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Determination of compounds responsible for tempeh aroma

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ABSTRACT

Tempeh is a fermented food, popular mainly in south-east Asia, but also among vegetarians worldwide. It is produced by fermenting soybean or other beans with *Rhizopus* strains and usually eaten deep-fried, steamed or roasted. The flavour of tempeh depends upon the fermentation time, beans used and the (eventual) frying process. Our goal was to identify compounds responsible for the unique aroma of fermented and fried soy tempeh. Gas chromatography–olfactometry (GC–O) with the aroma extract dilution analysis (AEDA) approach, was used to determine key odorants after 1 and 5 days of fermentation and subsequent frying. Comprehensive gas chromatography–mass spectrometry (GC × GC–ToF-MS) was used for their quantitation using stable isotope dilution analysis (SIDA) or standard addition (SA) methods. Odour activity values (OAV) were calculated for 19 out of 21 key odorants. Tempeh was fermented for 5 days and fried, and the main aroma compounds were found to be the following: 2-acetyl-1-pyrroline, (FD = 1024, OAV 1380), 2-ethyl-3,5-dimethylpyrazine (FD = 512, OAV 338), dimethyl trisulfide, (FD = 512, OAV 900), methional (FD = 512, OAV 930), 2-methylpropanal (FD = 512, OAV 311) and (E,E)-2,4-decadienal (FD = 512, OAV 455). The frying process induced the increase or appearance of the main key odorants in tempeh.

1. Introduction

Tempeh (also known as tempe) is a traditional Indonesian food produced by the fermentation of soybeans using *Rhizopus* species. Apart from soybeans, other substrates used for fermentation have been utilised: chickpeas (Angulo-Bejarano et al., 2008), barley (Feng, Eriksson, & Schnürer, 2005) and beans (Ashenafi & Busse, 1991). The tempeh of a specific flavour is produced from pressed copra (Hachmeister & Fung, 1993). Tempeh is normally consumed fried, boiled, steamed or roasted. During the fermentation process, the enzymatic digestion of substrates, leads to an increased amount of free aminoacids, water-soluble nitrogen compounds, free fatty acids, and to the development of characteristic flavour. During the fermentation of tempeh, a decrease in the amount of crude lipids is observed, as lipids serve as the main source of energy for the microorganisms during the fermentation process. The protein hydrolysis may amount to 25% of the initial soy protein (Sparringa & Owens, 1999).

In addition to being a source of proteins and lipids in a vegetarian diet, tempeh became of interest due to its interesting nutritional/functional properties. It has been observed that fermented soybean products, such as tempeh, miso and natto, are more resistant to lipid oxidation than unfermented soybeans. The isoflavone levels in tempeh are relatively high compared to other soybean products. Raw tempeh contains the highest levels of daidzein and genestein, compared to tofu or soybean drinks. However, the process of tempeh deep frying significantly (up to 45%) reduces the total isoflavones contents (Haron, Ismail, Azlan, Shahar, & Peng, 2009). The potential use of tempeh as a functional food has increased. Methods of production of γ-aminobutyric acid enriched tempeh, which has antihipertensive effects (Aoki, Furuya, Endo, & Fujimoto, 2003a; Aoki et al., 2003b), as well as isoflavone-enriched tempeh (Nakajima, Nozaki, Ishihara, Ishikawa, & Tsuji, 2005) have been reported.

The properties, described above, of tempeh are mainly a result of the microbial/enzymatic activity of microorganisms, used for its production and biotransformation of soy constituents. The main fungus used for the preparation of tempeh in Indonesia is *Rhizopus oligosporus,* which is considered a domesticated form of *Rhiyopus microspores* (Feng, 2006). More strains identified in tempeh, included *Rhizopus formosaensis, R.* and *Rhizopus oryzae* (Babu, Bhakyaraj, & Vidhyalakshmi, 2009). It has been postulated that the natural habitat for *Rhizopus* could be fresh leaves of the Hibiscus species, which is used to wrap cooked soybeans (Ogawa, Tokumasu, & Tubaki, 2004). Apart from *Rhizopus* species, various microorganisms, including filamentous fungi, yeasts and bacteria, are found in the traditional tempeh. Tempeh co-inoculation with lactic acid bacteria (LAB) is performed to improve the safety of the product and this process contributes also to the fermentation process (Ashenafi & Busse, 1991). The growth of LAB (*Lactobacillus plantarum*), coexisting with *R. oligosporus*, especially with the

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acidification of the media during the tempeh production process, could inhibit the growth of pathogenic bacteria (Feng et al., 2005).

Microorganisms are also responsible for the unique flavour properties of tempeh. Contrary to the data on the nutritional properties, microbiological aspects and raw materials used for the tempeh production, data on the volatile compounds and especially on the tempeh aroma are very limited (Feng, Larsen, & Schnürer, 2007). Data on key odorants in this product, especially regarding raw and fried tempeh is nonexistent. The goal of this work was to determine key odorants in fermented (raw) and fried tempeh, prepared from soybean using the strain of *R. oligosporus* NRRL 2710 often used for its production on a commercial scale.

2. Materials and methods

2.1. Tempeh preparation

Tempeh was prepared using fungus *R. oligosporus*, NRRL 2710 strain originating from Northern Regional Research Laboratory, Peoria Ill, USA. The inoculum, used for soy seeds inoculation, was prepared from suspension of *R. oligosporus* spores cultured on PDA medium for 72 h. Seeds of soy Noviko variety, after dehulling and cooking (40 min.), were cooled down and inoculated with the spore suspension, placed on Petri dishes with a 15 cm diameter and fermented for a period of up to 5 days at 37 °C. Once fermented, soy beans overgrown with *R. oligosporus* formed into a cake of the size of a Petri dish, and were fried in rapeseed oil for 10 min at 170 °C.

