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Role of the Fermentation Process in Off-odorant Formation in White Pepper: On-site Trial in Thailand

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In white pepper samples, directly taken from a retting batch at a pepper production plant in Thailand, 3-methylindole, 4-methylphenol, 3-methylphenol, and butanoic acid, recently confirmed to be responsible for the characteristic fecal off-odor frequently detected in white pepper powders, were quantified by stable isotope dilution analyses. The results clearly showed that, in particular, 3-methylindole (fecal, swine-manure-like), 4-methylphenol (fecal, horse-like), and butanoic acid (cheese-like) were biochemically formed during retting, thus indicating that fermentation is the crucial step for off-odorant formation during white pepper processing. Model fermentation experiments performed with different manufacturing regimens revealed that white pepper, containing no substantial amounts of these odorants, can be produced from ripe starting material by a short fermentation under water and with frequent exchange of water. The overall aroma of such pepper was superior as compared to the aroma of white pepper produced according to the traditional procedure.

KEYWORDS: Pepper; aroma; fecal off-flavor; skatole; 3-methylindole; *p*-cresol; 4-methylphenol; *m*-cresol; 3-methylphenol; stable isotope dilution analysis

INTRODUCTION

The fruits of the pepper plant *Piper nigrum* L., indigenous to the Malabar coast, southwestern India, represent the most popular spice worldwide. Pepper fruits are commercially available as black, white, green, and red pepper. Black pepper is the type mostly consumed (*1*) and is manufactured from unripe, green fruits by sun-drying.

White pepper, which makes up to ∼20% of the total world pepper production (*1*), is mainly produced from mature pepper fruits by removing the outer layers of the pericarp in a fermentation step. In Indonesia, the major white pepper exporting country (*1*), this is traditionally achieved by keeping the fruits in bags submerged in water for up to 15 days. After the outer parts are rubbed off, white pepper, the fruit's core, is obtained by sun-drying.

In the Western world, white pepper is mainly used in industrial food processing to season, for example, light colored sausages, soups, and convenience products.

A minor fraction of the world's pepper harvest is directly sold as immature green or mature red pepper, either freeze-dried or pickled.

In contrast to other pepper types, white pepper is frequently tainted with a distinct unpleasant odor note described as fecal, phenolic, and cheese-like or, more colorfully, as "cowshed-like". This off-flavor can be transferred to food products, thus causing a severe problem for the food industry.

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By application of gas chromatography−olfactometry (GC-O), quantitation by stable isotope dilution assays (SIDA) and sensory experiments, 3-methylindole, eliciting a typical fecal, swine-manure-like odor, and 4-methylphenol, with a fecal, horse-like smell, were recently confirmed as major off-odorants in white pepper (*2*). Further odorants, also contributing to this specific malodor, were identified as 3-methylphenol (phenolic) and butanoic acid (cheese-like) (*2*). Although these four odorants were shown to be present in 50 commercial samples of different origin and, thus, have to be regarded as aroma constituents of white pepper as it is produced today, these aroma compounds are also the cause of malodors in white pepper (*2*). The reason for this is that during storage of, in particular, white pepper powder some odor qualities are much reduced in their intensity due to the volatility of the respective pepper odorants, whereas 4-methylphenol and 3-methylindole were not substantially decreased, thus dominating the overall aroma after storage (*2*). Because in fresh, ripe pepper fruits as well as in black pepper the concentrations of these odorants were very low, it was suggested (*2*) that the retting process might be the crucial step for their formation.

The aim of this study was therefore (i) to prove this assumption by quantitative data acquired during an authentic trial on location in Thailand and (ii) to investigate possibilities to minimize their concentrations in the final product by performing fermentation trials with varying parameters. Furthermore, a sensory evaluation of pepper containing low amounts of the off-odorants should reveal their role in the overall pepper aroma.

