octanal	citrus-like	1279	1006	16	-
5	1-octen-3-one	mushroom-like	1293	976	1024	27-30
6	2-acetyl-1-pyrroline	popcorn-like, roasty	1329	926	16	-
7	(Z)-1,5-octadien-3-one	geranium, metallic	1364	983	32	-
10	(E)-2-octenal	fatty, nutty	1423	1062	128	-
11	acetic acid	vinegar-like	1443	613	128	-
12	1-octen-3-ol	mushroom-like	1445	982	512	27-30
13	3-(methylthio)propanal	cooked potato-like	1452	910	4096	-
14	2,3-diethyl-5-methylpyrazine	earthy	1477	1154	64	-
15	2-isobutyl-3-methoxypyrazine	earthy, green bell pepper	1514	1182	64	-
16	(E)-2-nonenal	fatty, green	1530	1164	32	-
18	butanoic acid	sweaty	1630	807	8	-
19	phenylacetaldehyde	flower-like	1638	1049	128	-
20	2- and 3-methylbutanoic acid	sweaty	1658	858/846	64	-
26	(*E*,*E*)-2,4-decadienal	fatty, fried	1808	1319	8	-
27	unknown	sweaty	1824	n.b.	32	-
28	2-methoxyphenol[e]	phenolic	1861	1086	64	-
29	2-methoxy-5-methylphenol[e]	phenolic	1944	1191	16	-
30	*trans*-4,5-epoxy-(*E*)-2-decenal	metallic	2000	1380	256	-
32	4-hydroxy-2,5-dimethyl-3(2*H*)-furanone	caramel-like	2029	1049	128	-
34	2-methoxy-4-propylphenol	caramel-like	2109	1366	8	-
35	unknown	seasoning-like	2142	n.b.	8	-
39	3-ethylphenol	clove-like	2182	1165	32	-
40	3-hydroxy-4,5-dimethyl-2(5*H*)-furanone	seasoning-like	2213	1105	128	-

Table 2. continued

41	1,2,4,5-tetrathiane	sulfury, burnt	2226	1484	32	*2*
47	unknown	burnt	2361	n.b.	64	-
51	phenylacetic acid	honey-like	2561	1240	32	-
52	vanillin	vanilla-like	2586	1409	256	-
53	1,2,3,5,6-pentathiepane	sulfury, burnt	>2600	1679	64 *g*	*1*

a Identification was performed by comparing retention indices (RI) on capillaries FFAP and DB-5, odor quality, and mass spectra (MS-EI) with data of reference compounds.
b Odor quality perceived at the sniffing-port.
c Flavor dilution (FD) factor determined by application of the AEDA.
d Reference reporting the respective compound as volatile compound in raw Shiitake mushroom.
e No unequivocal mass spectra could be obtained. Identification is based on the remaining criteria given in footnote *a*.
f nd, not determined.

Table 3. Concentrations of Nineteen Key Aroma Compounds in Raw Shiitake.

odorant	concn (μg/kg fresh weight)	RSD [a] (%)
1-octen-3-ol	3380	2.9
phenylacetaldehyde	97	5.1
phenylacetic acid	51	2.1
1-octen-3-one	47	1.8
3-methylbutanal	38	1.9
3-(methylthio)propanal	34	3.5
2-methoxyphenol	24	0.2
3-ethylphenol	15	0.5
3-methylbutanoic acid	15	0.5
vanillin	7.9	0.8
trans-4,5-epoxy-(*E*)-2-decenal	4.9	10.2
1,2,3,5,6-pentathiepane	2.8	2.0
1,2,4,5-tetrathiane	2.8	9.3
2-methylbutanoic acid	0.95	7.4
4-hydroxy-2,5-dimethyl-3*(2H)*-furanone	0.87	8.1
3-hydroxy-4,5-dimethyl-2(5*H*)-furanone	0.85	0.8
1,2,4,6-tetrathiepane	0.65	9.9
2,3-diethyl-5-methylpyrazine	0.1	7.4
2-isobutyl-3-methoxypyrazine	0.02	3.0

[a] number of replicates: n = 3.

Table 4. Orthonasal Odor Thresholds and Odor Activity Values (OAVs) of Nineteen Aroma Compounds in Raw Shiitake.

odorant	odor threshold in water[a] (µg/kg)	OAV[d]
1-octen-3-one	0.016[a]	2913
3-(methylthio)propanal	0.43[a]	80
3-methylbutanal	0.5a	76
1-octen-3-ol	45a	75
trans-4,5-epoxy-(*E*)-2-decenal	0.22[c]	23
phenylacetaldehyde	5.2[b]	19
3-ethylphenol	0.85[a]	18
2,3-diethyl-5-methylpyrazine	0.43[c]	17
1,2,4,5-tetrathiane	0.23[c]	12
1,2,3,5,6-pentathiepane	0.27[c]	10
2-methoxyphenol	2.5[a]	10
1,2,4,6-tetrathiepane	1.16[c]	9
2-isobutyl-3-methoxypyrazine	0.005[2]	5
3-hydroxy-4,5-dimethyl-2(5*H*)-furanone	0.49[a]	2
4-hydroxy-2,5-dimethyl-3(2*H*)-furanone	40[a]	<1
vanillin	53[a]	<1
2-methylbutanoic acid	2200[a]	<1
3-methylbutanoic acid	490[a]	<1
phenylacetic acid	6100[a]	<1

[a] Czerny et al. [25], [b]in-house data base, [c]newly determined in this study.

[d]Odor activity value (ratio of odor concentration to odor threshold).

Table 5: Concentrations and ^{12}C /^{13}C ratio in C-8 compounds formed after incubation of a shiitake homogenate with added $[^{13}C_{18}]$-linoleic acid

incubation [a] [min]	1-octen-3-ol			1-octen-3-one			3-octanone			(*E*)-2-octenal		
	conc. [b] [mg/kg d.m.]		$[^{12}C/^{13}C]$ [c] (µmol/µmol)	conc. [mg/kg d.m.]		$[^{12}C/^{13}C]$ (µmol/µmol)	conc. [mg/kg d.m.]		$[^{12}C/^{13}C]$ (µmol/µmol)	conc. [mg/kg d.m.]		$[^{12}C/^{13}C]$ (µmol/µmol)
	$[^{12}C_8]$	$[^{13}C_8]$		$[^{12}C_8]$	$[^{13}C_8]$		$[^{12}C_8]$	$[^{13}C_8]$		$[^{12}C_8]$	$[^{13}C_8]$	
0.3	214	73.0	2.9	0.88	0.24	3.7	2.12	0.03	70.6	0.77	0.29	2.7
2	220	124	1.8	1.66	0.70	2.4	2.31	0.08	28.9	1.41	0.71	2.0
4	230	178	1.4	1.73	0.76	2.3	2.91	0.27	10.8	1.99	1.25	1.6
8	243	219	1.3	1.75	0.94	1.9	3.42	0.84	4.1	2.78	2.19	1.3
15	224	213	1.05	1.78	1.26	1.4	2.45	1.01	2.4	2.49	2.30	1.1
30	156	177	0.9	1.40	0.98	1.4	0.35	0.16	2.2	2.47	2.15	1.1

[a] Time between homogenization of the mushroom tissue and addition of hydrochloric acid to stop enzymatic reactions.

