Main parameters and the dynamics of volatile compounds during the fermentation of Chinese *Mao-tofu* from Huangshan region

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Abstract The physico-chemical parameters and kinetics of the volatile compounds during the fermentation of Chinese *Mao-tofu* was investigated. The levels of water soluble proteins, amino nitrogen and acidity increased gradually throughout the fermentation process, while that of reducing sugars peaked after 2 days before gradually declining. The protease activity increased significantly and reached the maximum levels at day 3, then it slightly decreased. The hardness and firmness decreased during the first 3 days, but both increased subsequently. The viscosity and adhesiveness was inversely related to the hardness and firmness. Forty-four volatile compounds were identified and quantified. Correlation analysis revealed significant correlation of the content of water soluble proteins and amino nitrogen with most volatile compounds. Acidity levels were positively correlated with that of ethyl caproate and hexyl alcohol, and negatively with 2-methyl butyric acid, palmitic acid, ethyl laurate, 1-octen-3-ol and 1-nonanol. Reducing sugar content was also positively correlated with ethyl caproate.

Keywords *Mao-tofu* · Fermentation · Headspace solid phase microextraction · Volatile flavor compounds

Introduction

Soybean, a species of legume, is a natural source of high quality plant-derived protein, and contains a higher amount of dietary fiber and isoflavones compared to other legumes (Mamta et al., 2015). Proteins account for 40% of the dry weight of soybeans, while fats (soybean oil), soluble and insoluble (dietary fiber) carbohydrates and ash constitute the remaining 20%, 35% and 5% respectively (Jooyandeh, 2011). In east Asian countries, soybeans are routinely consumed as soymilk, soy cheese or tofu, and fermented products like miso, soy sauce, tempeh, *sufu* and *Mao-tofu*, a soft textured tofu with cheese-like consistency and a distinctive flavor. *Mao-tofu* originated in China, and is a highly nutritious and easily digested fermented soy-product that has been consumed for centuries in the Huangshan region as a delicious and healthy snack. Recent studies have shown anti-oxidative, anti-mutagenic, antihypertensive and hypo-cholesterolemic functions of *Mao-tofu* (Hang and Zhao, 2011), and have largely attributed them to the isoflavones (Chung et al., 2011).

In Huangshan, *Mao-tofu* is traditionally produced by the fungal solid-state fermentation of tofu. Freshly prepared tofu is inoculated with a starter mold culture, which can either be a pure culture suspension or the mixed flora from utensils. The inoculated tofu is incubated at 12–30 °C for 2–7 days, after which white mycelium-covered fermented pieces of tofu, now called “*Mao-tofu*”, are obtained. During fermentation, several proteolytic, lipolytic and other enzymes produced by the molds degrade the proteins, starch, lipids etc. of the tofu, and thus soften its texture and

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help develop the flavor. Therefore, the fermentation parameters influence the quality of the final product. Studies conducted on *Mao-tofu* production (Li and Wang, 2014) have mostly focused on the fermentation technology, while no study has been published so far on the physico-chemical factors affecting the process. In addition, no reports are available regarding the volatile compounds, as well as their physicochemical and biochemical properties during the fermentation process.

The aim of the present study was to determine the changes in the physicochemical parameters during the natural Huangshan fermentation process of producing *Mao-tofu*. In addition, the volatile compounds produced during fermentation were isolated and characterized by solid phase microextraction-gas chromatograph-mass spectrometry (SPME-GC-MS), and their correlation with the fermentation parameters were also analyzed. Our findings can improve our understanding of *Mao-tofu* production, and help design a more efficient fermentation process.

Materials and methods

Fermentative production of *Mao-tofu* and sampling

Tofu fermentation was carried out in an industrial scale manufacturing facilities (Anhui Wanwan Xiang soybean processing Co. Ltd., Huainan, China) during the month of November 2016, which is the season of *Mao-tofu* production. After thorough cleaning, the soybeans were soaked in an adequate amount of water for 12 h at room temperature (25 °C). The hydrated soybeans were filtered and blended with 6 times the amount of water (w/w). The mixture was then ground and filtered to obtain soy milk, which was heated in a water bath at 95 °C with continuous stirring for 10 min, and cooled to 80 °C. To prepare the tofu, MgCl_2 solution (0.2% w/v) was added to the hot soymilk, which was stirred for 5 min and then left undisturbed for 15 min to coagulate the proteins. The coagulated milk was then filtered through cheese cloth placed over tofu casts, pressed into sheets and cut into roughly 2 cm $\times$ 2 cm $\times$ 2 cm pieces. The tofu pieces were inoculated with a pure culture of *Mucor rouxianus* (CICC 3154), and incubated at 20 °C for 4 days. The relative humidity was maintained at $\sim$ 90%, and air was circulated to ensure proper aeration. After 4 days of incubation, the mycelium-covered *Mao-tofu* pieces were obtained.