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MATERIALS AND METHODS

Pepper Samples. Dried peppercorns from ripe fruits used for commercial white pepper production were taken from the local market at Chantaburi province in Thailand. Fresh pepper, used as starting material for the model fermentations, was purchased from a local farmer. After fermentation, the samples were immediately dried and transported to Germany by air freight.

Retting Process on an Industrial Scale. The bottom of a concrete basin (5 m × 5 m, 1 m depth) was covered with dried pepper (3 t; ~15 cm high). Groundwater was added, until the water level was well above the peppercorns. After 24, 48, and 72 h, the water was exchanged. After 96 h, the water was drained and the pepper was covered with jute bags. The jute bags were permanently kept wet by sprinkling with water. After 15 days, the bags were removed and the peppercorns were peeled. For that purpose, the pepper was transferred to a peeling machine in which rubber rollers abraded the outer parts. The pulp was washed off with water in a strainer, and the pepper was dried in the sun (12 h; frequent turn-over). Intermediate samples, taken from the batch during the retting process, were manually peeled by rolling between the palms. After the pulp had been washed off through a bamboo sieve, these peppercorns were dried in stainless steel trays in the sun.

Model Fermentations. Freshly harvested, ripe pepper fruits (10 kg) were placed in plastic barrels with covers (height = 70 cm; i.d. = 40 cm; models I−III) or open plastic bowls (height = 20 cm; i.d. = 60 cm; models IV and V). The water used for soaking was fresh, local groundwater. In model I, a continuous water flour (1 L/min) was maintained by a flexible tube, ending at the bottom of the barrel. Manual peeling and drying were performed as described above. Further details on the fermentation conditions are given under Results and Discussion.

Chemicals. Deuterium oxide, 3-methylindole, and palladium on barium sulfate (5%) were from Aldrich, Sigma-Aldrich Chemie, Taufkirchen, Germany. Anhydrous sodium sulfate and diethyl ether were from Merck, Darmstadt, Germany. Diethyl ether was freshly distilled before use. Sources of other chemicals and reference compounds are given in the previous publicaction (*2*).

Synthesis. *[$^2H_{6-8}$]-3-Methylindole.* In a tailor-made small autoclave (stainless steel, 80 mm, 10 mm i.d., 3 mm wall thickness) with screw cap and PTFE sealing, 3-methylindole (0.5 mmol; 65.5 mg) was heated (160 °C; 48 h) in deuterium oxide (1.5 mL) in the presence of palladium on barium sulfate (5%; 250 mg) by means of a metal block thermostat 2090 (Liebisch Labortechnik, Bielefeld, Germany). After cooling, the mixture was filtered through a glass filter (G4), and the residue was washed with diethyl ether (total volume = 50 mL). The combined filtrates were washed with water (3 × 50 mL), dried over anhydrous sodium sulfate, and topped up to 100 mL (stock solution). The yield was determined by GC.

Yield: 35.6 mg (51%); MS-EI (*m*/*z*: intensity in %) 137 (100), 136 (56), 139 (49), 138 (44), 82 (10), 81 (8), 109 (8), 54 (6), 108 (6), 69 (5), 140 (5), 70 (4), 110 (4), 80 (4), 68 (3), 107 (3), 66 (3), 135 (3).

Stable Isotope Dilution Analyses. The quantitation of 3-methylindole, 4-methylphenol, 3-methylphenol, and butanoic acid was performed by SIDA as detailed recently (*2*). For the determination of 3-methylindole the new, deuterium-labeled internal standard [$^2H_{6-8}$]-3-methylindole was used. Accordingly, in the mass chromatograms obtained by MS/CI, mass traces selected for quantitation were *m*/*z* 132 for 3-methylindole and *m*/*z* 140 for the isotropically labeled 3-methylindole.

