

Article

Effects of Media and Processes on the Aromas of White Truffle Solid-State Fermented Products

Chih-Yuan Cheng and Su-Der Chen * 

Department of Food Science, National Ilan University, Number 1, Section 1, Shen-Lung Road, Yilan City 260007, Yilan County, Taiwan; yuan860312@gmail.com

* Correspondence: sdchen@niu.edu.tw; Tel.: +886-9317761; Fax: +886-39351892

Abstract: This study aimed to formulate a black bean soy sauce using black beans and black rice as media for the solid-state fermentation of white truffle. Various proportions of these media (4:0, 3:1, 2:2, 1:3, and 0:4) were prepared, with methionine concentrations (0, 0.3, 0.6, 0.9, 1.2, and 1.5%) serving as precursors for a 4-week solid-state fermentation to analyze the aroma profiles. GC-MS analysis showed that samples with 1.5% methionine exhibited significantly higher levels of sulfur-containing volatile compounds compared to those without methionine. GC-IMS analysis revealed that a 2:2 ratio of black beans to black rice produced the most enriched aroma. Lower methionine levels improved mycelial growth, with 0.3% methionine yielding the richest aroma components. After fermentation, the white truffle products were sterilized using autoclaving, hot air assisted radio frequency (HARF), and high pressure processing (HPP), followed by freeze drying. GC-IMS analysis showed that HPP samples had an aroma closest to fresh samples, whereas HARF and autoclave resulted in similar aromas. However, 24 h freeze drying significantly diminished the aroma, resulting in no significant difference in aroma among the freeze-dried products treated with different sterilization methods.

Keywords: truffle; aroma; fermentation; GC-MS; GC-IMS; HPP; radio frequency



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1. Introduction

Truffles belong to the fungal kingdom and the *Tuber* genus. They are highly prized for their unique aroma. However, truffle fruiting bodies require specific and harsh climatic conditions to grow. In addition to climate and soil, environmental microorganisms are crucial for their successful growth [1]. Typically, it takes over six years for artificially cultivated truffles to reach the fruiting stage, with gradual yield increases of up to ten years. If the production of cultivated truffles increases and becomes sustainable, the supply of truffles causes price decline [2]. Truffles are one of the most expensive edible fungi due to their rarity, unique aroma, and high nutritional value as an antioxidant, anti-inflammatory, antiviral, immunomodulatory, antitumor, and antimicrobial [3]. Freshly harvested truffle fruiting bodies have a short shelf life and quickly lose their flavor intensity due to spoilage. Therefore, researchers have explored ways to extend the shelf life of truffles, using methods such as canning, hot air drying, freezing, and freeze-drying. GC-O (gas chromatography–olfactometry) analyses showed that the aroma of truffles decreased with all preservation methods compared to fresh truffles. Among these methods, freeze drying most closely retained the fresh truffle aroma. In contrast, sulfur compounds were not detected in canned and frozen samples, while hot air-dried samples had stronger alcohol and ketone aromas [4]. Additionally, Sorrentino et al. [5] found that treating truffles with gallic acid and refrigerating them effectively inhibited the growth of indicator microorganisms on the truffle surface, thereby extending their shelf life.

To address the time-consuming nature of cultivating truffle fruiting bodies, recent research has focused on liquid fermentation, which can develop truffle mycelium in about a week. Tang et al. [6] found that liquid fermentation of black truffle mycelium produced

higher amounts of 3-methylbutanol and 2-methylbutanol in the fermentation broth, while 2-phenylethanol was more abundant in the mycelium. The levels of 3-methylbutanol and 2-phenylethanol increased with fermentation time, reaching their peak on the ninth and tenth days. Liu et al. [7] enhanced the sulfur compound content of black truffle fermentation products by adding methionine to the fermentation broth, with dimethyl disulfide (DMDS) being the main metabolite. Liu et al. [8] discovered that a medium containing 60 g/L glucose and 15 g/L yeast extract, at 28 °C and pH 7, was optimal for black truffle fermentation, yielding the best sensory scores. Vahdatzadeh and Splivallo [9] studied sulfur-containing volatile compounds in *Tuber borchii* (spring white truffle), and they found that adding methionine as a precursor increased the content of sulfur compounds such as DMDS, confirming that this aroma is formed via the Ehrlich pathway. While liquid fermentation offers the advantage of shorter fermentation times, the processing of fermentation broth increases drying costs. To solve this issue, white truffles were subjected to solid-state fermentation using grains as the media for four weeks, and their antioxidant activity was analyzed [10].

The process of eliminating microorganisms on surfaces, solids, and liquids is called sterilization. In the traditional food industry, heat is commonly used to extend shelf life by killing microorganisms or inactivating enzymes. However, high-temperature sterilization may lead to loss of texture, taste, nutrition, and aroma. Therefore, non-thermal sterilization through high-pressure processing (HPP) is advantageous compared to traditional thermal methods, as it helps preserve food flavor and nutrition while reducing energy consumption. Verret et al. [11] utilized HPP (300 and 550 MPa for 10 min) for sterilizing black truffle, showing changes in volatile compounds compared to fresh samples after high-pressure treatment.

Currently, the most widely used instruments for aroma analysis include gas chromatography–mass spectrometry (GC-MS), gas chromatography–olfactometry–mass spectrometry (GC-O-MS), and electronic noses [12]. Tang et al. [13] established a distillation solid-phase extraction method combined with GC to detect key compounds in black truffle fungi. Pennazza et al. [14] used an electronic nose and GC-MS with headspace analysis to study the sensory attribute changes in white truffle fungi during storage. Splivallo and Ebeler [15] analyzed specific sulfur-containing volatile compounds (thiophene derivatives) in *T. borchii* (spring white truffle) using gas chromatography–olfactometry (GC-O), finding that freezing led to a decrease in these thiophene derivatives, indicating that the freezing process caused a loss of such aromas.

Due to the complex nature of food matrices and the need for cumbersome pretreatment steps, along with long analysis times, GC-MS cannot meet the requirements for multi-component analysis and rapid detection. Although GC-O-MS can effectively scan aroma compounds from complex mixtures, it requires prolonged and repetitive work, making it unsuitable for large-scale analysis. On the other hand, gas chromatography–ion mobility spectrometry (GC-IMS) can heat sample vials to obtain headspace aroma, eliminating the need for tedious pre-extraction processing. This makes GC-IMS an emerging method for analyzing volatile compounds [16].

Ion mobility spectrometry (IMS) separates ions in the gas phase under weak electric fields at atmospheric pressure, using a drift tube to separate ions based on their mass, charge, size, and shape [17]. Consequently, GC-IMS combines the high selectivity of gas chromatography (GC) with the high sensitivity of IMS, allowing for the analysis of headspace aroma compounds in 20–30 min without sample pretreatment, making it convenient and time-saving. The system includes automatic analysis and high resolution, minimizing human intervention, and presenting the results as tangible fingerprint maps of the aroma compounds [16,18]. Currently, GC-IMS is increasingly used for quality control analysis of food flavors and for monitoring changes during processing, such as the aroma changes in matsutake mushrooms after drying [19] and aroma changes in yak milk powder with different drying methods [18].

In this experiment, different proportions of black beans and black rice were used as media, with the addition of methionine, to perform solid-state fermentation of white truffles. The aroma components were analyzed to determine the optimal formulation of the solid-state medium. Subsequently, HPP non-thermal sterilization and traditional thermal sterilization were used, and GC-IMS was employed to analyze the effects of different sterilization methods and freeze-drying on the aroma of the white truffle solid-state fermented products.