On days 0, 1, 2, 3 and 4 at the end of the shift, ten tofu blocks each were randomly taken from the top, middle and bottom layers for sampling. The blocks were ground to powder in an alcohol disinfected grinder, and

approximately 20 g of each powdered sample was sealed into sterile bags, and stored at -20 °C until analysis.

Physio-chemical analysis

The total acidity of the fermented samples was measured by the alkali titration method, using acetic acid as the indicator (Fang and Zhang, 2012). The content of reducing sugars was measured using Fehling's test (Tang et al., 2013), while that of water soluble proteins was determined by the Kjeldahl method. The total protein content was calculated by multiplying the nitrogen content with 6.25 (AOAC, 2000). Protease activity was measured by the Folin-phenol method in pH 9.7 buffer (Zhen et al., 2013), using casein as the substrate and tyrosine release as the indicator. The total amount of free amino acids was determined by the ninhydrin method (Rosen, 1957).

Texture profile analysis (TPA) of *Mao-tofu* was conducted using the TA-XT plus texture analyzer (Micro Stable System, Godalming, UK) to evaluate textural properties like firmness, hardness, adhesiveness and viscosity. The *Mao-tofu* pieces were diced into 2 $\times$ 2 $\times$ 2 cm³ cubes, and compressed with a cylinder plunger (ϕ 35-mm) at the constant speed of 2 mm/s for 3 s, at 5 g trigger force and 45% deformation.

Extraction of volatile flavor compounds by HS-SPME

Headspace solid-phase micro-extraction (HS-SPME) was used to extract the volatile compounds with a 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber (2 cm, Supelco, Inc., Bellefonte, USA) according to the procedure described by Zheng et al. (2013). One gram of each *Mao-tofu* sample was put in the headspace bottle along with 100 μl 70 mg/L 2-octanol in absolute ethanol as the internal standard. After tightly capping the vials, they were equilibrated by ultrasonic vibration in a water bath at 60 °C for 10 min. An extraction head was then inserted into each vial, and the sample was extracted at 60 °C for 30 min. Finally, the extraction was analyzed using GC-MS system.

Gas chromatography-Mass spectroscopy (GC-MS) analysis

After HS-SPME, the extraction head was inserted into the injection port of the GC-MS system (Agilent 6890 GC and Agilent 5975 mass selective detector (MSD); Agilent, San Diego, USA) to separate and analyze the different compounds in the extracts. The extracts were thermally desorbed from the DVB/CAR/PDMS fiber at 250 °C (temperature of the injector and detector) for 3 min, and

then separated through a DB-Wax column (60 m length, 0.25 mm internal diameter, 0.25 µm film thickness) using helium as the carrier gas at a constant flow rate of 1 ml/min. The temperature program of GC was set as follows: 40 °C for 1 min, increased by 5 °C/min to 180 °C for 1 min, and again increased by 8 °C/min to 230 °C for 7 min. The individual compounds were then identified using the MSD using the following settings: electron impact energy 70 eV, ion source temperature 230 °C, and quadrupole temperature 230 °C. The electron impact (EI) MS was recorded for compounds ranging from 20–550 atomic mass units (amu). Each sample was analyzed in triplicates.

Identification and quantitative analysis of volatile compounds

The mass spectrum data were compared against the NIST 05a MS database (Agilent Technologies, Inc. Santa Clara, California, USA) to identify the volatile flavor compounds in *Mao-tofu* samples, and were quantified by comparing their peak areas to that of the 2-octanol internal standard. The amount of each compound was performed using the following formula:

$$C (\mu\text{g/g}) = \frac{A_c}{A_{is}} \times C_{is} (\mu\text{g/g})$$

C , the relative concentration of analyte; C_{is} , the concentration of internal standard in samples; A_c , peak area of analyte; A_{is} , peak area of internal standard. The results were expressed as the mean value of three replicates.

Statistical analysis

The MS data were analyzed using the SPSS version 21.0 statistical package for windows (SPSS Inc., Chicago, USA). Principal component analysis (PCA) was used to determine the differences between *Mao-tofu* samples.

Results and discussion

Content of water soluble proteins and amino nitrogen during *Mao-tofu* fermentation process

Water soluble proteins and amino nitrogen are the main nutrients of Huangshan *Mao-tofu* and therefore affect its quality. As shown in Fig. 1(A), the unfermented tofu had the lowest water soluble nitrogen content of 1.36%. With fungal growth and metabolism during fermentation, protease levels gradually increased which degraded the proteins into peptones, peptides, amino acids and other intermediates for microbial growth. Therefore, the overall

content of water soluble proteins increased as the fermentation progressed. Similarly, the amino nitrogen content also increased during fermentation (Fig. 1(A)) due to the fungal protease mediated hydrolysis of tofu proteins. *M. rouxianus* is a major producer of proteases that hydrolyze soy protein into peptides. In addition, the concentration of water soluble proteins was significantly positively correlated with the amino nitrogen content, with a correlation coefficient of 0.918. Therefore, amino nitrogen content could be used as an index for measuring the amount of water soluble proteins produced during soybean protein hydrolysis by *M. rouxianus* proteases.