RESULTS AND DISCUSSION

Commercial Retting Process. In the Chantaburi province, situated at the Gulf of Thailand, substantial amounts of pepper are grown, and a major part is processed to white pepper, which is predominantly sold on the domestic market. Because authentic pepper material is scarcely available, one of us (M.S.) was permitted by local providers of pepper to supervise several pepper manufacturing trials on a production plant located near the city of Chantaburi. The authentic material obtained was immediately transported to Germany by air freight for chemical analyses.

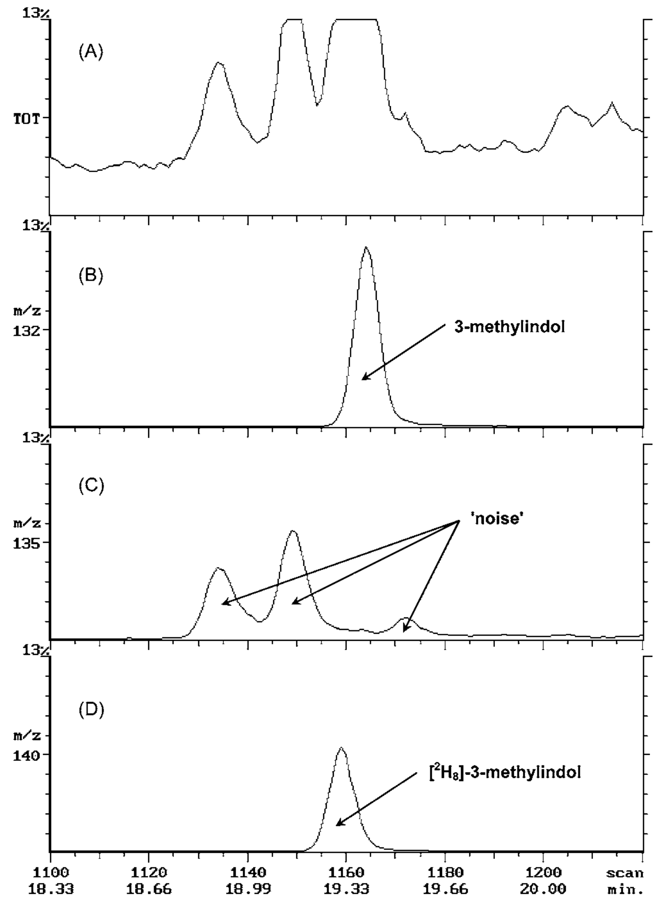

Figure 1. Mass chromatograms (MS/CI) obtained during quantitation of 3-methylindole via [$^2H_{6-8}$]-3-methylindole in white pepper based on the newly developed SIDA.

The pepper fruits for the industrial scale study (3 t) were supplied by local farmers. They consisted predominantly of mature fruits, which had been sun-dried on nets for 3 days in the sun. For retting, these brownish peppercorns were poured into a concrete basin and submerged in water. Retting was performed following the protocol given under Materials and Methods.

Quantitation. Samples, taken from the same batch at the beginning of the fermentation and after 15 days of processing, were analyzed for 3-methylindole, 4-methylphenol, 3-methylphenol, and butanoic acid.

During quantitation of 3-methylindole, we found that a coelution of an unknown compound occurred, showing the same molecular mass as the triply labeled [$^2H_3$]-3-methylindol (*3*) usually used as internal standard (**Figure 1**). For this reason, a new isotopically labeled internal standard had to be synthesized. Because no "noise" could be detected at mass traces *m*/*z* 138, 139, and 140, the use of a higher deuterated 3-methylindole should solve this problem.