2. Materials and Methods

2.1. Materials

The truffle strain used was the Italian white truffle strain (*Tuber magnatum*) obtained from Professor Hong-Dao Hu's laboratory at the Department of Forestry and Resource Conservation, National Taiwan University. Black beans and black rice were purchased from Ji Yuan Farm (Yilan, Taiwan). The nutritional analysis of the black beans and black rice was conducted by the Bio-Resources Product Testing and Technology Promotion Center at National Ilan University. Black beans contained 40.5% protein, 12.6% total fat, and 30.5% carbohydrate. Black rice contained 6.4% protein, 2.9% total fat, and 78.3% carbohydrate. Potato dextrose agar (PDA) and potato dextrose broth (PDB) were purchased from Difco Co. (Sparks, MD, USA).

2.2. Equipment

The following equipment were used: shaking incubator (LM-600R, Yihder Technology Co., Ltd., Taipei, Taiwan), high-temperature steam vertical autoclave (TM-329, Tomin Medical Equipment Co., Ltd., New Taipei, Taiwan), oven (DCM45, Channel, Sci-Mistry Co., Ltd., Yilan, Taiwan), electronic precision balance (HDW-15L, Hengxin Weighing Technology Co., Ltd., Yilan, Taiwan), horizontal sterile operating table (4HT-24, Taiwan), high-pressure food sterilization machine (HPP 600 MPa/6.2 L, Kun Tai International Co., Ltd., Yunlin, Taiwan), batch-type radio frequency heating equipment combined with a hot air blower (power: 5 kW, frequency: 40.68 MHz, Yida Biotech Co., Yilan, Taiwan), shelf-type freeze dryer (FD-80-4B, Ivorist International Co., Ltd., New Taipei, Taiwan), high-speed grinder (RT-04, Sci-Mistry Co., Ltd., Yilan, Taiwan), gas chromatography–mass spectrometer (Varian Saturn GC-MS 2200, Louisville, KY, USA), solid phase microextraction fiber (50/30 µm DVB/CAR/PDMS), capillary column DB-5MS (60 m × 0.25 mm I.D., 0.25 mm film thickness; Supelco Inc., Bellefonte, PA, USA), gas chromatography–ion mobility spectrometer (GC-IMS, Flavour Spec[®], G.A.S. Dortmund, Germany), and gas chromatography separation capillary column (OV-5, 5% diphenyl, 95% dimethylpolysiloxane, non-polar, length: 20 m).

2.3. Cultivation and Pre-Activation of Strains

The white truffle strain *Tuber magnatum* was the white truffle used in this study. It was cultured on potato dextrose agar (PDA) plates at 25 °C in a constant temperature incubator and subcultured once a month. For pre-activation, the white truffle was placed on PDA plates and incubated at 25 °C for 7 days. Three 1 cm² PDA mycelial blocks were then inoculated into 500 mL baffled Erlenmeyer flasks containing 150 mL of PDB medium. These were incubated at 25 °C and shaken at 120 rpm for 3 days.

2.4. White Truffle Solid-State Fermented Products

Using black beans and black rice as substrates in ratios of 4:0, 3:1, 2:2, 1:3, and 0:4, 500 g polypropylene (pp) bags were prepared with a substrate-to-water ratio of 6:4. A total of 1.5% L-methionine was added, and the bags were sterilized in an autoclave at 121 °C for 1 h. After cooling, 10 mL of pre-activated white truffle culture (incubated for 3 days) was inoculated, and the bags were incubated at 25 °C for four weeks to obtain the solid-state fermentation products. Subsequently, black beans and black rice in a 2:2 ratio were used as the fermentation substrate, with varying concentrations of methionine (0%,

0.3%, 0.6%, 0.9%, 1.2%, and 1.5%) added as precursors. After sterilization, the substrates were inoculated with white truffle culture and incubated at 25 °C for four weeks.

2.5. Sterilization of White Truffle Solid-State Fermented Products

Using a substrate ratio of 2:2 for black beans and black rice, 500 g pp bags were prepared with a substrate-to-water ratio of 6:4. L-methionine (0.3%) was added as a precursor. The bags were sterilized in an autoclave at 121 °C for 1 h. After cooling, 10 mL of pre-activated white truffle culture (incubated for 3 days) was inoculated, and the bags were incubated at 25 °C for four weeks. The white truffle solid-state fermentation products were then collected.

Additionally, referencing Hsu et al. [10], the completed white truffle solid-state fermentation products were sterilized using three different methods: High-pressure processing (HPP) at 500 MPa for 5 min, autoclaving at 121 °C for 1 h, 5 kW radio frequency hot air heater with a 12 cm electrode plate gap, supplemented with 100 °C hot air heating for 1 min to reach 100 °C. The sterilized products were then frozen at −20 °C and freeze-dried using a shelf freeze dryer for 24 h (shelf temperature set to 35 °C, condenser chamber temperature set to −78 °C, and vacuum pressure below 0.1 torr). The experimental design of this study is shown in Figure 1.

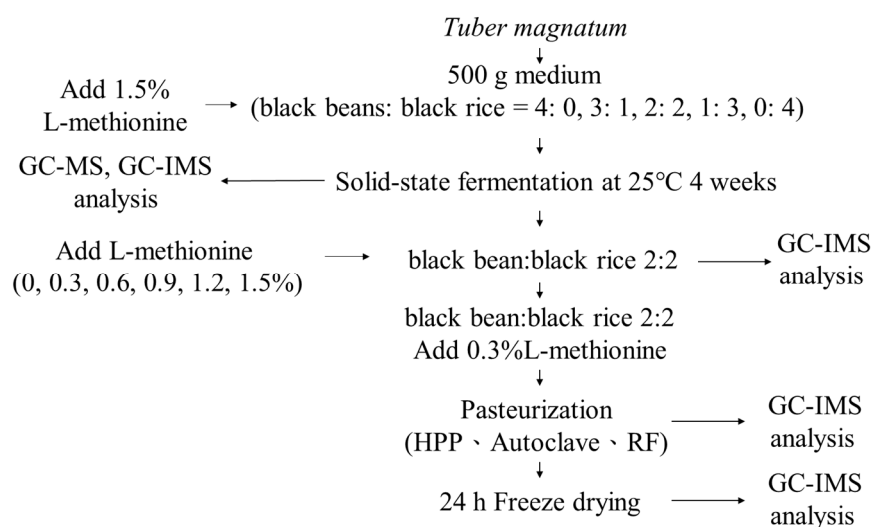


Figure 1. Experimental design of aroma study in *Tuber magnatum* solid-state fermentation.

2.6. Aroma Analysis of Truffles

2.6.1. Headspace Solid-Phase Microextraction

Referencing and modifying the approach by Diaz et al. [20], 1 g of sample was placed in a 20 mL headspace vial. A total of 100 µL of 10 ppm n-butanol was added as an internal standard. The vial was tightly sealed with a cap containing a PTFE/white silicone septum and equilibrated at 53 °C for 5 min. A 50/30 µm DVB/CAR/PDMS solid-phase microextraction fiber was then exposed to the headspace above the sample for 13.6 min to adsorb the volatile organic compounds.

