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# Quantification of key odor-active compounds of a novel nonalcoholic beverage produced by fermentation of wort by shiitake (*Lentinula edodes*) and aroma genesis studies

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L-Isoleucine (PubChem CID: 6306)
Methional (PubChem CID: 18635)
2-Methylbutanoic acid (PubChem CID: 8314)
(*E*)-Methyl cinnamate (PubChem CID: 637520)
Methyl 2-methylbutanoate (PubChem CID: 13357)
2-Phenylethanol (PubChem CID: 6054)

ABSTRACT

A novel non-alcoholic beverage was produced by fermentation of wort with shiitake (*Lentinula edodes*), and the key aroma compounds were quantified. Reconstitution studies confirmed their contribution to the overall aroma of the beverage. Kinetic studies indicated that shiitake synthesized the key odor-active compounds imparting fruity and rose-like odors, while the typical odorants of the substrate wort were degraded during the fermentation. By means of the standard addition method, a good linearity ($R^2 \geq 0.983$) and degree of precision (RSD < 13%) were obtained for quantification of twelve aroma compounds. Their respective odor activity values (OAVs) were calculated to estimate their contribution to the overall aroma. Methyl 2-methylbutanoate, produced by shiitake, showed the highest OAV (30) and was responsible for the typical fruity odor of the beverage. A correlation between the concentration of methyl 2-methylbutanoate and the perceived fruitiness of the beverage was observed. The biosynthesis of this methyl ester by transformation of 2-methylbutanoic acid and L-isoleucine was confirmed by means of an isotopic labeling experiment.



## 1. Introduction

Shiitake (*Lentinula edodes*), as the second most popular edible mushroom in the world, represents a good source of carbohydrates, proteins, essential amino acids, poly-unsaturated fatty acids, and vitamins, especially vitamin D (Bisen, Baghel, Sanodiya, Thakur, & Prasad, 2010; Mattila, Suonpää, & Piironen, 2000). Furthermore, shiitake produces various secondary metabolites with potential pharmacologic relevance, such as lentinan, lectins, and eritadenine (Jasrotia, Sharma, Badhani, & Prashar, 2012). Last but not least, shiitake is highly valued because of its pleasant flavor.

Due to the worldwide declining consumption of beer (Colen & Swinnen, 2010), breweries are eagerly searching for innovative non-alcoholic beverages to broaden their product portfolio. A novel non-alcoholic beverage fermented by shiitake was developed using wort as substrate (Zhang, Fraatz, Horlamus, Quitman, & Zorn, 2014). It is nutritious and exhibits a pleasant fruity, slightly sour and plum like flavor. The key odor-active compounds of the novel fermented beverage were identified in our previous study by liquid–liquid extraction (LLE) and headspace solid phase microextration (HS-SPME) in combination with a gas chromatography system equipped with a tandem mass spectrometry detector and an olfactory detector port (GC–MS/MS–O) (Zhang et al., 2014). For further product development, i.e. up-scaling and controlling the quality of product, it is important to obtain quantitative data for the key aroma compounds and to perform aroma reconstitution studies. The latter is carried out by mixing pure aroma compounds in the concentrations determined in the food product in an appropriate matrix. Aroma reconstitution is widely accepted to finally proof the typical food aroma in regard to interactions between key odor-

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active constituents (Grosch, 2001). It has been successfully applied to a variety of food, such as wine (Ferreira, Ortín, Escudero, López, & Cacho, 2002), orange and grapefruit juices (Buettner & Schieberle, 2001), roasted pistachio (Aceña, Vera, Guasch, Busto, & Mestres, 2010), and strawberry (Schieberle & Hofmann, 1997).

In the present study, twelve key odor-active compounds including 2-acetylpyrrole, 2-acetylthiazole, *o*-aminoacetophenone, β-damascenone, 2,5-dimethylpyrazine, methional, (*E*)-methyl cinnamate, methyl hexadecanoate, methyl 2-methylbutanoate, (*Z*)-3-nonenol, 2-phenylethanol, and 2-phenylethyl acetate with flavor dilution factors of 4 to 64, were selected based on the data of an aroma extract dilution analysis (Zhang et al., 2014). A kinetic study was conducted to investigate the changes of the concentrations of the key odorants during the fermentation process. Furthermore, the corresponding odor activity values were calculated, and reconstitution studies were carried out. Finally, the biosynthetic pathway of the most important odorant was elucidated by means of a labeling experiment.

## 2. Materials and methods

### 2.1. Chemicals and materials

*L. edodes* (shiitake) was obtained from the *Centraalbureau voor Schimmelcultures* (CBS, Utrecht, Netherlands).

L-isoleucine (U-$^{13}$C) (98%) was purchased from Euriso-Top GmbH (Saarbrücken, Germany). 2-Acetylpyrrole (98%), L-isoleucine (99%), methional (98%), 2-methylbutanoic acid (98%), and (*E*)-methyl cinnamate (99%) were obtained from Alfa Aesar (Karlsruhe, Germany). *o*-Aminoacetophenone (98%), and thymol (99%) were bought from TCI (Eschborn, Germany). Ethanol (99.5%), (*Z*)-3-nonenol (95%), and L-phenylalanine (99%) were obtained from Carl Roth (Karlsruhe, Germany). 2-Acetylthiazole (99%), β-damascenone (1.1–1.3 wt.% in 190 proof ethanol), 2,5-dimethylpyrazine (98%), methyl 2-methylbutanoate (98%), β-ionone (95%), and 2-phenylethyl acetate (99%) were purchased from Sigma-Aldrich (Steinheim, Germany). Methyl hexadecanoate (99%) was obtained from Cayman Chemical (Ann Arbor, USA). 2-Phenylethanol (99%) and (*E*)-cinnamic acid (98%) were purchased from Acros Organics (Nidderau, Germany). For gas chromatography (GC), helium 5.0 and nitrogen 5.0 were used.