Fermentation often results in the production of bio-active or functional compounds from the raw food substrates, which are easier to digest and absorb. Although soybeans can provide abundant nutrients for the human body, its utilization rate is comparatively low. In addition, unfermented soybeans have been known to result in diarrhea and flatulence. However, during fermentation of soybean products like tofu, the proteins are decomposed into low-molecular-weight polypeptides and peptones, which are more easily digested and utilized, and are also non-toxic. Furthermore, fermented soybean products have stronger anti-oxidative, anti-neoplastic and anti-metastatic properties compared to raw soybeans and their non-fermented products (Jiang et al., 2011). Studies have also reported significant therapeutic effects of fermented soybean products in hypertension, diabetes and inflammatory conditions, in addition to immune functions (Hang and Zhao, 2011). The strong umami taste of *Mao-tofu* is attributed to its amino nitrogen content, as well as various amino acids like glutamic acid. An increase in amino nitrogen content greatly improves the flavor of *Mao-tofu*.

Protease activity during the fermentation process

The protease activity also increased significantly as fermentation progressed, peaking at 3.68 U/g *Mao-tofu* on day 3 (Fig. 1(B)). This was likely due to the rapid proliferation of *M. rouxianus* during the early days of fermentation, as the nutrients are relatively abundant, and the temperature and humidity are also optimum at this stage. However, after 3 days of fermentation, the protease activity slightly decreased due to aging of the fungal mycelium, as well as the high acidity of the medium which inhibited protease production and activity.

The fungi used in *Mao-tofu* production are harmless and enzymatically break down food macromolecules into simpler, non-toxic compounds using proteases, lipases and amylases (Yan et al., 2011). *Mucor spp* are the most commonly used fungi in *Mao-tofu* production, due to the high levels of protease this fungi produces. Furthermore, the mycelia of *Mucor spp* surrounding the tofu act as a

Fig. 1 Changes in water soluble protein and amino nitrogen (A), protease activity (B), acidity (C) and reducing sugar (D) during *Mao-tofu* fermentation process

layer of protective film which helps retain the shape of the *Mao-tofu*. Therefore, the strain of *Mucor* used for fermentation affects the quality of the final product. Apart from *Mucor*, *Bacillus spp* and lactic acid bacteria are also used for *Mao-tofu* production, and are associated with substrate utilization, flavor enhancement, probiotic activities, and food preservation (Zou et al., 2008).

Acidity levels during the fermentation process

The acidity of *Mao-tofu* is mainly due to the organic acids, free amino acids and fatty acids that are produced during the fermentation process. As shown in (Fig. 1(C)), the total acidity of *Mao-tofu* increased as fermentation progressed, peaking at 0.32 g/100 g *Mao-tofu* on the fourth day. As already described, the rapid fungal proliferation during this period led to large amounts of secreted proteases, glucoamylases and lipases, which respectively degraded the proteins, carbohydrates and fats into organic acids, free amino acids and fatty acids. The organic acids not only give *Mao-tofu* its distinctive flavor but are also converted

to esters, which further increase the aroma of the fermented product. The free amino acids, especially glutamate and aspartic acid, are the main sources of *Mao-tofu* flavor, and higher content of free amino acids greatly improves the flavor. Fatty acids present in *Mao-tofu* can also be esterified with ethanol when *Mao-tofu* is further fermented into bean curd. Although high acidity improves flavor, too much acidity is not only disadvantageous to the preservation of the product but also affects the flavor. In China, the maximum acceptable acidity of fermented bean curd originating from *Mao-tofu* is 1.30 g/100 g (Anonymous, 2011).

Content of reducing sugars during the fermentation process

The reducing sugar content of *Mao-tofu* gradually increased during fermentation, and reached the maximum level of 2.53 g/100 g *Mao-tofu* on day 2, followed by a steady decline (Fig. 1(D)). The initial increase may be due to the breakdown of tofu starches by the glucoamylases. As the fungi proliferate, the reducing sugars become the major

source of carbon and energy. In addition, the reducing sugars are also involved in the production of amino acids such as glutamic acid, which lead to a decline in their content after 2 days of fermentation.

The total carbohydrate content in soybeans is about 25–30% and mainly includes starch, oligosaccharides and dietary fiber, of which the oligosaccharides account for 10% of the total carbohydrates. The major sugars of soybean oligosaccharides are raffinose and sucrose, which cannot be directly digested and absorbed by the human body. However, the fungal enzymes such as α -amylase, glucoamylase and α -galactosidase eventually decompose the polysaccharides into dextrin and oligosaccharides, which are further broken down into glucose and other easily utilized sugars.

Textural changes in *Mao-tofu* during the fermentation process

Soon after inoculation of the *Mucor* starter culture, the fungi start to colonize and the mycelium spread over the surface of *tofu*. This fungal growth, along with the degradative action of the fungal enzymes greatly influences the physical characteristics of *Mao-tofu*. We therefore studied the changes in the physical properties of Huangshan *Mao-tofu* during the fermentation (Fig. 2).