[b] median value of a threefold assay (RSD ≤ 20%); conc. Related to dry mass (d.m.) of mushroom tissue (7.3%)

[c] ratio between inherent linoleic acid derived C_8 compound and $[^{13}C_{18}]$-linoleic acid derived $[^{13}C_{18}]$ isotopoloque in the mushroom homogenate

Tabelle 6: Concentrations of Unlabeled and Labeled C-8 compounds in a Shiitake Homogenate after Addition of [$^2H_4$]-1-Octen-3-ol

Incubation time[a] [min]	Concn. [b] [mg/kg d.m.]							
	educt		product					
	1-octen-3-ol		1-octen-3-one		3-octanone		(*E*)-2-octenal	
	unlabeled	[$^2H_4$][c]	unlabeled	[$^2H_4$]	unlabeled	[$^2H_4$]	unlabeled	[$^2H_4$]
0.3	87	140	0.70	0.03	1.10	0.03	4.60	0.20
4	119	141	2.40	0.03	2.40	0.16	11.1	1.30
8	130	140	3.20	0.03	4.50	0.45	17.0	2.0
15	128	137	2.60	0.03	8.70	1.0	15.0	1.60
30	185	138	2.60	0.03	7.70	1.60	15.9	2.20
60	120	121	2.60	0.03	9.30	2.0	14.0	1.60

[a] time between homogenization of the mushroom tissue and addition of hydrochloric acid to stop enzymatic reactions

[b] mean value of a threefold assay (RSD ≤ 20%); Conc. Calculated in dry mass (d.m.) of mushroom tissue (7.3%)

[c] Concentration of the labeled precursor (mg/kg dry mass) left after incubation

Table 7. **Most Odor-Active Compounds (FD ≥ 8) Identified in Pan-Fried Shiitake Mushrooms.**

no.	odorant [a]	odor quality [b]	RI on		FD [c]	previously identified [d]
			FFAP	DB-5		
1	3-methylbutanal	malty	965	640	16	-
2	2,3-butanedione	buttery	992	600	32	-
5	1-octen-3-one	mushroom-like	1294	979	64	*26,27*
6	2-acetyl-1-pyrroline	popcorn-like	1332	919	128	-
8	dimethyl trisulfide	cabbage-like	1374	976	32	*26,25*
9	2-propionyl-1-pyrroline[e]	popcorn-like, roasty	1416	1025	8	-
11	acetic acid	vinegar-like	1441	613	16	-
13	3-(methylthio)propanal	cooked potato-like	1454	908	1024	-
16	(*E*)-2-nonenal	fatty, green	1531	1164	32	-
17	3,5-dimethyl-2-ethenylpyrazine[e]	earthy	1550	1084	64	-
18	butanoic acid	sweaty	1620	808	8	-
20	2- and 3-methylbutanoic acid	sweaty	1658	845	64	-
21	(*E*,*E*)-2,4-nonadienal	fatty, green	1693	1219	16	-
22	3-methylnonan-2,4-dione[e]	hay-like, fishy	1713	1229	16	-
23	pentanoic acid	sweaty	1728	895	512	-
24	1,2,4-trithiolane	sulfury, onion-like	1747	1109	8	*18,27,25*
25	2-acetyl-2-thiazoline	roasty	1757	1106	8	-
26	(*E*,*E*)-2,4-decadienal	fatty, fried	1804	1323	256	-
28	2-methoxyphenol	phenolic	1863	1090	64	-
29	2-methoxy-5-methylphenol[e]	phenolic	1947	1180	16	-
30	*trans*-4,5-epoxy-(*E*)-2-decenal	metallic	2000	1384	1024	-
31	γ-nonalactone[e]	coconut-like	2024	1369	8	-
32	4-hydroxy-2,5-dimethyl-3(2*H*)-furanone	caramel-like	2036	1055	2048	-
33	δ-nonalacton	peach-like	2091	1395	16	-
34	2-methoxy-4-propylphenol	phenolic	2112	1357	16	-
36	4-allyl-2-methoxyphenol	clove-like	2147	1345	8	-
37	γ-decalactone	peach-like	2158	1469	8	-
38	δ-decalactone	peach-like	2178	1511	128	-
40	3-hydroxy-4,5-dimethyl-2(5*H*)-furanone	seasoning-like	2206	1100	8192	-

Table 7. continued

41	1,2,4,5-tetrathiane	sulfury, burnt	2225	1380	4096	*18,26,27*
42	4-*tert*-butylphenol	carrot, phenolic	2238	1297	16	-
43	γ-undecalactone	peach-like	2270	1578	8	-
44	1,2,3,5-tetrathiane	sulfury, burnt	2280	1412	16	*27*
45	unknown	seasoning-like	2308	nd	8	-
46	unknown	metallic, wood-like	2343	nd	8	-
47	indole	faecal, mothball-like	2440	1293	32	-
48	δ-dodecalactone	peach-like	2457	1717	16	-
49	unknown	seasoning-like	2497	nd	8	-
50	3-methylindole	faecal, mothball-like	2511	1399	8	-
51	phenylacetic acid	honey-like	2565	1244	2048	-
52	vanillin	vanilla-like	2582	1412	64	-
53	1,2,3,5,6-pentathiepane	sulfury, burnt	>2600	1673	256[g]	*18,26,27*

[a] Identification was performed comparing retention indices (RI) on capillaries FFAP and DB-5, odor quality, and mass spectra (MS-EI) with data of reference compounds.
[b] Odor quality at the sniffing-port.
[c] Flavor dilution factor determined by means of AEDA on capillary FFAP.
[d] Previously identified as volatile in fresh Shiitake mushrooms.
[e] No unequivocal mass spectra could be obtained. Identification is based on the remaining criteria given in footnote *a*.
[f] nd, not determined.
[g] FD factor determined on DB-5 capillary column.