Recently, the usefulness of a palladium-catalyzed protium−deuterium exchange for the synthesis of labeled compounds to be used in SIDA was demonstrated in the synthesis of [$^2H_4$]folic acid vitamers from 4-aminobenzoic acid via [$^2H_4$]-4-aminobenzoic acid (*4*). Accordingly, unlabeled 3-methylindole was thermally treated in D_2O and Pd on carbon as the catalyst to yield deuterated 3-methylindole. After aqueous workup, [$^2H_8$]-3-methylindole was obtained in 100% isotopic purity, but low yield (1.4%). It was assumed that the adsorption of 3-methylindole at the carbon support accounted for that. Consequently, the synthesis was repeated using Pd on barium sulfate as the

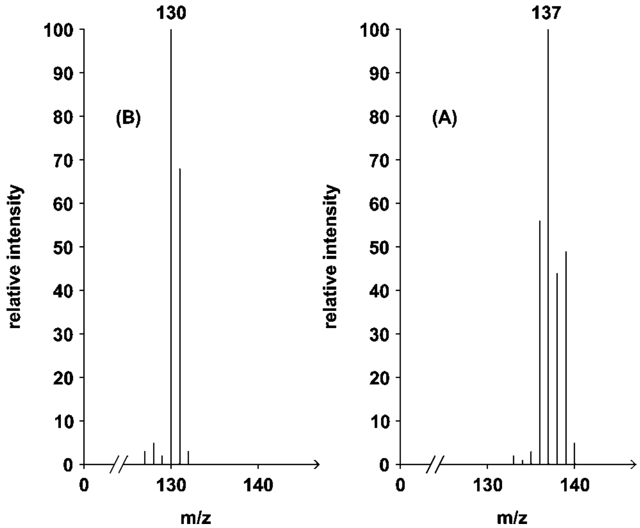

Figure 2. Relevant parts of mass spectra (MS/EI) of 3-methylindole (**A**) and [$^{2}H_{6-8}$]-3-methylindole (**B**).

Table 1. Concentrations of Four Off-odorants in White Pepper before and after Retting (Commercial Process; Same Batch)

odorant	concn (mg/kg) before retting	concn (mg/kg) after retting
3-methylindole	0.00	1.5
4-methylphenol	≤0.07	8.4
3-methylphenol	≤0.08	≤0.23
butanoic acid	16	188

catalyst. After aqueous workup, deuterated 3-methylindole was obtained in sufficient yield (54%). Mass spectral data (EI) showed the incorporation of six to eight deuterium atoms (**Figure 2**). This labeled 3-methylindole was then used as internal standard for the quantitation of 3-methylindole in the pepper samples. In the mass chromatograms (MS/CI), the peak-to-noise ratios obtained were good for the relevant mass traces *m/z* 132 (3-methylindole) and *m/z* 140 ([$^{2}H_{8}$]-3-methylindole) (**Figure 1**).

The results obtained (**Table 1**) clearly proved the formation of, particularly, 3-methylindole, 4-methylphenol, and butanoic acid during the retting process. 3-Methylindole, which was not present in the sample before the fermentation process, was found with a concentration of 1.5 mg/kg after fermentation. The concentration of 4-methylphenol also markedly increased from ≤0.07 to 8.4 mg/kg and that of butanoic acid from 16 to 188 mg/kg. The concentrations of 3-methylindole and 4-methylphenol in the fermented sample were well within the concentration ranges found earlier (*2*) for a larger number of commercial white pepper samples, whereas that of butanoic acid was slightly higher. Interestingly, 3-methylphenol, which was found previously in ~50 samples of white pepper, was not detectable in the Thai samples (**Table 1**).

To get an insight into the time course of the odorant formation, 3-methylindole and 4-methylphenol were additionally determined in samples taken after 1, 2, 3, 4, 5, 7, and 10 days of retting. These peppercorns were manually peeled, washed, and dried in the sun. The results (**Figure 3**) showed that both aroma compounds were very low until the third day of fermentation. However, a very pronounced generation was observable between days 4 and 7 of the trial.

3-Methylindole and 4-methylphenol are well-known microbial metabolites of the amino acids tryptophan and tyrosine (*5*, *6*). Thus, the data suggest that the activity of these microorganisms is highest between the fourth and seventh days of the fermentation.

Model Fermentations. In a next series of experiments, batches of the same pepper fruits were fermented in different models aimed at minimizing the concentrations of the odorants by modifying the fermentation parameters.