### 2.2. Pre-culture and wort fermentation

Wort (Kölsch type, 13° Plato) was provided by the University of Applied Sciences (Giessen, Germany) (Zhang et al., 2014). The agar plug (1 × 1 cm) from the leading mycelial edge of the stock culture was transferred to flasks containing standard nutrition solution (100 mL/250 mL, medium volume/flask volume) and homogenized using an Ultra Turrax homogenizer (IKA, Staufen, Germany) as published previously (Fraatz et al., 2009). The pre-culture was incubated on a rotary shaker (24 °C, 150 rpm, 25 mm shaking diameter) for 9 days in the dark. The mycelium of 10 mL pre-culture broth was precipitated by means of centrifugation (4000 rpm, 2150 × *g*, 10 min, 20 °C) and washed three times with sterile water. The fungal pellets were resuspended in 10 mL sterilized wort, and the suspension was transferred into an Erlenmeyer flask (250 mL) containing 100 mL wort. To identify potential precursors of the biotransformation products, (*E*)-cinnamic acid (0.7 mg/100 mL), L-isoleucine (61.0 mg/100 mL), 2-methylbutanoic acid (0.4 μL/100 mL), and L-phenylalanine (62.6 mg/100 mL) were supplemented to the wort prior to the fermentation. The fermentation was carried out at 24 °C for 48 h under aerobic conditions on a rotary shaker (150 rpm). To elucidate the biogenetic pathway of methyl 2-methylbutanoate formation, labeled L-isoleucine (U-$^{13}$C) (0.45 mM) was added to wort, and the culture supernatant was analyzed by HS-SPME–GC–MS/MS–O after fermentation for 48 h by shiitake.

### 2.3. HS-SPME

For HS-SPME, CAR/PDMS (75 μm carboxen/polydimethylsiloxane, fiber length 1 cm; Supelco, Steinheim, Germany) and DVB/CAR/PDMS fibers (50/30 μm divinylbenzene/carboxen/polydimethylsiloxane, fiber length 1 cm) in combination with a GERSTEL MPS 2XL multipurpose sampler (GERSTEL, Mülheim an der Ruhr, Germany) were used. Ten mL of the culture broth (2.2) were transferred into a headspace vial (20 mL). The sample was agitated for 20 min (250 rpm) at 60 °C, followed by headspace extraction for 45 min at the same temperature. Afterwards, the analytes were directly desorbed in the split/splitless inlet (250 °C; SPME liner, 0.75 mm i.d.; Supelco) of the GC–MS/MS–O system for 1 min (2.4).

After desorption, the fiber was heated at 300 °C for the CAR/PDMS fiber and at 270 °C for the DVB/CAR/PDMS fiber, respectively, in the fiber conditioning station for 20 min.

### 2.4. GC

GC was carried out using an Agilent 7890A gas chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with an Agilent 7000B triple quadrupole mass spectrometry (MS/MS) detector (Agilent Technologies). A polar Agilent J&W VF-WAXms column (30 m × 0.25 mm × 0.25 μm) was used for analysis. He at a constant flow rate of 1.56 mL/min was used as the carrier gas. At the end of the capillary column, the carrier gas was split 1:1 into the triple quadrupole mass spectrometer and into an olfactory detector port (ODP 3, GERSTEL, Mülheim an der Ruhr, Germany). Further conditions were as follows: temperature program, 40 °C (3 min)/5 °C/min to 240 °C (5 min); injector temperature, 250 °C; septum purge flow rate, 3 mL/min; MS modes, selected ion monitoring (SIM) and scan mode in Q1; scan range, *m/z* 33–

**Table 1**
Compounds, internal standards, MS modes, *m/z* fragments, and fiber type used for quantitative analysis.

| Compound | MS mode | Quantifier ion (*m/z*) | Qualifier ion (*m/z*) | Fiber type | Internal standard |
|---|---|---|---|---|---|
| Methyl 2-methylbutanoate | Scan | 33–300 | – | CAR/PDMS | Thymol |
| (*E*)-methyl cinnamate | Scan | 33–300 | – | CAR/PDMS | Thymol |
| (*Z*)-3-nonenol | Scan | 33–300 | – | CAR/PDMS | Thymol |
| 2-Phenylethanol | Scan | 33–300 | – | CAR/PDMS | Thymol |
| 2-Acetylpyrrole | SIM | 109 | 94 | CAR/PDMS | Thymol[a] |
| 2-Acetylthiazole | SIM | 99 | 43 | CAR/PDMS | Thymol[a] |
| Methional | SIM | 104 | 76 | CAR/PDMS | Thymol[a] |
| *o*-Aminoacetophenone | SIM | 120 | 92 | DVB/CAR/PDMS | β-ionone[b] |
| β-Damascenone | SIM | 69 | 190 | DVB/CAR/PDMS | β-ionone[b] |
| 2,5-Dimethylpyrazine | SIM | 108 | 42 | DVB/CAR/PDMS | β-ionone[b] |
| Methyl hexadecanoate | SIM | 143 | 129 | DVB/CAR/PDMS | β-ionone[b] |
| 2-Phenylethyl acetate | SIM | 104 | 43 | DVB/CAR/PDMS | β-ionone[b] |

[a] : Quantifier ion and qualifier ion of thymol were 135 and 120, respectively.
[b] : Quantifier ion and qualifier ion of β-ionone were 177 and 43, respectively.