2.6.2. GC-MS Analysis

Referencing the method by Diaz et al. [20], the fiber with the adsorbed compounds was placed in the GC-MS injection port and thermally desorbed at 200 °C for 10 min in splitless mode. Volatile organic compounds were analyzed using a DB-5 capillary column (60 m × 0.25 mm I.D., 0.25 mm film thickness; Supelco, USA). The oven's initial temperature was set at 40 °C, increased to 60 °C at a rate of 10 °C per minute, and then raised to 200 °C at a rate of 3 °C per minute. Helium was used as the carrier gas at a flow rate of 1.0 mL/min. The mass spectrometer was set with a transfer line temperature of

280 °C and an ion source temperature of 170 °C, and ionization was performed using a 70 eV electron beam with a scan range of 40–650 m/z .

2.6.3. GC-IMS Analysis

A total of 1 g of sample was weighed and transferred to a 20 mL headspace vial. The sample was treated at 53 °C and 500 rpm for 14 min. Using a heated syringe at 85 °C, 600 µL of headspace was automatically injected into an OV-5 (5% diphenyl, 95% dimethylpolysiloxane) capillary column (20 m × 0.53 mm ID). Nitrogen gas (purity 99.999%) was used as the carrier gas with the following flow rate program: started at 2 mL/min and increased to 15 mL/min, maintained this rate for 5 min and 30 s, then increased to 30 mL/min and maintained for 13 min, then decreased to 2 mL/min until stopping at 20 min. The analytes were separated in the chromatographic column at 60 °C and then ionized in the IMS ionization chamber at 45 °C. The drift gas flow rate was set at 150 mL/min.

2.7. Data Compilation and Statistical Analysis

Data analysis was performed using the built-in software of the GC-IMS, Laboratory Analytical Viewer (LAV), along with its plug-ins, which include Reporter, Gallery, and Dynamic Principal Component Analysis (PCA).

3. Results and Discussion

3.1. Effect of Different Ratios of Black Bean and Black Rice as Media on Aroma in Solid-State Fermented White Truffle Products

This study, aimed at the preparation of black bean soy sauce, primarily used black beans and black rice as raw materials. Therefore, black beans and black rice were chosen as media for the solid-state fermentation of white truffle, with different black beans and black rice ratios prepared as follows: (A) 4:0, (B) 3:1, (C) 2:2, (D) 1:3, and (E) 0:4. The moisture content was controlled at 40%, and 500 g of the media was placed in a PP bag. They were sterilized in an autoclave at 121 °C for 1 h, then cooled and inoculated with 10 mL of pre-activated culture broth. The samples were incubated at 25 °C for four weeks.

The nutritional composition analyses of black beans and black rice were provided by inspection and certification agencies; black beans contained 40.5% crude protein, 12.6% crude fat, and 30.5% carbohydrates, and black rice contained 6.4% crude protein, 2.9% crude fat, and 78.3% carbohydrates. It could be understood that in the solid-state media for white truffle fermentation, the protein in black beans serves as the nitrogen source, while the carbohydrates in black rice serve as the carbon source. Furthermore, the carbohydrate and protein content of the prepared solid-state media were estimated (Table 1). It was found that the medium with a black bean to black rice ratio of 4:0 had the highest protein content at 40.5 g/100 g. As the proportion of black beans decreased, the protein content showed a decreasing trend, while the carbohydrate content increased with the rise in black rice proportion. The medium with a black bean to black rice ratio of 0:4 had the highest carbohydrate content at 78.3 g/100 g. The ratio of carbohydrates to protein significantly increased with the rise in black rice proportion, from 0.75 in the 4:0 ratio to 12.23 in the 0:4 ratio. This indicated that the proportion of black beans and black rice in the media affected the subsequent solid-state fermentation of white truffle.

Regardless of the medium formulation, mycelium (the white part) was successfully generated after fermentation (Table 1). The growth area of the mycelium increased with fermentation time. To enhance the aroma of sulfur-containing compounds in the solid-state fermentation products of white truffle, 1.5% methionine was added to each pp bag [9]. The growth area of the mycelium increased with fermentation time. However, compared to the groups without methionine, the mycelium growth in the methionine-added groups was not as robust (Table 1). This may be due to the addition of methionine altering the carbon-to-nitrogen ratio, thus affecting the growth of the white truffle fungi. Nevertheless,

after mixing, the internal medium was still fully colonized with mycelium, and a four-week fermentation was selected as the endpoint for subsequent analysis and research.

Table 1. Nutrition facts analyses and *Tuber magnatum* solid-state fermented products by different ratios of black bean (BB) and black rice (BR).

Items of Media (per 100 g)	BB:BR (4:0)	BB:BR (3:1)	BB:BR (2:2)	BB:BR (1:3)	BB:BR (0:4)
Total carbohydrate (g)	30.50	42.45	54.40	66.35	78.3
Total protein (g)	40.50	31.975	23.45	14.925	6.40
Carbohydrate/Protein	0.75	1.33	2.32	4.45	12.23
4-week solid-state fermented white truffle					
4-week solid-state fermented white truffle with 1.5% Met					

Further analysis of the aroma components of various white truffle solid-state fermentation products was conducted using GC-MS. Figures 2 and 3 show the aroma fingerprint profiles of white truffle solid-state fermentation products without and with added 1.5% methionine, respectively. Overall, the group with added methionine had higher levels of volatile compounds compared to the group without methionine. According to the Ehrlich metabolic pathway, methionine can act as a precursor, being biosynthesized into dimethyl sulfide (DMS), dimethyl disulfide (DMDS), and dimethyl trisulfide (DMTS) during white truffle fermentation. Additionally, the literature indicates that the addition of 20 mM methionine can promote the content of DMDS in *T. borchii* [9]. The aromatic attributes proposed for the trained panel evaluation are usually a combination of some molecules, i.e., DMS and DMDS for truffle aromas [21].

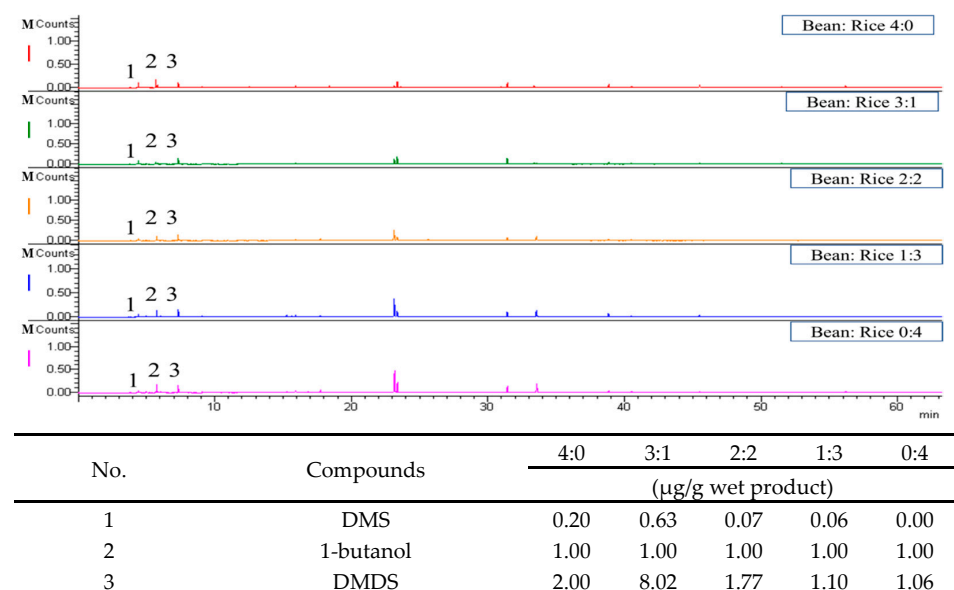


Figure 2. GC-MS spectrum of *Tuber magnatum* four-week solid-state fermented products using different ratios of black bean and black rice as media.