Therefore, five different model fermentations were set up. Each model started with 10 kg of mature peppercorns from the same batch. Variations basically addressed access of air to the samples (aerobic/anaerobic) and water throughput. Peppercorns for models I–III were placed in plastic barrels and submersed in water, thereby providing mainly anaerobic conditions. In model I, a steady flow of water was maintained, in model II the water was daily exchanged, and in model III no water exchange was performed during the whole fermentation period.

Models IV and V were fermented in open plastic bowls. In model IV, peppercorns were soaked in water for 4 days with a daily exchange of water. Then, the water was removed and the peppercorns were covered with wet jute bags, thus providing mainly aerobic conditions. Peppercorns in model V underwent aerobic fermentation under wet jute bags without preliminary soaking in water. After a total fermentation time of 15 days, the outer layers of the pericarp were manually rubbed off and the material obtained was sun-dried.

Not only the odor characteristics but also the appearance of the individual model peppers were quite different (**Table 2**). Models I and II yielded appealing light fawnish gray peppercorns with a typical peppery odor. In contrast, peppercorns obtained in models III–V had a displeasing dark brownish color. The smell of peppercorns obtained in model III (no exchange of water for 15 days) was dominated by an intense fecal odor note. By contrast, the aerobically fermented model peppers from IV and V showed distinct moldy off-odors. In addition, insects had entered the bowls, resulting in a high number of maggots and cocoons.

Quantitations before fermentation and after fermentation of the five models (**Table 3**) confirmed that 3-methylindole, 4-methylphenol, and butanoic acid were clearly formed during the retting process.

By far the highest concentrations of 3-methylindole, 4-methylphenol, and butanoic acid were found in model III, the batch that was fermented nearly anaerobically without any exchange of water. Especially the values for 3-methylindole (5.1 mg/kg) and butanoic acid (480 mg/kg) were exceptionally high as compared to our previous data on commercial pepper samples (*2*). By contrast, the concentrations determined in models I (steady flow) and II (daily water exchange) were much lower and in the lowest ranges previously determined in commercial samples (*2*). Accordingly, a sufficient throughput of water is highly desirable for an appropriate white pepper production.

Interestingly, the pepper from model V did not show any formation of 3-methylindole or 4-methylphenol. Obviously, the soaking process prior to wet storage is important for the development of the two odorants. Model IV, which was kept under water during the first 4 days and was then aerobically fermented, showed slightly higher concentrations of the off-odorants. Therefore, anaerobic conditions in the first days of the fermentation seem to be a prerequisite for the formation of the methylphenols and 3-methylindole.

However, aerobic fermentation (model V) is no alternative to the traditional fermentation under water, because of the dark color, the generation of a musty off-odor, and the problem of insect attack.

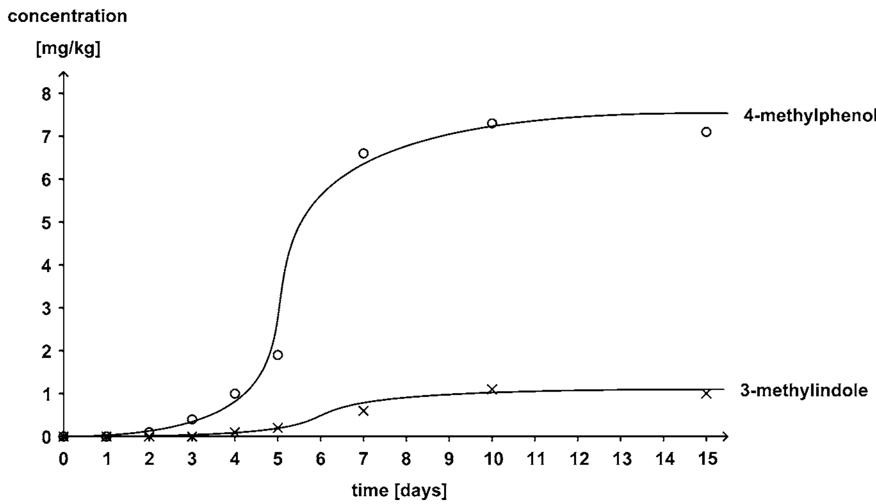

Figure 3. Time course of the formation of 3-methylindole and 4-methylphenol during retting (industrial scale).