The hardness and firmness of the tofu substrate decreased during the initial 3 days of fermentation, and both subsequently increased till the end of the incubation period. In contrast, the viscosity and adhesiveness increased during the first 3 days of the fermentation, reaching the highest values of 1001.2 J and 95.87 g respectively, after which both declined till the end of the process. The hardness and firmness of *Mao-tofu* were significantly correlated during the course of fermentation, with Pearson correlation coefficient of 0.982. Similarly, a high positive correlation (coefficient 0.962) was seen between viscosity and adhesiveness.

Fig. 2 Changes in hardness, firmness, stickiness and adhesiveness during *Mao-tofu* fermentation process

Identification and quantification of volatile compounds in Huangshan *Mao-tofu*

The Huangshan *Mao-tofu* has a distinctive flavor due to its unique fermentative process, which sets it apart from that produced in other regions (Li and Wang, 2014). The compounds that contribute to this flavor are produced as a result of various enzymatic reactions during fermentation. Five *Mao-tofu* samples were profiled by HS-SPME/GC-MS analysis, making this the first study to characterize the volatile fraction formed during Huangshan *Mao-tofu* fermentation.

Forty-four volatile compounds, including 9 esters, 9 acids, 8 alcohols, 5 aldehydes, 4 alkenes, 3 ketones, 2 benzenes, 2 phenols, 1 furan and 1 indole were identified and quantified in Huangshan *Mao-tofu* (Table 1). The total amount of the volatile compounds ranged from 3.156 to 16.016 $\mu\text{g/g}$ *Mao-tofu* across the samples, and alcohols and acids were the dominant compounds in all the samples during fermentation. Most volatile compounds were identified on the third and fourth days of fermentation, 46 different compounds for each time point, whereas only 19 compounds were detected in the tofu samples prior to fermentation. The majority of the compounds identified in our study had been reported previously for other fermented foods such as soy sauces, doenjang and Korean soybean paste (Kim et al., 2012; Yoo et al., 2000; Zheng et al., 2013).

Nine acids were identified in Huangshan *Mao-tofu*, and were the most abundant compounds seen during the fermentation process with total concentration ranging from 1.071 to 6.81 $\mu\text{g/g}$ *Mao-tofu*. Acetic acid was the most abundant among these nine acids, followed by butyric acid and propionic acid. Studies have shown that the presence of organic acids in fermentation medium inhibits the growth of contaminating bacteria, increases the mellowness and aftertaste, and enhances the flavor of the fermented products (Ledauphin et al., 2003). Therefore, acidic compounds have an important influence on the savory characteristics of Huangshan *Mao-tofu*.

Esters formed during the process of fermentation, either via the chemical esterification of alcohols and fatty acids or by de-novo microbial synthesis using alcohol acetyltransferase on acetyl-CoA and alcohol substrates (Ledauphin et al., 2003), bestow floral or fruity odors to many fermented foods (Zheng et al., 2013). Nine esters were identified in Huangshan *Mao-tofu*, which increased sharply during fermentation and peaked on the fourth day. The major esters were hexyl acetate, ethyl oenanthate, ethyl laurate, ethyl palmitate and ethyl oleate, of which hexyl acetate was most abundant ester (24%) post fermentation.

Eight different alcohols were identified, of which 1-octen-3-ol, phenylethyl alcohol and enanthol were the

Table 1 The volatile aroma compounds identified and quantified during Huangshan *Mao-tofu* fermentation