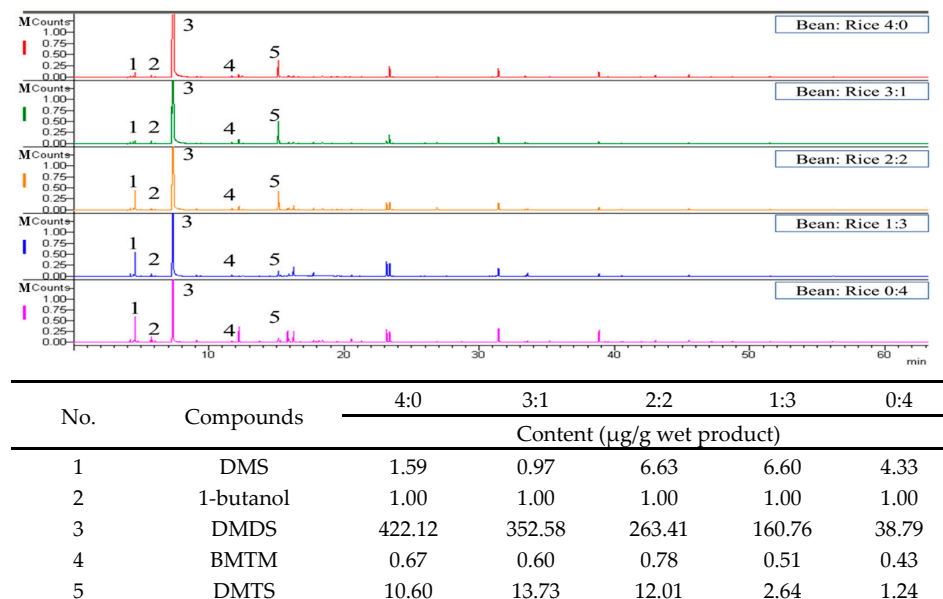


Figure 3. GC-MS spectrum of *Tuber magnatum* four-week solid-state fermented products using different ratios of black bean and black rice as media with 1.5% methionine.

Since sulfur compounds are key aroma components of truffles with low thresholds that humans easily detect, Tejedor-Calvor et al. [21] focused on the aroma because truffles are appreciated worldwide for their volatile sulfur-containing aroma compounds. In Figure 2, for the group without added methionine, compound number 1, DMS, had a content of 0~0.6 µg/g wet weight, and compound number 3, DMDS, had a content of 1.06~8.02 µg/g wet weight, with DMDS having a higher content. However, the unique truffle aroma compound BMTM was not detected. In contrast, in Figure 3, for the group with 1.5% added methionine, there was a noticeable increase in the peaks of sulfur compounds in the GC chromatogram. Compounds 1, 2, 3, and 4 represented DMS, DMDS, BMTM, and DMTS, respectively, with contents of 0.97~6.63 µg/g wet weight, 38.79~422.12 µg/g wet weight, 0.43~0.78 µg/g wet weight, and 1.24~13.73 µg/g wet weight, which were significantly higher than those in the group without added methionine.

Truffles possess significant variability in their aroma profiles from species to species. In general, sulfur compounds such as dimethyl sulfide (DMS) and dimethyl disulfide (DMDS), 1-octen-3-ol, and 2-methyl-1-propanol have been identified in most truffle species [3]. Liu et al. [7] suggested that adding methionine as a precursor during the liquid fermentation of black truffle mycelium can effectively increase the content of sulfur compounds such as DMS and DMDS. Vahdatzadeh and Splivallo [9] also confirmed through liquid fermentation that adding methionine could enhance the sulfur compound content in spring white truffles via the Ehrlich pathway.

When the ratio of black beans to black rice was 4:0, the DMDS content was the highest, reaching 422.12 µg/g wet weight, but the DMS content was relatively low, resulting in a simpler composition. As the proportion of black rice increased, the DMS content also increased. However, when the ratio of black beans to black rice was 1:3, the DMDS content significantly decreased, and there was a general downward trend in the overall sulfur compound content. Additionally, the characteristic aroma of white truffle, BMTM, was higher in the methionine-added group compared to the group without methionine, as shown in Figure 2. The highest BMTM content was observed at a black bean-to-black rice ratio of 2:2.

GC-MS analysis has already shown that adding methionine can effectively produce sulfur-containing aroma compounds in white truffle. Next, rapid aroma analysis was conducted using GC-IMS to investigate the impact of different ratios of black bean and black rice media on the aroma of white truffle solid-state fermented products. From the

fingerprint profiles in Figure 4, slight differences were observed among different media. Each red dot in the figure represented a volatile compound. When the color appeared yellow or red, it indicated a higher concentration, whereas blue indicated a lower concentration.

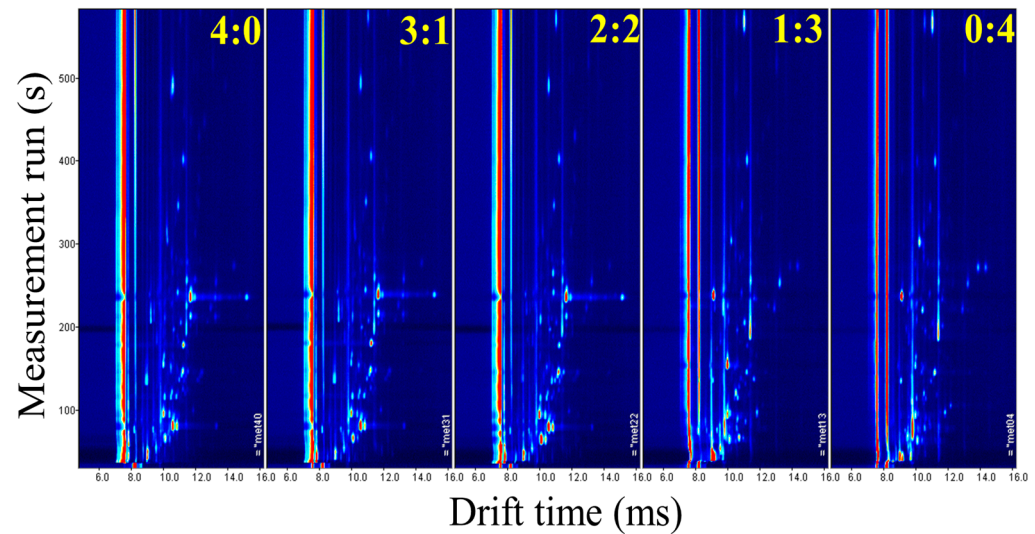


Figure 4. Aroma fingerprints of *Tuber magnatum* solid-state fermented products using different ratios of black bean and black rice as media.

A further selection of characteristic volatile compounds based on their differences is shown in Figure 5. A 2:2 ratio of black beans to black rice had the richest aroma content. The fingerprint profiles of the 4:0 and 3:1 ratios of black beans to black rice were very similar, with the main aroma components being numbered 3-1 to 16. However, the fingerprint profiles of the 1:3 and 0:4 ratios of black beans to black rice were more similar, with characteristic aromas numbered 8, 10, 17, 19, and 20. Compared to the groups with higher black bean content, these groups had fewer and different aroma components.