Table 2. Sensory Characteristics of White Peppers Obtained in Five Different Model Fermentations

model	color	odor: whole peppercorns	odor: pepper powder
I submersed, steady flow	light gray to light fawn	peppery, weak	peppery, typical
II submersed, daily change	light gray to light fawn	peppery, weak	peppery, typical
III submersed, no change	light brown	fecal, strong	fecal, strong
IV soaking, then aerobic	light brown	moldy	moldy
V aerobic	dark brown	moldy	moldy, strong

Table 3. Concentrations of Characteristic Off-odorants in White Pepper Obtained by Model Fermentations[a]

	concn (mg/kg)					
odorant	before fermentation	model I	model II	model III	model IV	model V
3-methylindole	0.0	0.9	0.5	5.1	0.4	0.1
4-methylphenol	0.0	3.8	1.6	6.1	1.3	0.1
3-methylphenol	0.1	0.2	0.1	0.1	0.0	0.2
butanoic acid	1.6	83	84	480	53	22

[a] Total fermentation time: 15 days.

Again, no formation of 3-methylphenol was observed. It may, thus, be assumed that the Thai genotype of pepper, in contrast to Indonesian, Malaysian, and Brazilian peppers (*2*), does not provide the precursor from which 3-methylphenol is generated during fermentation.

Anaerobic fermentation under water with a daily change of the water seems to be the most practical way to produce white pepper with tolerable off-odorant concentrations. Nevertheless, a further reduction of the off-odorants would be desirable, because the concentrations of, in particular, 3-methylindole and 4-methylphenol were still clearly above their breakthrough thresholds (*2*).

Therefore, the influence of the fermentation time was studied by quantitation of 3-methylindole and 4-methylphenol in intermediate stages of model II.

The results (**Table 4**) indicated that under these conditions, a pronounced formation of 3-methylindole and 4-methylphenol started after day 7 of the trial. Compared to the traditional process (**Figure 3**), the curves were not as steep and the induction periods were longer. Consequently, a reduction of the fermentation time to <7 days under these conditions could be the method of choice to suppress off-odorant formation and, therefore, to produce white pepper without any potential of generating off-flavor after storage.

Table 4. Time Course of the Formation of 3-Methylindole and 4-Methylphenol during Fermentation of Model II

	concn (mg/kg)	
fermentation time (days)	3-methylindole	4-methylphenol
0	0.00	0.03
1	0.00	0.02
2	0.00	0.04
3	0.00	0.03
4	0.00	0.02
5	0.00	0.07
7	0.10	0.21
10	0.40	1.29
15	0.52	1.58

The reduction of fermentation time, however, requires uniformly ripe starting material, because the pericarp of green, unripe pepper fruits needs more time to soften. This is a problem, because pepper fruits do not mature simultaneously, not even at the same spike. A delay of the harvest is not an alternative for farmers, as early maturing pepper fruits would drop off or decay, thus substantially reducing crop yield and quality. Therefore, methods for a mechanical separation of pepper fruits of different ripening stages should be developed. Such an alternative, which was observed by us at Thai farms, involves soaking mixed mature material for 4 days. Gentle rubbing then turns the softened pericarps of the ripe fruits into sludge, which is easily removed by rinsing, while immature green fruits stay unaltered. After the pulp has been washed off, separation of green and white peppercorns can be achieved by flotation according to their different specific gravities. Recovered green material can further be used, for example, for making black pepper.

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