Number	Aroma compounds	Retention time (min)	Identification	Contents of volatile aroma compounds in 5 samples/(μg/g)				
				0 days	1 days	2 days	3 days	4 days
Acids								
AC1	Acetic acid	11.307	MS, RI	1.028 ± 0.012	1.503 ± 0.113	2.70 ± 0.232	3.182 ± 0.383	5.139 ± 0.075
AC2	Pentanoic acid	17.919	MS, RI	0.016 ± 0.008	0.018 ± 0.002	0.020 ± 0.003	0.041 ± 0.002	0.044 ± 0.005
AC3	Caproic acid	20.171	MS, RI	0.027 ± 0.083	0.035 ± 0.005	0.047 ± 0.009	0.071 ± 0.002	0.075 ± 0.006
AC4	Butyric acid	15.484	MS, RI	ND	ND	0.014 ± 0.003	0.220 ± 0.033	0.613 ± 0.078
AC5	Propionic acid	13.452	MS, RI	ND	0.012 ± 0.003	0.093 ± 0.002	0.395 ± 0.031	0.412 ± 0.072
AC6	2-Methyl butyric acid	31.059	MS, RI	ND	0.045 ± 0.005	0.060 ± 0.009	0.081 ± 0.005	0.085 ± 0.007
AC7	3-Methyl butanoic acid	16.424	MS, RI	ND	ND	0.017 ± 0.003	0.097 ± 0.006	0.155 ± 0.003
AC8	Palmitic acid	27.672	MS, RI	ND	0.067 ± 0.006	0.146 ± 0.063	0.258 ± 0.014	0.261 ± 0.014
AC9	9-Decyl acetic acid	14.96	MS, RI	ND	ND	0.014 ± 0.002	0.025 ± 0.002	0.026 ± 0.006
	Σ			1.071 ± 0.051	1.680 ± 0.008	3.111 ± 0.171	4.370 ± 0.018	6.81 ± 0.013
Esters								
ES1	Ethyl caproate	5.617	MS, RI	0.217 ± 0.013	0.102 ± 0.015	0.035 ± 0.004	0.059 ± 0.008	0.048 ± 0.018
ES2	Hexyl acetate	6.525	MS, RI	0.075 ± 0.008	0.161 ± 0.013	0.214 ± 0.013	0.327 ± 0.009	0.354 ± 0.018
ES3	Ethyl oenanthate	7.996	MS, RI	ND	ND	0.022 ± 0.004	0.024 ± 0.003	0.129 ± 0.017
ES4	Ethyl laurate	13.107	MS, RI	ND	0.010 ± 0.005	0.082 ± 0.003	0.095 ± 0.006	0.105 ± 0.013
ES5	1-Cyclopentene-1-carboxylic acid, methyl ester	14.468	MS, RI	ND	ND	ND	0.014 ± 0.002	0.066 ± 0.014
ES6	Ethyl palmitate	26.672	MS, RI	ND	ND	ND	0.106 ± 0.011	0.165 ± 0.016
ES7	Ethyl oleate		MS, RI	ND	ND	ND	0.100 ± 0.013	0.164 ± 0.023
ES8	Ethyl linoleate	32.094	MS, RI	ND	ND	0.024 ± 0.003	0.247 ± 0.023	0.359 ± 0.036
ES9	Ethyl 3-phenylpropionate	20.638	MS, RI	ND	ND	ND	0.015 ± 0.004	0.089 ± 0.012
	Σ			0.292 ± 0.009	0.273 ± 0.008	0.377 ± 0.015	0.987 ± 0.013	1.479 ± 0.026
Alcohols								
AL1	Hexyl alcohol	8.878	MS, RI	0.354 ± 0.031	0.288 ± 0.035	0.211 ± 0.006	0.164 ± 0.015	0.151 ± 0.013
AL2	1-Octen-3-ol	11.08	MS, RI	0.664 ± 0.072	0.707 ± 0.068	0.756 ± 0.112	0.761 ± 0.075	0.782 ± 0.064
AL3	Enanthol	11.213	MS, RI	0.113 ± 0.014	0.118 ± 0.015	0.124 ± 0.002	0.134 ± 0.012	0.369 ± 0.034
AL4	Isooctanol	12.007	MS, RI	0.028 ± 0.022	0.035 ± 0.006	0.045 ± 0.006	0.047 ± 0.007	0.051 ± 0.006
AL5	Octanol	13.597	MS, RI	0.020 ± 0.023	0.022 ± 0.006	0.031 ± 0.006	0.051 ± 0.008	0.057 ± 0.007
AL6	1-Nonanol	15.952	MS, RI	0.163 ± 0.018	0.178 ± 0.003	0.181 ± 0.015	0.186 ± 0.011	0.190 ± 0.016
AL7	Isoamyl alcohol	5.831	MS, RI	ND	0.011 ± 0.002	0.021 ± 0.007	0.134 ± 0.012	0.141 ± 0.016
AL8	Phenylethyl alcohol	21.325	MS, RI	ND	ND	0.081 ± 0.009	0.439 ± 0.046	0.440 ± 0.045
	Σ			1.342 ± 0.023	1.359 ± 0.034	1.450 ± 0.012	1.916 ± 0.022	2.181 ± 0.023