Table 8. Concentrations of Twenty-one Key Aroma Compounds in Pan-Fried Shiitake Mushrooms.

odorant	concn (μg/kg fresh weight)	RSD[a] (%)
1-octen-3-ol	1050	12.6
4-hydroxy-2,5-dimethyl-3(2*H*)-furanone	470	3.0
2,3-butanedione	300	4.7
1,2,3,5,6-pentathiepane	265	8.0
2-methylbutanoic acid	245	0.4
1,2,4,5-tetrathiane	200	2.1
phenylacetaldehyde	100	1.4
3-(methylthio)propanal	87	1.5
3-methylbutanal	59	6.8
phenylacetic acid	22	4.2
vanillin	18	1.2
3-methylbutanoic acid	13	5.7
dimethyl trisulfide	9.9	1.4
(*E,E*)-2,4-decadienal	4.6	3.2
3-hydroxy-4,5-dimethyl-2(5*H*)-furanone	3.2	2.2
1,2,4,6-tetrathiepane	2.8	1.0
2-acetyl-1-pyrroline	2.4	0.9
1-octen-3-one	1.8	4.3
trans-4,5-epoxy-(*E*)-2-decenal	1.7	6.2
2-methoxyphenol	1.6	4.6
3,5-dimethyl-2-ethenylpyrazine	0.1	7.4

[a] number of replicates: n = 3.

Table 9. Orthonasal Odor Thresholds and Odor Activity Values (OAVs) of Twenty-one Key Aroma Compounds in Pan-Fried Shiitake Mushrooms.

odorant	odor threshold in water[a] (µg/kg)	OAV[b]
1,2,3,5,6-pentathiepane	0.27[3]	981
1,2,4,5-tetrathiane	0.23[3]	891
2,3-butanedione	1.0[2]	300
3-(methylthio)propanal	0.43[1]	202
(*E,E*)-2,4-decadienal	0.027[1]	170
3-methylbutanal	0.5[1]	119
1-octen-3-one	0.016[1]	114
dimethyl trisulfide	0.099[2]	100
2-acetyl-1-pyrroline	0.053[1]	44
1-octen-3-ol	45[2]	23
phenylacetaldehyde	5.2[2]	19
4-hydroxy-2,5-dimethyl-3(2*H*)-furanone	40[1]	12
trans-4,5-epoxy-(*E*)-2-decenal	0.22[3]	8
3-hydroxy-4,5-dimethyl-2(5*H*)-furanone	0.49[1]	7
1,2,4,6-tetrathiepane	1.16[1]	2
phenylacetic acid	6100[1]	<1
2-methoxyphenol	2.5[2]	<1
3-methylbutanoic acid	490[1]	<1
2-methylbutanoic acid	2200[1]	<1
vanillin	53[1]	<1
2-ethenyl-3,5-dimethylpyrazine	0.1[2]	<1

[a] Detection threshold in water:1: Czerny et al. [25], 2: in-house data base, 3: newly determined in this study).

[b] Odor activity value (ratio of odor concentration to odor threshold).

Table 10. Comparison of Aroma Compound Concentrations in Raw and Pan-Fried Shiitake Mushrooms.

Aroma Compound	Concn.[a] [µg/kg dry weight]		factor [b]
	raw	pan-fried	
4-hydroxy-2,5-dimethyl-3(2*H*)-furanone	8.0	1240	154
dimethyl trisulfide	0.5	26	57
1,2,3,5,6-pentathiepane	26	697	27
1,2,4,5-tetrathiane	26	539	21
(*E,E*)-2,4-decadienal	6	26	4.7
2-acetyl-1-pyrroline	2.3	6	2.6
3-(methylthio)propanal	315	229	0.7
3-methylbutanal	352	229	0.7
vanillin	73	48	0.7
2-phenylacetaldehyde	894	266	0.3
1-octen-3-ol	31296	2763	0.1
trans-4,5-epoxy-(*E*)-2-decenal	47	4	0.1
phenylacetic acid	472	58	0.1
2-methoxyphenol	225	4	0.02
1-octen-3-one	431	5	0.01

[a] Concentration of odorant related to dry matter
[b] Ratio of odorant concentration in pan-fried vs. raw shiitake.

Table 11: **Comparison of the Concentrations of Selected Aroma Compounds in Dry and Dry/Rehydrated Shiitake**

Aroma Compound	Concn. [a] [µg/kg dry weight]		ratio [b]
	dry	dry/rehydrated	
1,2,4-trithiolane	52.0	1730	33
1,2,4,6-tetrathiepane	1.5	45.0	30
phenylacetaldehyde	12.4	313	25
1-octen-3-ol	508	9160	18
3-(methylthio)propanal	0.7	11.5	18
1,2,3,5,6-pentathiepane	231	2850	12
1-octen-3-one	2.7	23.0	10
3-methylbutanal	150	1270	9
3-methylbutanoic acid	1050	6630	6
1,2,4-tetrathiane	681	3430	5
trans-isoeugenol	6.1	8.3	1
2-acetyl-1-pyrroline	1.6	1.6	1
2-methylbutanoic acid	4730	3080	0.7

[a] concentration related to the dry matter (d.m.) of shiitake mushroom
[b] ratio between the concentrations of odor compounds in dry and dry/rehydrated shiitake mushrooms