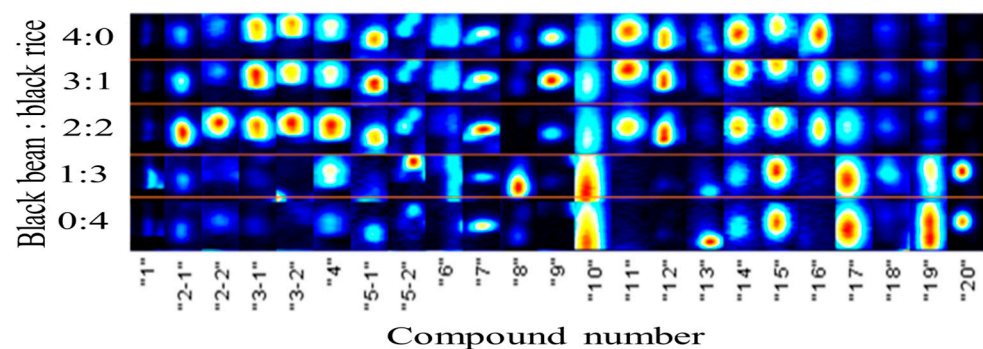


Figure 5. The characteristic aroma fingerprints of *Tuber magnatum* solid-state fermented products using different ratios of black bean and black rice as media.

Figure 6 shows the principal component analysis (PCA) of the selected characteristic volatile compounds performed using the software provided with the GC-IMS. The aim was to explore the aroma differences in the white truffle solid-state fermentation products with different ratios of black beans and black rice substrates. The figure shows that when the proportion of black beans was higher, the data points fell in the third quadrant. When the proportion of black rice was higher, the points were located in the first and fourth quadrants. Due to its more balanced aroma components, the 2:2 ratio of black beans to black rice fell in the second quadrant. Comparing these results with the GC-MS data, it was evident that the 4:0 and 3:1 ratios of black beans to black rice have higher total sulfur compound content but higher amounts of individual sulfur compounds, resulting in a less rich overall aroma. The

2:2 ratio had moderate intensity and a more balanced distribution of aroma components. The 1:3 and 0:4 ratios had lower aroma content and richness. The results from GC-IMS and GC-MS are similar. Therefore, considering the intensity and richness of the aroma, the 2:2 ratio of black beans to black rice, with the addition of methionine, was the most suitable medium ratio for white truffle fermentation.

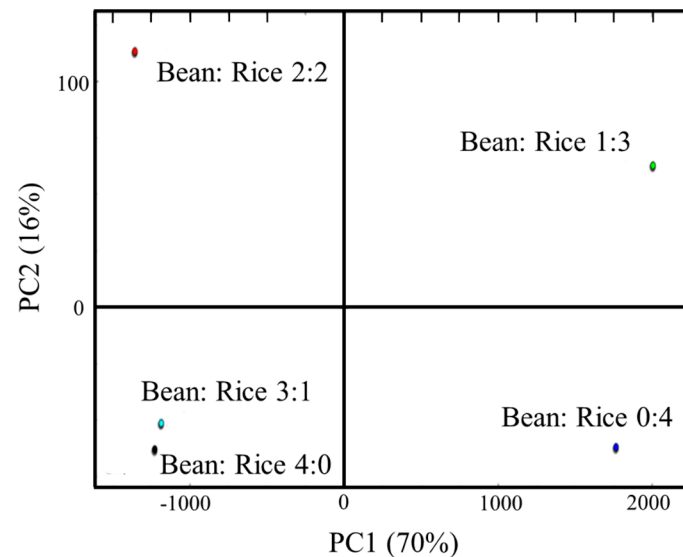


Figure 6. Principal component analysis of *Tuber magnatum* solid-state fermented products using different ratios of black bean and black rice as media.

3.2. Effect of Methionine Addition in a Medium on the Aroma of White Truffle Solid-State Fermented Products

Vahdatzadeh and Splivallo [9] demonstrated that adding 20 mM of methionine to the liquid fermentation substrate of *T. borchii* significantly increased the content of DMDS. Splivallo and Maier [22] also mentioned in their patent that adding 50 mM methionine can induce the production of a large amount of DMDS in black truffles. Therefore, the addition of methionine affects the generation of sulfur-containing aroma compounds through the Ehrlich biosynthesis pathway. From the previous results, it was known that a 2:2 ratio of black beans to black rice with 1.5% methionine was an optimal condition for the solid-state fermentation of white truffles. Although the aroma of the group with 1.5% methionine was superior to the group without methionine, the mycelium growth was poorer. To improve mycelium growth, the methionine addition levels were adjusted to 0.3%, 0.6%, 0.9%, 1.2%, and 1.5%, and growth conditions and aroma components were observed and analyzed. Table 2 shows the 4-week growth conditions with different concentrations of methionine. The mycelium growth area increased with time, and compared to the original 1.5% methionine addition, reducing the addition amount increased the mycelium growth area.

Table 2. *Tuber magnatum* 4-week solid-state fermentation using 1:1 black bean and black rice as medium with different concentrations of Met (0.3, 0.6, 0.9, 1.2, and 1.5%).

Met (%)	0.3	0.6	0.9	1.2	1.5
Pictures					

Further aroma analysis was conducted using GC-IMS. Figure 7 shows the fingerprint profiles of white truffle fermentation products at different methionine concentrations. Characteristic aromas were selected for comparison (Figure 8). The fingerprint profiles of the group without methionine fell within numbers 12 to 21, while the profiles of the groups with methionine addition fell within numbers 1 to 11. Notably, the group with 0.3% methionine also included numbers 22 to 35, indicating a richer aroma profile.

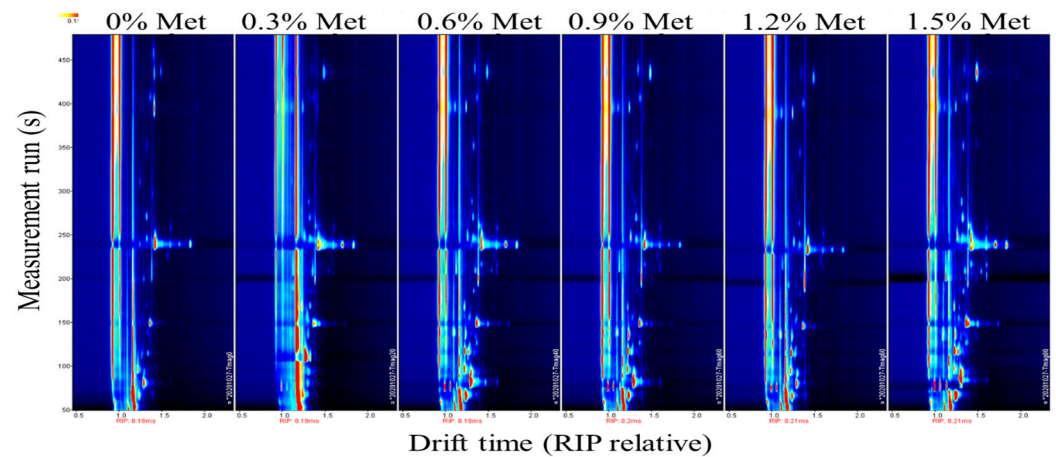


Figure 7. Aroma fingerprints of *Tuber magnatum* solid-state fermented products using different concentrations of methionine in media.