Table 1 continued

Number	Aroma compounds	Retention time (min)	Identification	Contents of volatile aroma compounds in 5 samples/(μg/g)				
				0 days	1 days	2 days	3 days	4 days
Aldehydes								
AD1	(E)-2-Heptenal	7.875	MS, RI	0.015 ± 0.002	0.018 ± 0.003	0.025 ± 0.003	0.041 ± 0.004	0.055 ± 0.008
AD2	2-Undecenal	17.742	MS, RI	ND	ND	0.010 ± 0.002	0.021 ± 0.006	0.025 ± 0.009
AD3	Nonaldehyde	9.44	MS, RI	ND	ND	ND	0.034 ± 0.003	0.170 ± 0.043
AD4	Benzaldehyde	12.676	MS, RI	ND	ND	0.205 ± 0.004	0.500 ± 0.012	0.593 ± 0.065
AD5	2-Phenyl-2-butenal	21.59	MS, RI	ND	0.014 ± 0.002	0.021 ± 0.001	0.072 ± 0.018	0.230 ± 0.032
	Σ			0.015 ± 0.002	0.032 ± 0.003	0.261 ± 0.003	0.668 ± 0.016	1.073 ± 0.045
Alkenes								
AK1	Phenylethylene	6.203	MS, RI	0.041 ± 0.008	0.046 ± 0.004	0.079 ± 0.004	0.139 ± 0.027	0.154 ± 0.046
AK2	Caryophyllene oxide	11.086	MS, RI	ND	ND	0.063 ± 0.002	0.082 ± 0.009	0.091 ± 0.009
AK3	2-Methyl-2-butene	5.762	MS, RI	ND	ND	0.012 ± 0.001	0.054 ± 0.006	0.208 ± 0.029
AK4	Dodecamethyl cyclohexasiloxane	8.784	MS, RI	0.021 ± 0.007	0.120 ± 0.005	0.035 ± 0.002	0.079 ± 0.003	0.283 ± 0.021
	Σ			0.062 ± 0.005	0.166 ± 0.004	0.189 ± 0.003	0.354 ± 0.006	0.736 ± 0.024
Ketones								
KE1	2-Octanone	6.961	MS, RI	0.332 ± 0.031	0.345 ± 0.036	0.401 ± 0.011	0.515 ± 0.083	0.526 ± 0.078
KE2	2-Nonanone	9.352	MS, RI	ND	0.064 ± 0.017	0.077 ± 0.007	0.085 ± 0.012	0.615 ± 0.073
KE3	3-Octanone	6.216	MS, RI	ND	ND	0.011 ± 0.002	0.081 ± 0.011	0.118 ± 0.024
	Σ			0.332 ± 0.031	0.409 ± 0.053	0.489 ± 0.008	0.681 ± 0.073	1.259 ± 0.075
Benzenes								
BE1	n-Butylbenzene	7.352	MS, RI	0.110 ± 0.005	0.160 ± 0.037	0.035 ± 0.002	0.388 ± 0.036	0.160 ± 0.027
BE2	Amylbenzene	9.787	MS, RI	0.076 ± 0.004	0.145 ± 0.016	0.032 ± 0.004	0.182 ± 0.024	0.071 ± 0.015
	Σ			0.186 ± 0.006	0.305 ± 0.022	0.067 ± 0.003	0.57 ± 0.03	0.231 ± 0.021
Phenols								
PH1	Guaiacol	20.284	MS, RI	ND	ND	0.011 ± 0.003	0.035 ± 0.004	0.072 ± 0.015
PH2	Phenol	23.35	MS, RI	ND	0.064 ± 0.012	0.071 ± 0.005	0.282 ± 0.016	0.320 ± 0.098
	Σ			0	0.064 ± 0.012	0.082 ± 0.004	0.317 ± 0.015	0.392 ± 0.014
Furans								
FU1	2-Amyl furan	5.491	MS, RI	0.794 ± 0.098	1.152 ± 0.024	1.161 ± 0.031	1.578 ± 0.137	1.585 ± 0.133
	Σ			0.794 ± 0.098	1.152 ± 0.024	1.161 ± 0.031	1.578 ± 0.137	1.585 ± 0.133
Indoles								
IN1	Indole	31.06	MS, RI	0.062 ± 0.006	0.086 ± 0.009	0.021 ± 0.003	0.196 ± 0.035	0.270 ± 0.076
	Σ			0.062 ± 0.006	0.086 ± 0.009	0.021 ± 0.003	0.196 ± 0.035	0.270 ± 0.076

MS, compounds were identified by MS spectra; RI (Retention index), compounds were identified by comparison with RI from literature; ND, not detected

most prevalent. The total alcohol content increased from 1.342 $\mu\text{g/g}$ *Mao-tofu* to 2.181 $\mu\text{g/g}$ during the 4 days of fermentation, and 1-octen-3-ol was especially abundant. It is responsible for the mushroom, green or cooked soybean character, and it has been isolated from rice wine, wheat Qu and sake koji (Ledauphin et al., 2003; Mo et al., 2009; Takahashi et al., 2006).

Aldehydes are produced as a result of the transamination and decarboxylation of amino acids, or by Strecker degradation, and are reduced to alcohols. Five aldehydes and 3 ketones were identified, which attained the peak concentrations of 1.073 $\mu\text{g/g}$ and 1.259 $\mu\text{g/g}$ *Mao-tofu* respectively, at the end of fermentation. Benzaldehyde, which imparts the aroma of bitter almonds, is the most important aldehyde in Huangshan *Mao-tofu*. It is either a result of benzyl alcohol oxidation, or the microbial decomposition of aromatic amino acids like phenyl hydroxybenzoic acid and p-acetic acid. Furthermore, both aldehydes and ketones are also formed by linoleic acid oxidation (Mo et al., 2009).