2.2. Volatile compounds isolation

Tempeh cakes from Petri dishes were cut into approximately 1 cm squares, frozen in liquid nitrogen, and ground to obtain a homogenous sample (200 g). In the next step, 100 g of ground tempeh was transferred into an Erlenmeyer flask and extracted separately with two solvents of different polarities – diethyl ether (200 ml) and methylene chloride (200 ml), for 2 h each. Both fractions were filtered and combined prior to distillation, which was performed using a solvent-assisted flavour evaporation (SAFE) apparatus described by Engel, Bahr, and Schieberle (1999). During this procedure, the temperature of the water bath was held at 40 °C, at reduced (<300 m Torr) pressure, obtained using an Edwards RV5 rotary vane pump. The distillate of the aroma compounds was collected in a flask cooled with liquid nitrogen. After 30 min of distillation, the solution was dried over anhydrous Na_2SO_4, and the fraction was concentrated to about 400 μl using a Kuderna Danish concentrator.

2.3. Gas chromatography–olfactometry (GC–O)

GC–O was performed using a HP 5890 chromatograph and the following capillary columns: SPB-5 (30 m × 0.32 mm × 0.5 μm) and Supelcowax 10 (30 m × 0.32 mm × 0.5 μm; both columns from Supelco, Bellefonte, PA). The GC was equipped with a Y splitter, dividing effluent between an olfactometry port with humidified air as a makeup flow and a flame ionisation detector. The operating conditions were as follows for the SPB-5 column: initial oven temperature, 40 °C (1 min), raised at 6 °C/min to 180 °C and at 20 °C/min to 280 °C. Operating conditions for the Supelcowax-10 column were as follows: initial oven temperature, 40 °C (2 min), raised to 240 °C at 8 °C/min rate, held for 2 min isothermally. For all peaks and flavour notes, retention indices (RI) were calculated to compare results obtained by GC–O with that obtained by GC–MS and with literature data. Retention indices were calculated for each compound using a homologous series of C6-C24 n-alkanes.

2.4. Gas chromatography–mass spectrometry (GC–MS)

Compound identification was performed using two instruments. Agilent Technologies 7890A gas chromatograph was coupled to a 5975C TAD quadrupole mass spectrometer of the same producer. This instrument was equipped with a Supelcowax-10 column (30 m × 0.25 mm × 0.25 μm). Operating conditions for GC–MS were as follows: helium flow 32.2 cm/s; oven conditions were the same as for GC–O. Mass spectra were recorded in an electron impact mode (70 eV) in a scan range of m/z 33–350. Samples were also run on GC × GC-ToF-MS (Pegasus IV, Leco) running in both one- and two-dimension modes to quantify odorants. The GC was equipped with a DB-5 column (25 m × 0.2 mm × 0.33 μm) and a Supelcowax 10 (1.3 m × 0.1 mm × 0.1 μm) as a secondary one. For the one-dimensional analysis, the secondary oven was kept at a temperature that was 30 °C higher than the first oven, for which a temperature program was used from 40 °C (1 min) at 5 °C/min to 220 °C and kept for 5 min. Mass spectra were collected at a rate of 30 scans/s, and the detector voltage was 1750 V. For the two-dimensional analysis, the temperature of the second oven was kept 5 °C higher than the first oven. Modulation time was optimised and set at 7 s, and mass spectra were collected at a rate 200 scans/s.

Identification of volatiles was performed in two ways, depending on the availability of standard compounds: full identification comprising comparison of mass spectra, retention indices (RI), and odour notes on two columns of different polarities was performed when the reference standard of the investigated compound was available. In some cases, the MS signal of the analyte was too weak to facilitate mass spectra comparison. In these cases, RI and odour notes of the compounds were compared to a reference standard. When standards were not available, tentative identification was performed on the basis of the comparison of the mass spectrum of a compound with a NIST 05 library match and comparison of retention indices with those available in the literature. Also, the odour characteristics for an analysed compound was compared with the literature data and used in tentative identification.

2.5. Aroma extract dilution analysis (AEDA)

The flavour dilution factor (FD) of each of the odorants was determined by an AEDA method (Grosch, 1993). The flavour extract (2 μl) was injected into a GC column. Odour-active regions were detected by GC-effluent sniffing (GC–O), and three panelists (two of them with >5 year experience in GC–O analyses) determined the sensory description of the volatiles. The extract was then diluted stepwise by the addition of diethyl ether, and each sample of the dilution series was re-analysed until no odour was perceivable at the sniffing port. Retention data of the compounds were expressed as RI on both columns.

2.6. Quantification of aroma compounds

For quantification, stock solutions of internal standards of the respective isotopically labelled compounds, were prepared in diethyl ether and added to the tempeh samples in a concentration similar to that of the relevant analyte present (estimated in a preliminary experiments by peak area comparison). The suspension was stirred, and volatiles were isolated as described before using SAFE. Distillates were analysed by GC × GC-ToF-MS, monitoring the intensities of the respective ions listed in Table 1. For SIDA analysed compounds, response factors were calculated in the standard mixture of labelled and unlabeled compound in a concentration of