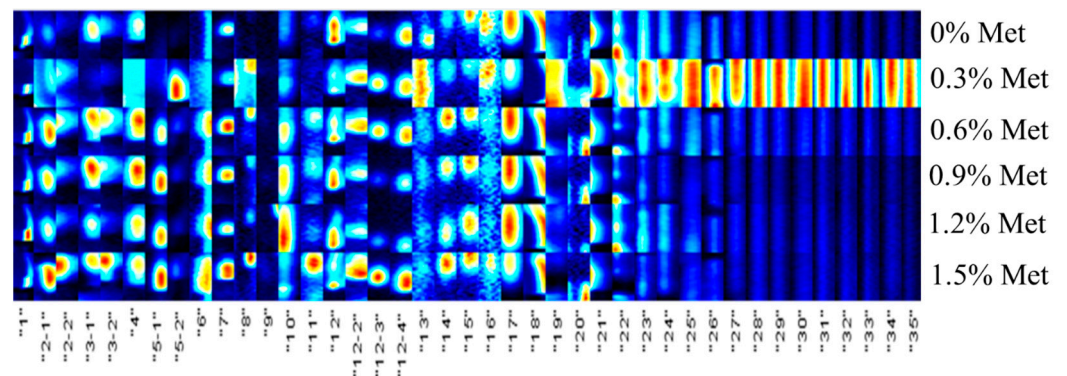


Figure 8. The characteristic aroma fingerprints of *Tuber magnatum* solid-state fermented products with different concentrations of methionine in media.

To confirm that these aroma components were indeed metabolized during fermentation, a medium with a 2:2 ratio of black beans and black rice was prepared. Different concentrations of methionine were added, followed by sterilization, but no fermentation was conducted. The aroma was then measured (Figure 9), where the numbers indicated the methionine concentration (%). Comparing these aroma fingerprints with those of the fermented products revealed significant differences in the fingerprint profiles (Figure 10).

Overall, the aroma fingerprints before and after solid-state fermentation of white truffle were not the same. Further PCA analysis showed that the overall PCA results before and after fermentation were completely different. Post-fermentation samples with 0.3% methionine addition showed distinct differences from other groups (Figure 11a,b). This indicated that the aroma formed solely through the Maillard reaction with methionine addition differs from the aroma produced after one month of white truffle solid-state fermentation.

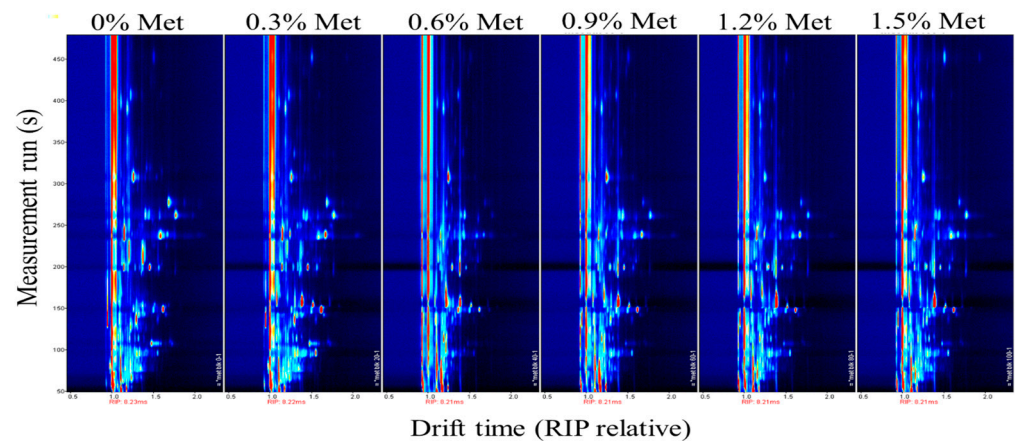


Figure 9. Aroma fingerprints of unfermented products with different concentrations of methionine in media.

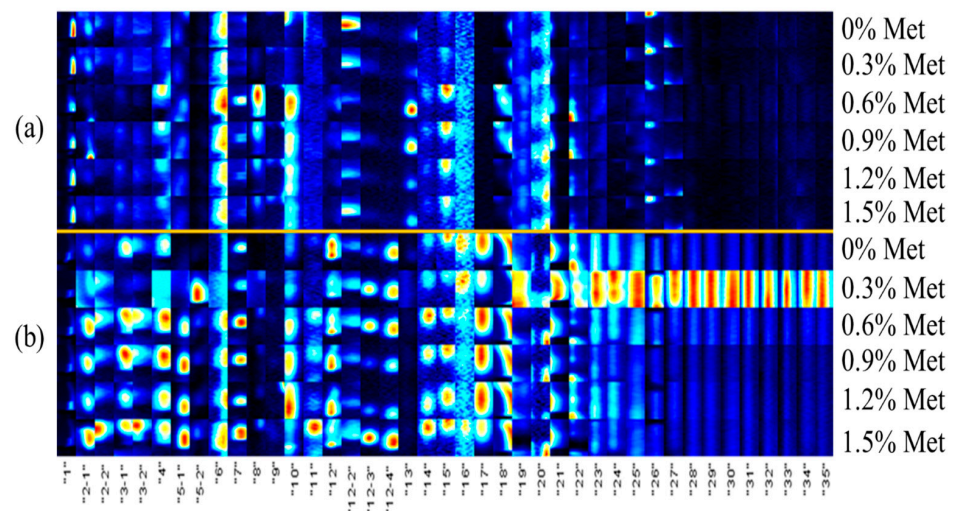
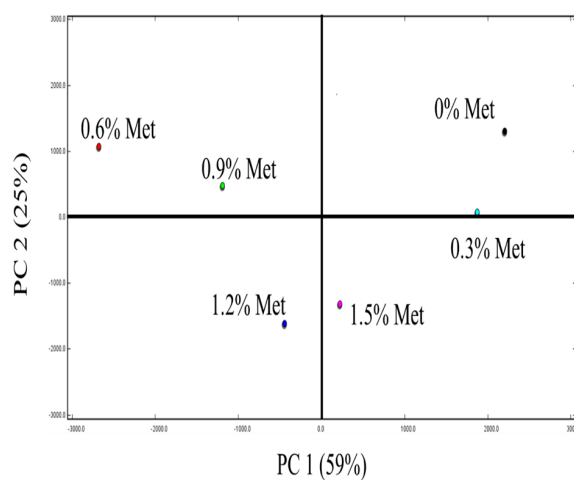
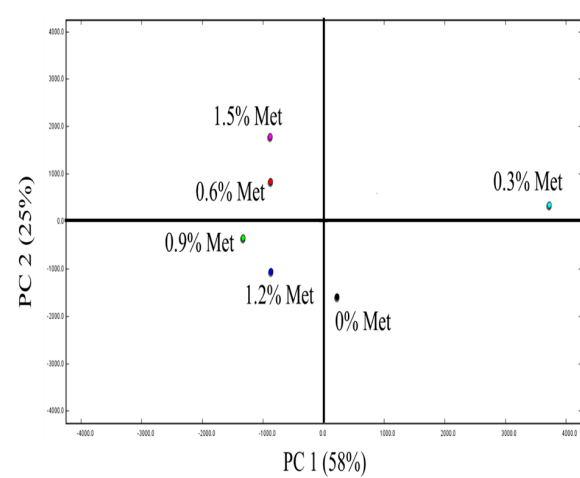


Figure 10. The characteristic aroma fingerprints of (a) before and (b) after *Tuber magnatum* solid-state fermented products with different concentrations of methionine in media.



(a)



(b)

Figure 11. Principal component analysis of (a) before and (b) after *Tuber magnatum* solid-state fermented products with different concentrations of methionine in media.