Fig. 3 Principal component analysis of 44 volatile compounds from 5 Huangshan *Mao-tofu* samples. Scores along the first 3 principal components explained 97.890% of the total variability. Each code was coincided with the specific volatile compound which was shown in Table 1

Two volatile phenols, guaiacol and phenol were detected post-fermentation (Table 1), and their content ranged from 0 to 0.392 $\mu\text{g/g}$ *Mao-tofu* throughout the process. Guaiacol imparts a spicy, clove-like and smoky aroma to the fermented tofu. The volatile phenols are formed by the enzymatic or thermal decarboxylation of p-coumaric and ferulic acids (Maria et al., 2012). In addition, 4 alkenes (Phenylethylene, caryophyllene oxide, 2-methyl-2-butene and dodecamethyl cyclohexasiloxane) and 2 benzenes (*n*-butylbenzene and amylbenzene) were also detected, although their contribution to *Mao-tofu* aroma and flavor needs further investigation. Finally, high levels of one furan and one indole were also detected (Table 1), which rose in the initial stages of fermentation and stabilized post fermentation.

PCA of the volatile compound data

Principal component analysis or PCA converts correlated variables of a data set into linearly uncorrelated variables known as principal components (PC), in order to determine the maximum variance of the original data set. In the context of the present study, the data set of volatile compounds were converted to 2 or 3 dimensions, to determine the variation between the different fermented samples, which were distinct as per the loading plots of the compounds (Fig. 3). The first three PCs of the quantitative data of volatile compounds (Table 2), PC1, PC2 and PC3, contributed 82.469%, 9.559% and 5.86% of the total variation respectively (Table 2).

The compounds contributing positively to the PC1 dimension (loading value > 0.6), i.e. located on the positive side of the axis, were AC1, AC2, AC4, AC6, AC7, ES3, ES5, ES6, ES7, ES8, ES9, AL3, AD1, AD3, AD5, AK3, AK4, KE2, KE3, PH1 and IN1. Likewise, AC3, AC5, AC8, AC9, ES2, ES4, AL2, AL4, AL5, AL6, AL7, AL8, AD2, AD4, AK1, AK2, KE1, PH2 and FU1 contributed positively to the PC2 axis (loading value > 0.6), while ES1 and AL1 contributed negatively (loading value > 0.9). The benzenes BE1 and BE2 were located in the positive direction of PC3 axis (loading value > 0.9).

PCA results also indicated that the different Huangshan *Mao-tofu* samples that correlated with certain groups of

Table 2 Cumulative variance contribution rates of principal components

Components	Characteristic value	Variance contribute rate (%)	The cumulative variance contribute rate (%)
1	36.286	82.469	82.469
2	4.206	9.559	92.027
3	2.579	5.862	97.890

Table 3 Correlation between physiochemical, biochemical indexes and volatile compounds of the Huangshan *Mao-tofu*

	Soluble protein	Amino nitrogen	Protease activity	Acidity	Reducing sugar
Acetic acid	0.874	0.836	0.294	− 0.682	− 0.429
Pentanoic acid	0.895*	0.882*	0.180	− 0.674	− 0.423
Caproic acid	0.972**	0.963**	0.390	− 0.816	− 0.587
Butyric acid	0.712	0.785	− 0.067	− 0.418	− 0.137
Propionic acid	0.922*	0.898*	0.264	− 0.724	− 0.468
2-Methyl butyric acid	0.966**	0.979**	0.575	− 0.924*	− 0.818
3-Methyl butanoic acid	0.843	0.869	0.096	− 0.585	− 0.303
Palmitic acid	0.995**	0.981**	0.503	− 0.887*	− 0.690
9-Decyl acetic acid	0.969**	0.946*	0.498	− 0.840	− 0.588
Ethyl caproate	− 0.852	− 0.881*	− 0.773	0.931*	0.904*
Hexyl acetate	0.982**	0.986**	0.437	− 0.855	− 0.665
Ethyl oenanthate	0.648	0.757	− 0.010	− 0.383	− 0.111
Ethyl laurate	0.963**	0.957*	0.652	− 0.899*	− 0.681
1-Cyclopentene-1-carboxylicacid,methylester	0.624	0.723	− 0.137	− 0.323	− 0.051
Ethyl palmitate	0.808	0.829	0.016	− 0.533	− 0.257
Ethyl oleate	0.799	0.826	0.005	− 0.521	− 0.243
Ethyl linoleate	0.842	0.857	0.082	− 0.583	− 0.306
Ethyl 3-phenylpropionate	0.598	0.703	− 0.153	− 0.297	− 0.028
Hexyl alcohol	− 0.992**	− 0.994**	− 0.604	0.928*	0.757
1-Octen-3-ol	0.959**	0.985**	0.640	− 0.916*	− 0.764
Enanthol	0.547	0.671	− 0.156	− 0.258	− 0.001
Isooctanol	0.970	0.991	0.618	− 0.909	− 0.735
Octanol	0.936*	0.932*	0.294	− 0.739	− 0.479
1-Nonanol	0.937*	0.972**	0.521	− 0.884*	− 0.785
Isoamyl alcohol	0.899*	0.878	0.196	− 0.687	− 0.441
Phenylethyl alcohol	0.910*	0.879*	0.245	− 0.708	− 0.450
(E)-2-heptenal	0.885*	0.918*	0.194	− 0.657	− 0.389
2-Undecenal	0.950*	0.943*	0.382	− 0.777	− 0.509
Nonaldehyde	0.617	0.717	− 0.142	− 0.316	− 0.044
Benzaldehyde	0.942*	0.934*	0.341	− 0.756	− 0.487
2-Phenyl-2-butenal	0.700	0.790	− 0.048	− 0.418	− 0.152
Phenylethylene	0.946*	0.937*	0.332	− 0.762	− 0.502
Caryophyllene oxide	0.959**	0.947*	0.594	− 0.865	− 0.622
2-Methyl-2-butene	0.669	0.761	− 0.075	− 0.378	− 0.101
Dodecamethyl cyclohexasiloxane	0.524	0.655	− 0.221	− 0.263	− 0.108
2-Octanone	0.955*	0.936*	0.357	− 0.784	− 0.534
2-Nonanone	0.584	0.710	− 0.100	− 0.313	− 0.074
3-Octanone	0.851	0.867	0.102	− 0.597	− 0.318
<i>n</i> -Butylbenzene	0.521	0.391	0.046	− 0.435	− 0.378
Amylbenzene	0.196	0.087	− 0.120	− 0.190	− 0.300
Guaiacol	0.810	0.863	0.085	− 0.548	− 0.262
Phenol	0.916*	0.913*	0.214	− 0.713	− 0.488
2-Amyl furan	0.958*	0.955*	0.390	− 0.842	− 0.697
Indole	0.710	0.734	− 0.153	− 0.415	− 0.178