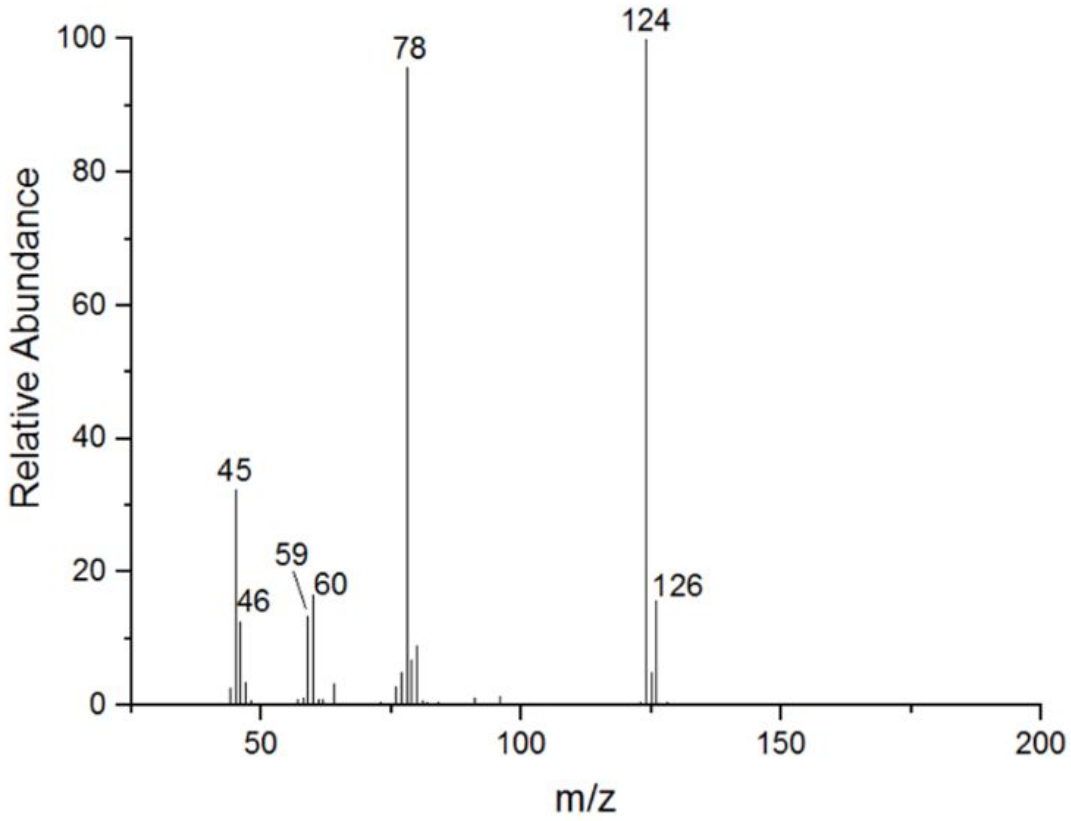


Figure 1 A

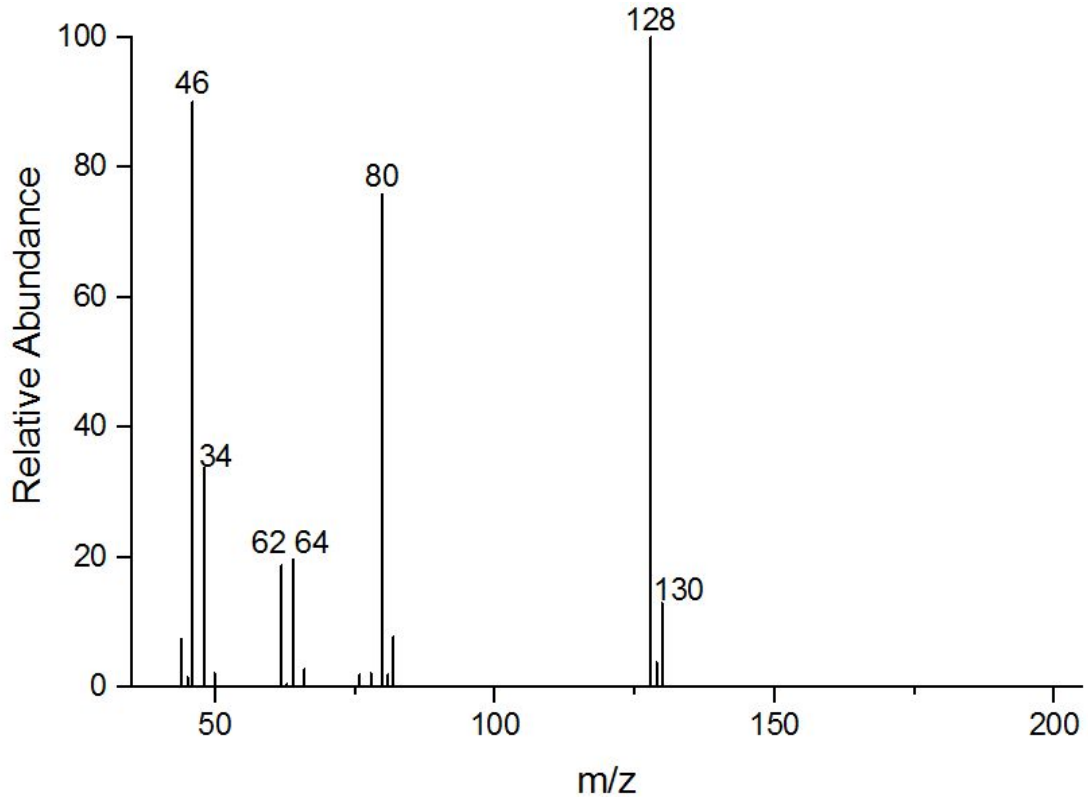


Figure 1B

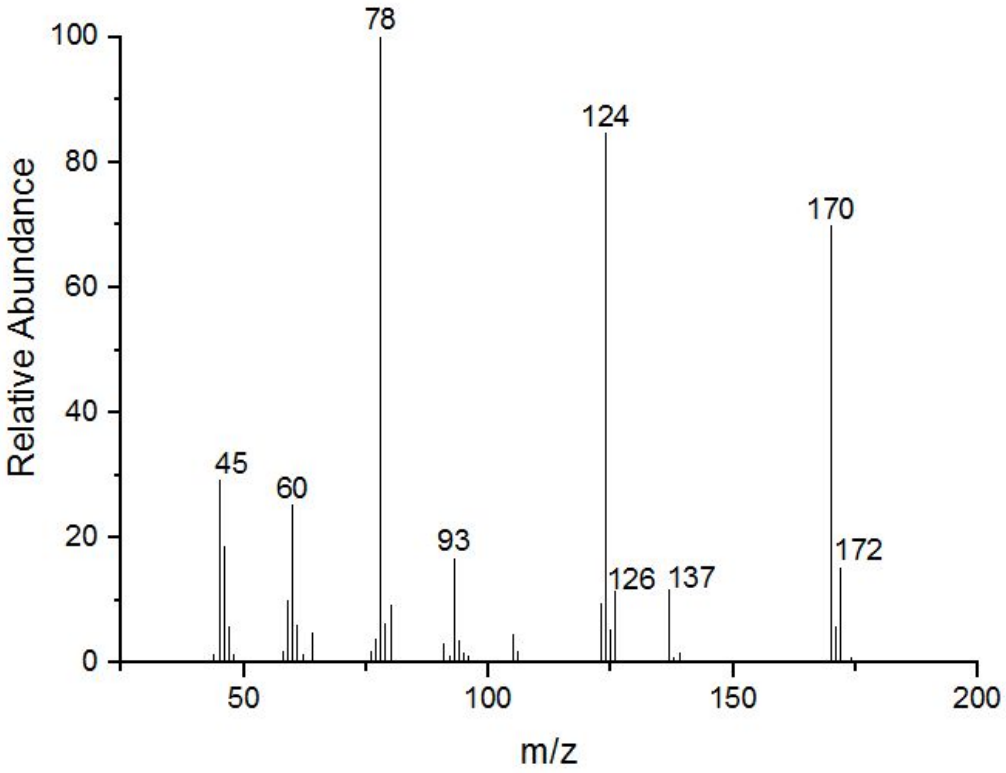


Figure 2A

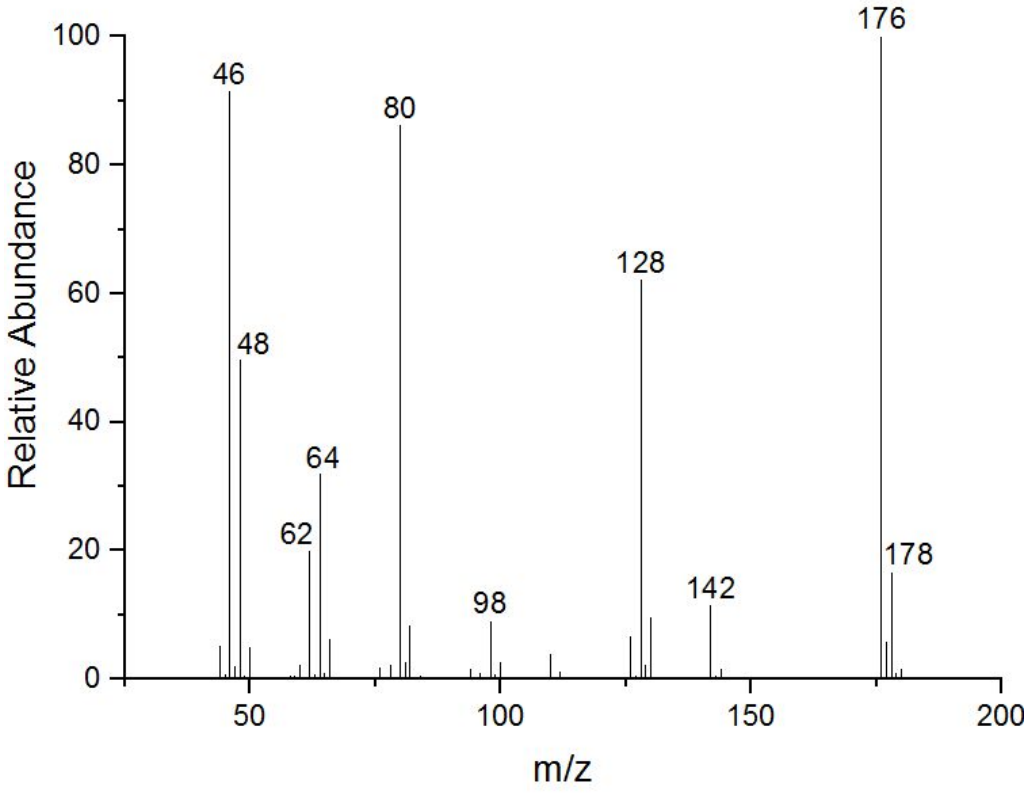


Figure 2B

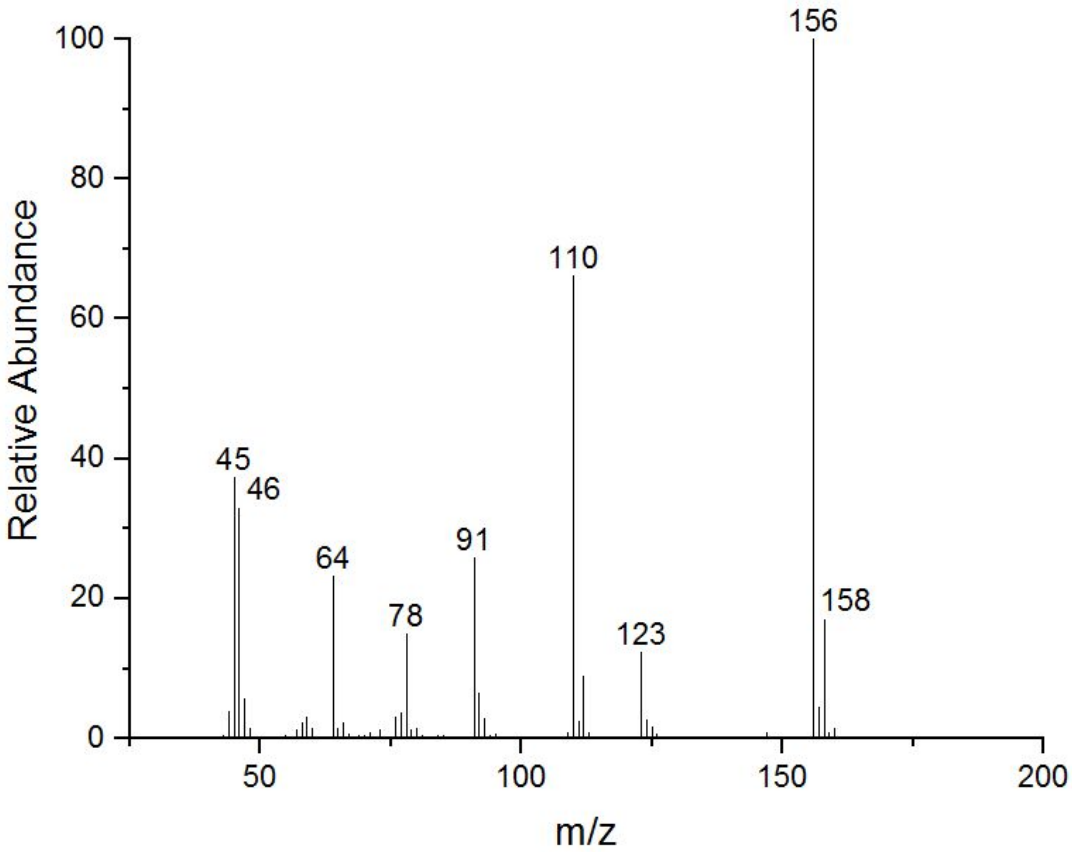