3.3. Effect of Sterilization and Freeze Drying on the Aroma of White Truffle Solid-State Fermented Products

White truffle solid-state fermentation substrates were prepared using a 2:2 ratio of black beans to black rice, with moisture content controlled at 40%. Methionine at a concentration of 0.3% was added as a precursor for the fermentation process. After four weeks of cultivation, three different sterilization methods (high pressure, autoclaving, and radiofrequency) were used to terminate the fermentation reaction. The samples were then dried using traditional freeze-drying and analyzed for aroma using GC-IMS. Figure 12a,b illustrate the impact of different sterilization methods and then freeze drying on the aroma of white truffle solid-state fermentation products. Overall, it was found that the HPP sterilization fingerprint profile was closer to that of fresh products, while the aroma profiles of autoclave and radiofrequency sterilization were more similar to each other.

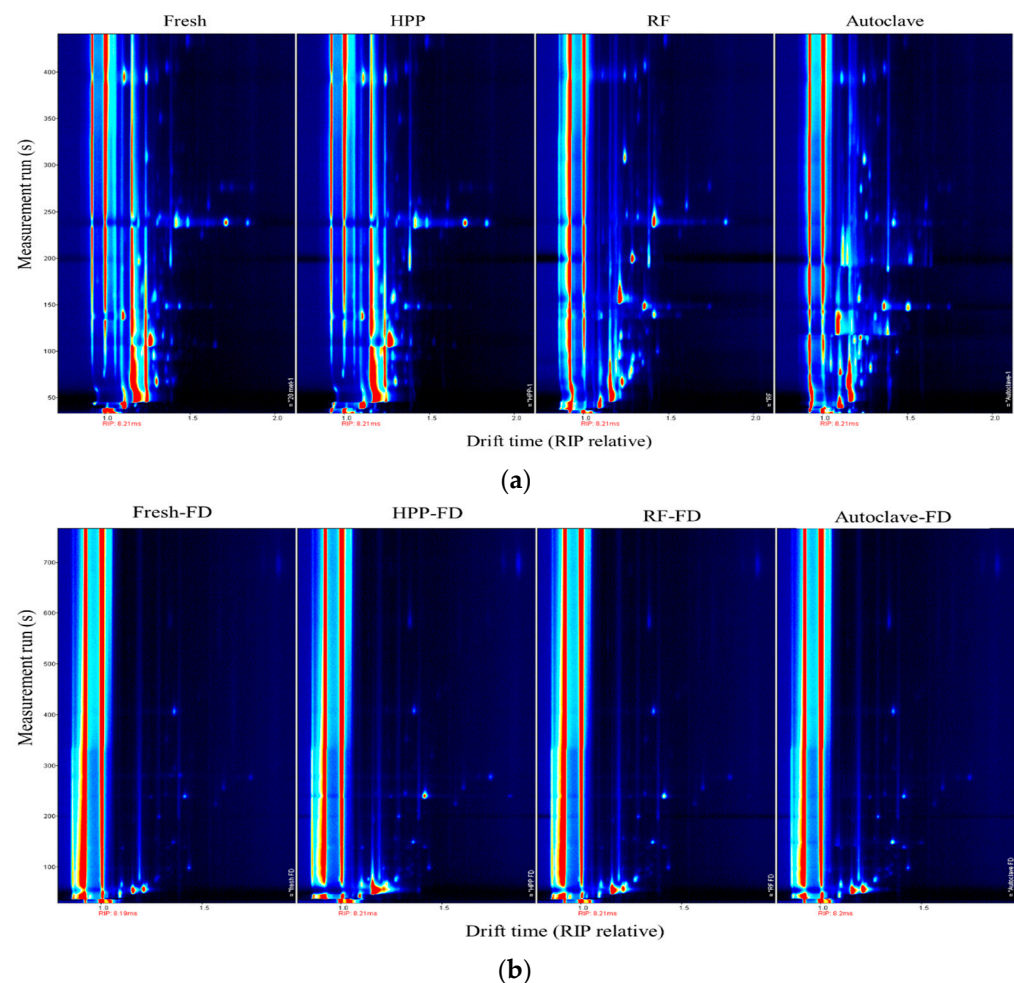


Figure 12. Aroma fingerprints of different pasteurization methods and their freeze-dried (a) before and (b) after *Tuber magnatum* solid-state fermented products.

To further understand the differences in these profiles, specific points from Figure 12 were selected to form characteristic aroma fingerprint profiles (Figure 13). Each number represents a compound, and if a number includes a “-” symbol, it indicates that the compound was detected at the same GC time but different drift times. To understand the changes in aroma after sterilization, the aroma of fresh samples was used as the baseline, and the selected points were applied to each treatment group for comparison. It was more evident that the aroma after HPP sterilization was almost identical to the fresh, non-sterilized samples, while the aromas of radio frequency and autoclave sterilization were more similar to each other. This indicates that heating causes changes in the aroma.

Since HPP is a non-thermal process, it does not generate a large amount of heat during sterilization, causing minimal damage to the aroma, thereby maintaining the original aroma of the solid-state fermentation products. Both radio frequency and autoclave sterilization are thermal processes that lead to Maillard reactions during heating, resulting in significant changes in aroma components. Compounds numbered 22–35 disappeared due to the heat during thermal sterilization.

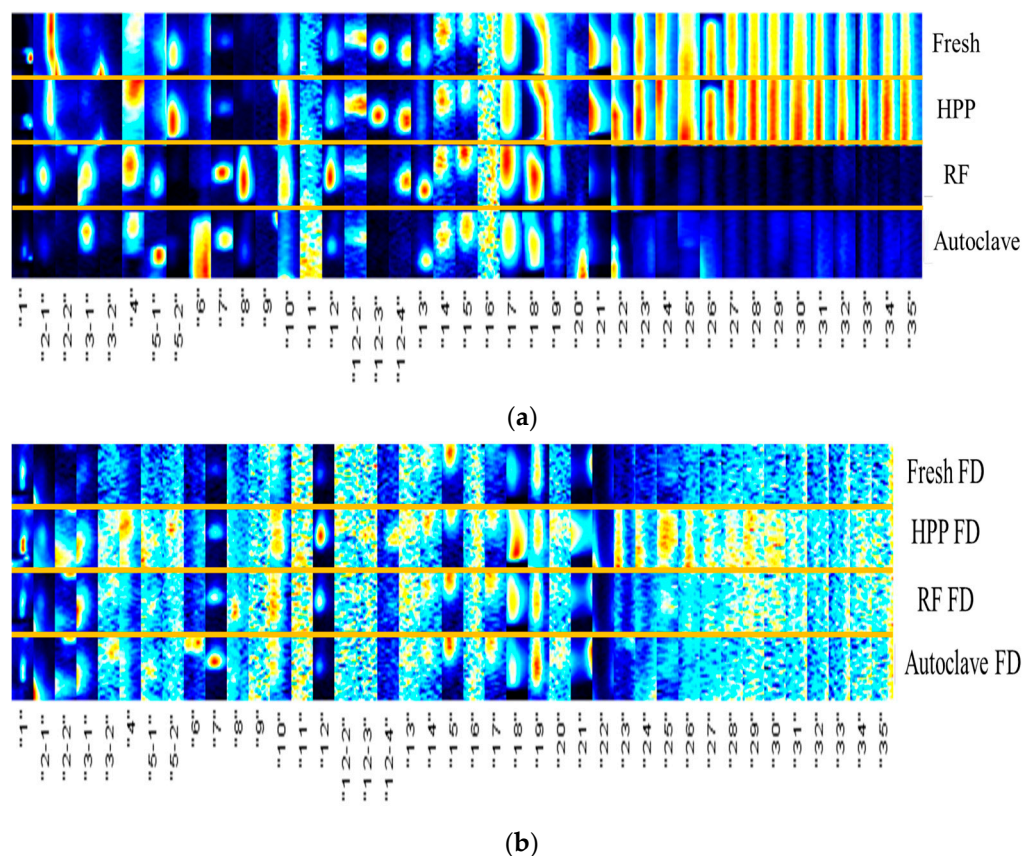


Figure 13. (a) Before and (b) after characteristic aroma compound fingerprints of *Tuber magnatum* solid-state fermented products by different pasteurization methods and freeze-drying.