*Correlation is significant at the 0.05 level (two-tailed)

**Correlation is significant at the 0.01 level (two-tailed)

volatile compounds were distinct (Fig. 3). At the positive direction in PC1 axis, the *Mao-tofu* samples of 4 days of fermentation were highly correlated with AC1, AC2, AC4, AC6, AC7, ES3, ES5, ES6, ES7, ES8, ES9, AL3, AD1, AD3, AD5, AK3, AK4, KE2, KE3, PH1 and IN1. The volatiles including esters from AC3, AC5, AC8, AC9, ES2, ES4, AL2, AL4, AL5, AL6, AL7, AL8, AD2, AD4, AK1, AK2, KE1, PH2 and FU1 were highly correlated with 3 days of fermented *Mao-tofu* samples at the positive position of PC2 axis, while benzenes BE1 to BE2 were highly correlated with the *tofu* samples at the negative position of PC3 axis. This demonstrates the important relationship between different volatile compounds and fermentation stages.

Correlation between physiochemical, biochemical indexes and volatile compounds

A correlation analysis was also performed to determine the relationships between the physiochemical and biochemical indices, and the volatile compounds of Huangshan *Mao-tofu* (Table 3). A strong positive correlation was seen between soluble protein levels with that of caproic acid, palmitic acid, 2-methyl butyric acid, 9-decyl acetic acid, hexyl acetate, ethyl laurate, 1-octen-3-ol and caryophyllene oxide during the fermentation process (Table 3). The soluble protein content was moderately positively correlated with pentanoic acid, propionic acid, octanol, 1-nonanol, isoamyl alcohol, phenylethyl alcohol, (E)-2-heptenal, 2-undecenal, benzaldehyde, phenylethylene, 2-octanone, phenol and 2-amyl furan. A significant negative correlation was seen with hexyl alcohol (correlation coefficient – 0.992).

Amino nitrogen levels showed similar correlation with the volatile compounds of Huangshan *Mao-tofu* as soluble protein, indicating that these volatile compounds are greatly influenced by soluble protein and amino nitrogen. Protease activity had no significant correlation with the volatile compounds (Table 3). Acidity levels on the other hand was positively correlated with ethyl caproate, hexyl alcohol, while negatively correlated with 2-methyl butyric acid, palmitic acid, ethyl laurate, 1-octen-3-ol and 1-nonanol. Reducing sugar content was positively correlated with ethyl caproate, but no significant correlation was seen with other volatile compounds.

Volatile flavor compounds are an important quality index of Huangshan *Mao-tofu*, and their formation is a very complex process that depends on the substrate ingredients and the microorganisms. The fermentation methods and conditions contribute to the distinctive flavors of Huangshan *Mao-tofu*, which are critical factors affecting the quality and composition, and eventually consumer satisfaction, of Huangshan *Mao-tofu*.

The *tofu* proteins are hydrolyzed to low-molecular-weight peptides by the microbial enzymes during fermentation, and these compounds are bio-active (Yan et al., 2011). In addition, some of the peptides and the concomitantly liberated ammonia could be transformed to various volatile flavor compounds such as acids, alcohols etc. (Jiang and Li, 2011), which was also confirmed by the correlation of soluble protein and amino nitrogen with various volatile compounds. However, the mechanism of the formation of these compounds during *Mao-tofu* fermentation is not clear, and needs further investigation.

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