Figure 3A

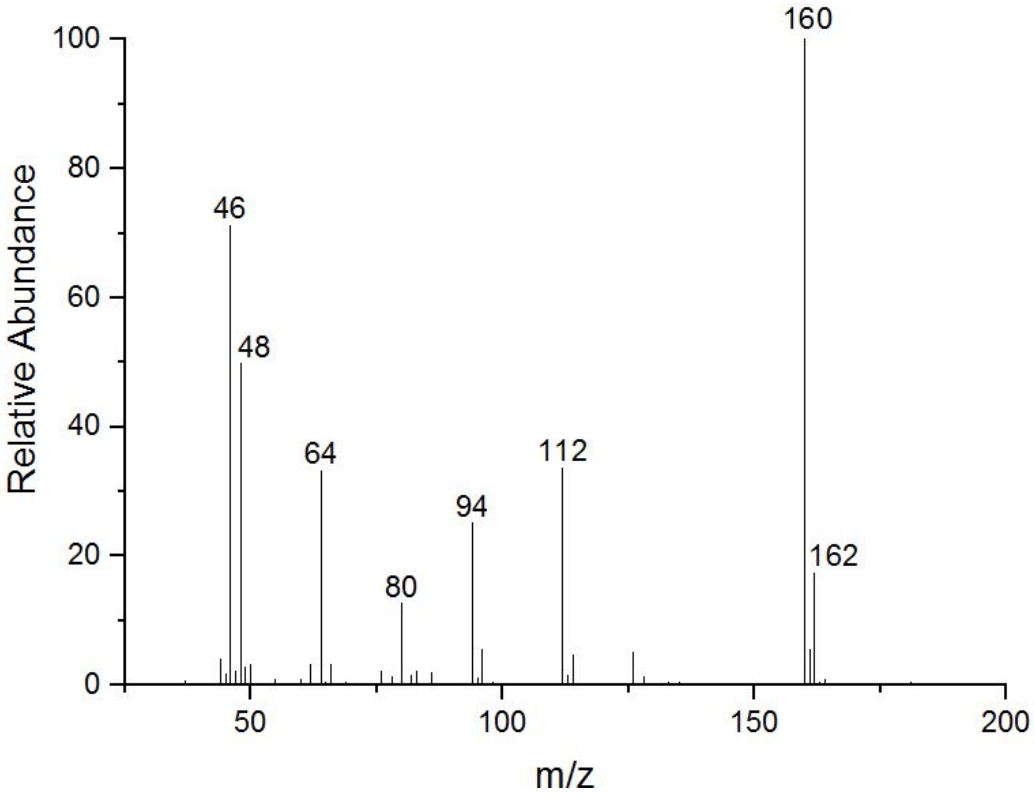


Figure 3B

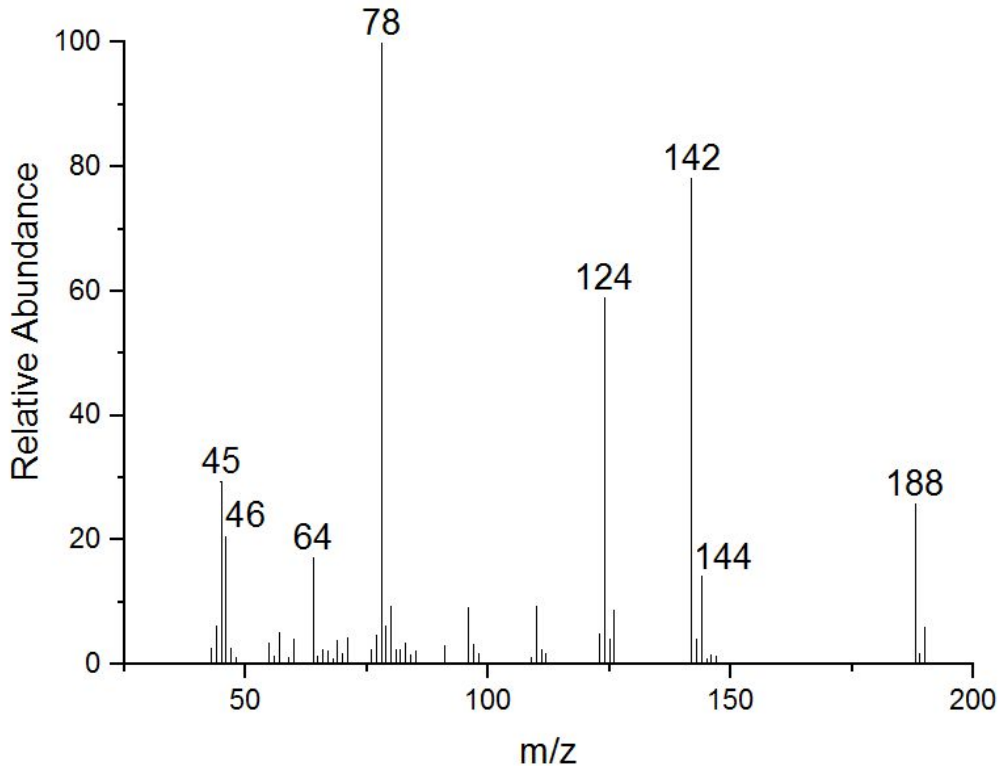


Figure 4A

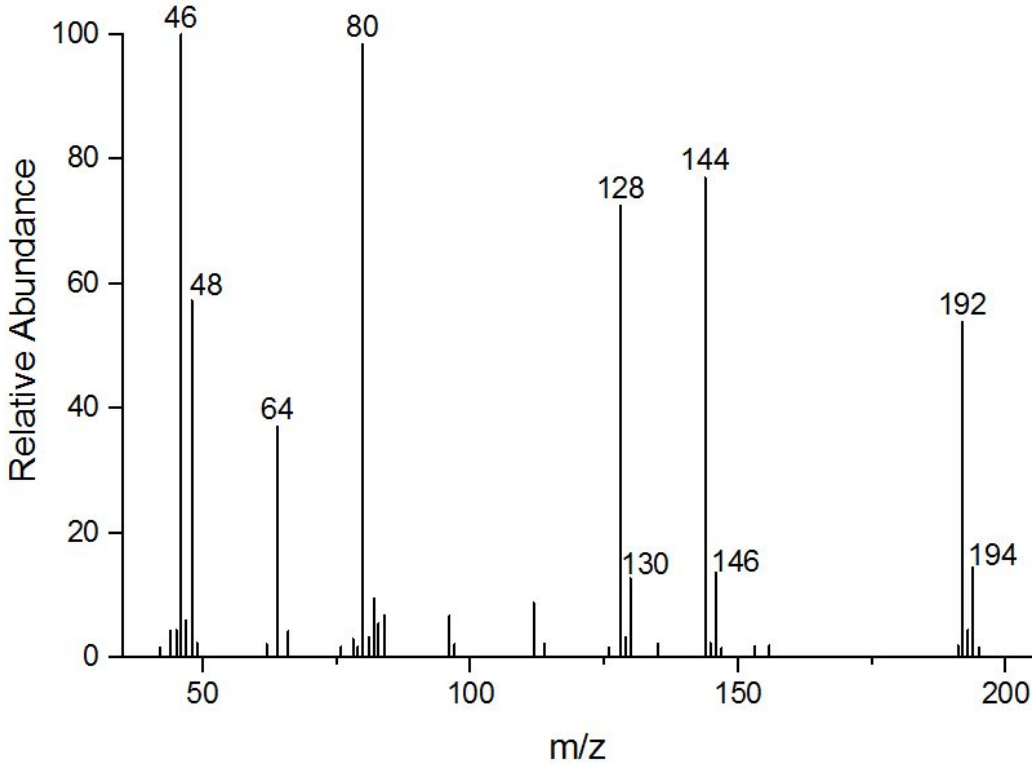
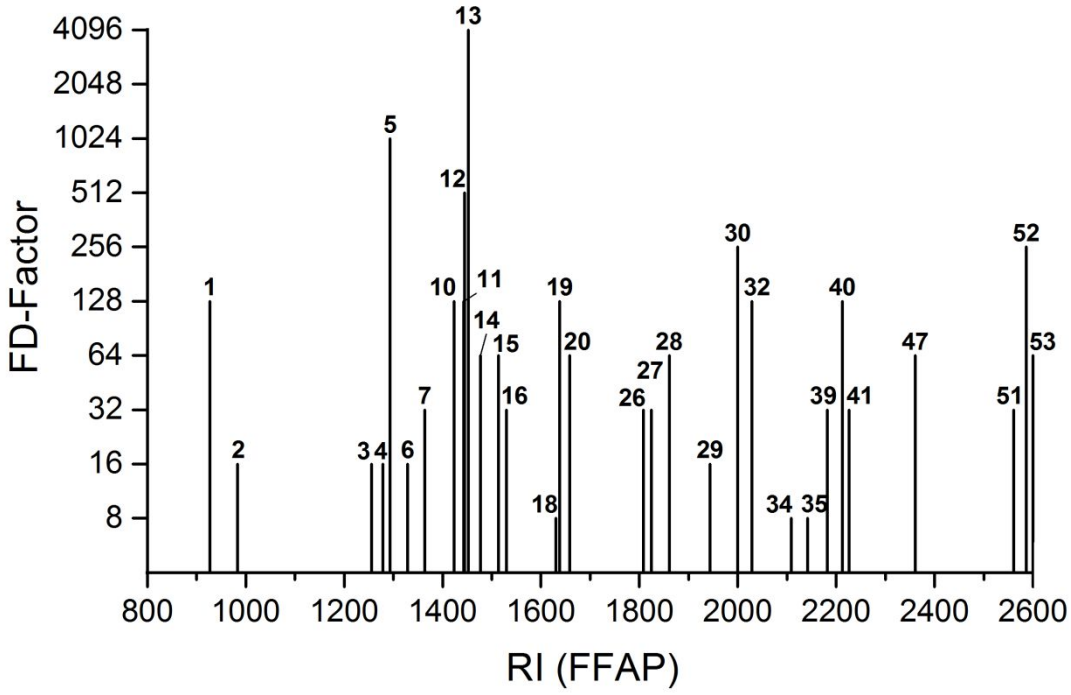


Figure 4B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1024
512
256
128
64
32
16
8
800
1000
1200
1400
1600
1800
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2200
2400
2600
RI (FFAP)
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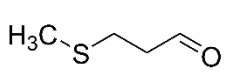
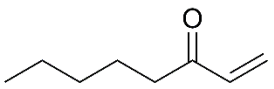
53

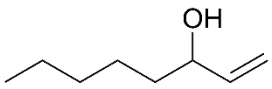
Figure 5



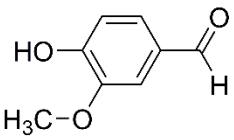
13 (cooked potato-like, FD 4096)



5 (mushroom-like, FD 1024)



12 (mushroom-like, FD 512)



52 (vanilla-like, FD 256)

30 (metallic, FD 256)