Verret et al. [11] treated black truffle fungi at 4 °C using 300 and 550 MPa for 10 min. The levels of ethanol, 2-methyl-1-propanol, 1-hexanol, dimethyl sulfide, and 2-butanone decreased compared to the untreated group. However, the contents of 1-octen-3-ol and benzaldehyde increased after HPP treatment. Overall, HPP treatment produced results closer to those of fresh samples compared to thermal sterilization.

Therefore, after treating the samples with high-pressure processing, the aroma components exhibited varying degrees of change, with no absolute good or bad results, as the outcomes differed under different operating conditions. However, most studies used GC-MS for aroma component analysis. While GC-MS can achieve qualitative and quantitative analysis, it cannot reveal the overall differences in aroma components. In this experiment, GC-IMS was used for analysis. The results showed that HPP caused slight losses in the aroma, and the overall aroma fingerprint was closer to that of fresh samples. In contrast, the thermal sterilization process caused more evaporation losses of volatile compounds, resulting in a significant difference compared to the fresh state.

Figure 12b shows the aroma fingerprint profile after freeze-drying, which indicates a significant decrease compared to before drying (Figure 12a). When applying the aroma template of fresh samples to compare different treatments (Figure 13b), it was found that the aroma fingerprints representing fresh samples almost disappeared after drying, with only compounds numbered 15 and 18 remaining. Therefore, whether thermal or non-thermal

sterilizations were applied, traditional freeze-drying for 24 h resulted in a significant loss of aroma compounds.

Kompany and René [23] indicated that when the chamber pressure dropped from 50 Pa to 5 Pa at the same operating temperature, the aroma retention rate of mushrooms (*Agaricus bisporus*) increased, illustrating that vacuum application affects aroma. In addition to vacuum levels influencing aroma loss after freeze-drying, the freezing process itself can also lead to aroma loss. Culleré et al. [24] analyzed the aroma compounds of black truffle after frozen storage using GC-MS and found a decrease in aroma compounds, which was similar to the results obtained using GC-IMS in this experiment. Campo et al. [4] also discovered that the aroma of truffle fruiting bodies after freeze-drying was closer to fresh fruiting bodies when analyzed by GC-O. However, further sensory description and analysis revealed that the intensity of the typical truffle aroma decreased after freeze-drying. Feng et al. [18] pointed out that yak milk, when processed into milk powder via freeze-drying after sterilization, had fewer aroma types compared to spray-dried milk powder. GC-IMS analysis revealed significant differences, consistent with the trend observed in this experiment.

Further principal component analysis (Figure 14) reveals that autoclave and radiofrequency sterilization were located in the first quadrant, while fresh samples and HPP were in the fourth quadrant. All freeze-dried samples were clustered tightly in the third quadrant. This indicated that the aroma components of white truffle solid-state fermented products were distinctly affected by whether heat was generated during processing when not sterilized. However, there were no significant differences after freeze-drying. It is speculated that although aroma compounds may increase or decrease due to heat during sterilization, traditional freeze-drying involves heating shelves to cause sublimation under vacuum, which, due to increased heat transfer resistance as the surface dries, significantly extends the drying time. Aroma compounds tend to sublime more easily than ice crystals, leading to aroma loss, which results in all sterilization treatment groups losing their aroma after drying with no significant differences.

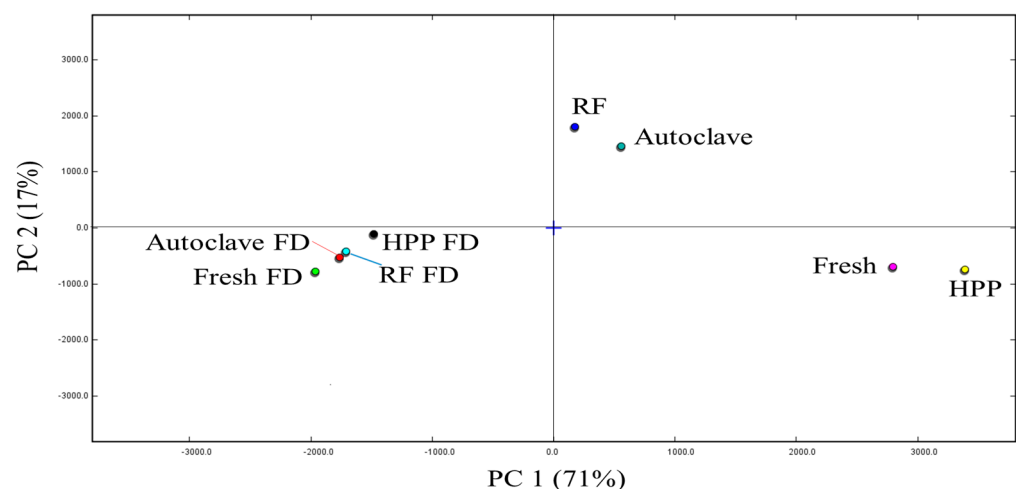


Figure 14. Principal component analysis of *Tuber magnatum* solid-state fermented products using different pasteurization methods and freeze-drying.

Pei et al. [25] combined freeze-drying with microwave vacuum drying and found that traditional freeze-drying of button mushrooms (*Agaricus bisporus*) required 8 h to reduce moisture to 4.19%. However, switching to 60 W/g microwave vacuum drying after 5 h of freeze-drying reduced moisture to 2.74% in 5.15 h. GC-MS and electronic nose analyses indicated that the aroma of the microwave vacuum-dried group was closer to that of fresh samples, thereby saving time and preserving the aroma. In addition, compared to cold air drying, using 30 W microwave freeze-drying for white truffle solid-state fermentation products reduced the traditional freeze-drying time from 24 h to 2 h. During the drying

process, a constant rate drying period was maintained. In terms of aroma retention, cold air drying and microwave freeze-drying reduced the retention rate of DMDS to 28.8% and 53.9%.

4. Conclusions

Using a 2:2 ratio of black beans to black rice, with the addition of 0.3% methionine and a moisture content controlled at 40%, four weeks of solid-state fermentation of white truffle was conducted at 25 °C. The GC-IMS analysis results indicated that this medium produced the richest aroma in the solid-state fermented products. Subsequently, sterilization using HPP (500 MPa, 5 min) was performed to terminate the fermentation reaction. Comparing characteristic aroma fingerprint profiles and PCA analysis showed that the aroma of the HPP-treated samples was most similar to that of fresh samples, better preserving the aroma. In contrast, radio-frequency heating and autoclave thermal sterilization methods produced similar results due to heat-induced loss or generation of other compounds. However, regardless of the sterilization methods, subsequent freeze-drying for 24 h resulted in all samples falling within the same quadrant in PCA analysis, indicating a significant decrease in aroma.

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