Figure 6

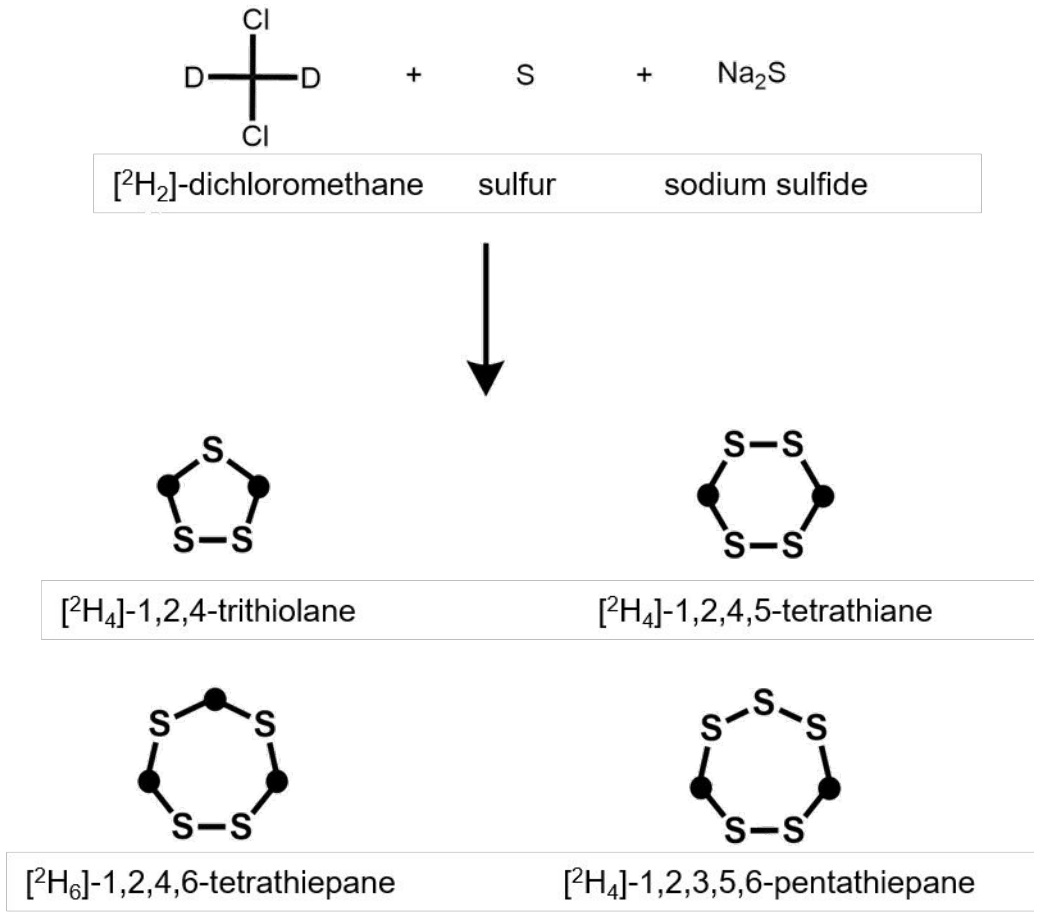

Cl
D
D
Cl
+
S
+
Na_2S
[$^2H_2$]-dichloromethane
sulfur
sodium sulfide
[$^2H_4$]-1,2,4-trithiolane
[$^2H_4$]-1,2,4,5-tetrathiane
[$^2H_6$]-1,2,4,6-tetrathiepane
[$^2H_4$]-1,2,3,5,6-pentathiepane

Figure 7

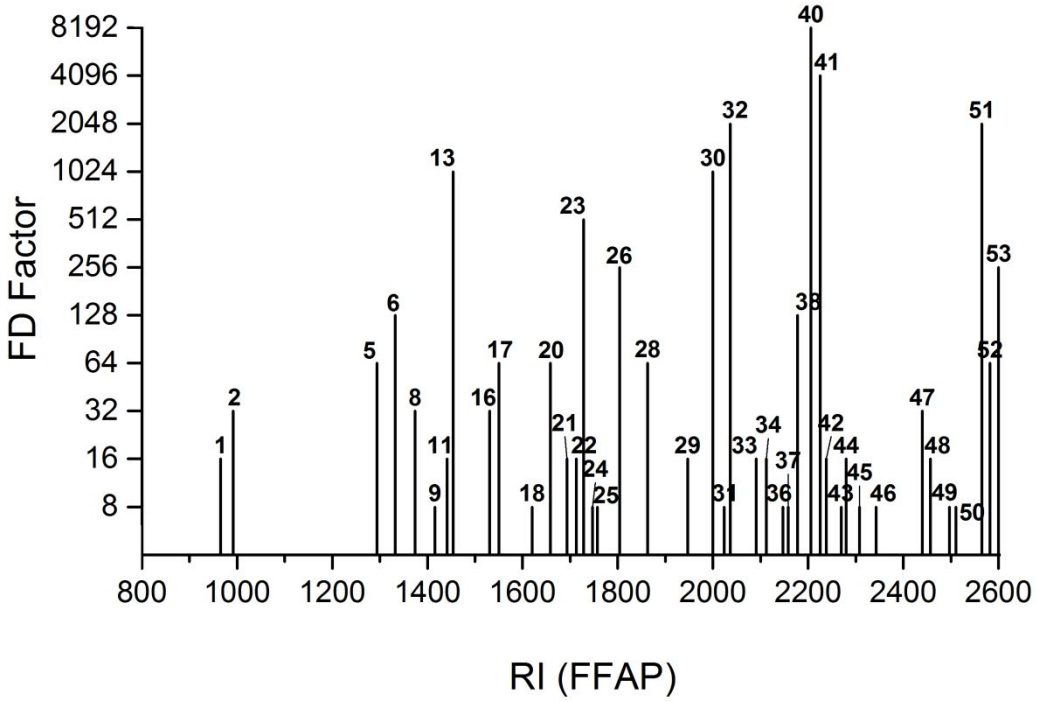

FD Factor
8192
4096
2048
1024
512
256
128
64
32
16
8
800
1000
1200
1400
1600
1800
2000
2200
2400
2600
RI (FFAP)
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Figure 8

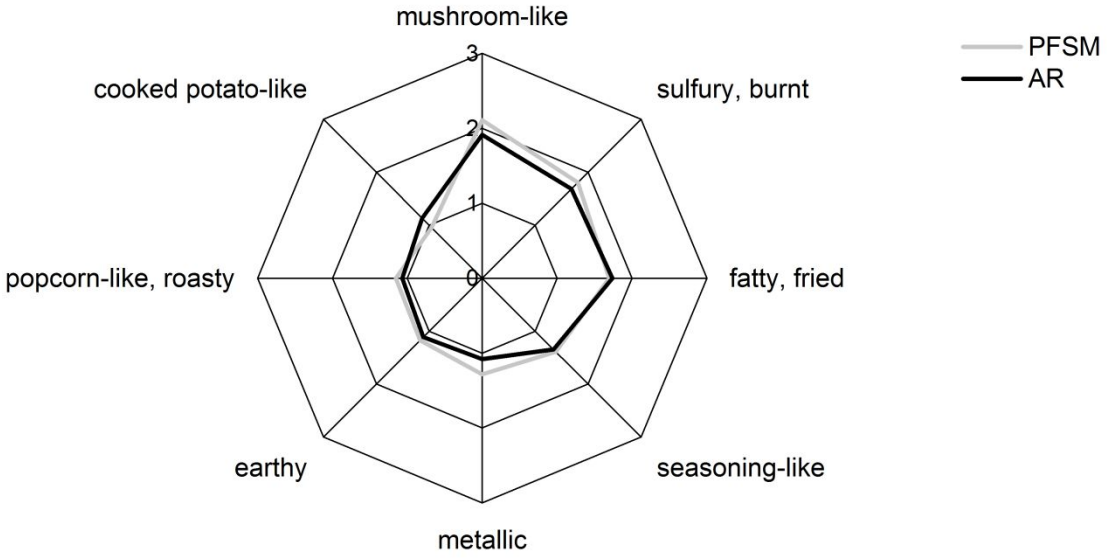
mushroom-like
sulfury, burnt
fatty, fried
seasoning-like
metallic
earthy
popcorn-like, roasty
cooked potato-like
3
2
1
0
PFSM
AR

Figure 9

TOC graphic