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Analysis of volatile components in beer containing *Cordyceps militaris* extract by electronic nose and GC-MS

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ABSTRACT: In the present study, the volatile flavor compounds in beer containing *Cordyceps militaris* extract (CMB) were analyzed by solid-phase microextraction combined with gas chromatography-mass spectrometry (SPME/GC-MS) and electronic nose compared with normal beer (NB) as control. The results showed that, through principal component analysis (PCA) and loading analysis (LA), ester content of CMB was less than that of NB while alcohol content was higher. A total of 27 volatile compounds were identified by SPME/GC-MS from CMB and NB, classified into acids, alcohols, esters and terpenes.

KEYWORDS: *Cordyceps militaris*, beer, volatile flavor compounds, electronic nose, gas chromatography-mass spectrometry (GC-MS)

INTRODUCTION

Cordyceps militaris is an entomopathogenic fungus that invades insect larvae and pupae commonly known as caterpillar fungus. It belongs to families Clavicipitaceae and Ascomycotina, and has been used in oriental medicine for many years [1]. The bioactive compounds in *C. militaris* such as cordycepin, cordycepic acid, cordyceps polysaccharide and novel carotenoids have been reported to possess many effects including, but not limited to, antitumour, antioxidant, antiaging, immunomodulating and hypoglycaemic activities [2]. Nowadays, the artificial cultivation of *C. militaris* has basically achieved industrialization [3] and gradually developed for large scale and economic impact, especially the solid cultivation of *C. militaris*. However, the application of *C. militaris* products is still severely limited.

Beer, as the third largest consumption beverage in the world, has great consumption potential. In virtue of the production of CMB, not only can it maintain beneficial components of *C. militaris* itself and enrich nutritional components of beer, but also improve the application of *C. militaris*, which responds to the emerging consumer demand for high quality, nutrition and safety. As we know, volatile compounds of beer play an important role for organoleptic evaluation. Beyond that, many studies have reported changes of aroma compounds in different health benefit beer, recently [4–7]. On the contrary, there have been no reports on the counterparts in CMB. Therefore, it is necessary to analyse volatile compounds in CMB.

The aim of our present work was to investigate the effect of extraction of *C. militaris* with soild fermentation on volatile components of beer by solid-phase microextraction combined with gas chromatography-mass spectrometry (SPME/GC-MS) and electronic nose.

MATERIALS AND METHODS

Materials

C. militaris products with solid fermentation were prepared by Qingdao Agricultural University laboratory (Shandong, China); two-row barely malt was purchased from a local market in Qingdao Country, Shandong, China; ALE514 dry yeast was from Australian Mauribrew company; and Cascade hop was from Yakima Chief-Hopunion LLC., USA.

Preparation of extract from *C. militaris* (CME)

CME was prepared as described by Yu et al [8]. Briefly, the solid fermentation product of *C. militaris* was dried in oven at 60 °C and crushed through

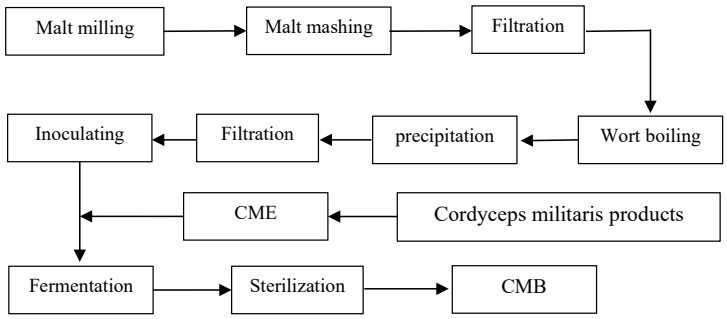

Fig. 1 Brewing technology process of CMB.

80 mesh sieve. Then samples was put in conical flask containing proper ethyl alcohol and placed in water bath (DK-S26 Shanghai Jing Hong Laboratory Instrument Co., Ltd., China) for 90 min at 45 °C. The supernatant, obtained by centrifugal filtration (3000 rpm, 10 min) through a 0.2 μm sterile Millipore filter to remove all cells was sterilized, then placed in the refrigerator after cooling.

Brewing technology of CMB

Brewing technology process of CMB is shown in Fig. 1. Broken malt was placed into mash tun followed by stirring at low temperature in case of agglomerate as heating leaching saccharification method and then placed into 50 °C water bath for 30 min. Next temperature was increased to 67 °C for 1 h. After that, temperature was increased to 78 °C for 20 min, then filter and obtain wort. 0.2 g/l and 0.4 g/l hops were used at the beginning and 30 min of the boiling process, respectively. By the end of boiling, 0.2 g/l hops were added again. After that, the wort is quickly stirred in one direction to facilitate impurity precipitation in the wort. Put the boiling pot in aseptic table to cool down until below 30 °C and filter with 6 layers of aseptic gauze. Wort is collected in a 1000 ml triangle bottle with a one-way valve for fermentation. Then crude CME was obtained by cyclotron precipitation and filtering. After that, 5–10 times dried yeast was sprinkled in 30 °C water and stirred gently for 15 min and let stand for 5 min. The activated yeast was poured into the wort after cooling. Every liter of wort was inoculated with 0.5 g dry yeast. With that, CME accounting for 10% of wort volume was added to bub followed by inoculating yeast and the main fermentation was completed at 20 °C for 7 days. Finally, the fermentation broth was then fermented in a pressurized brown bottle at 4 °C, pasteurized and centrifugated (4500 rpm, 15 min, 4 °C) to clarify beer.

NB was prepared as similar to CMB but without addition of ethanol extract of *C. militaris*.

Electronic nose

Electronic nose system (PEN3; AIRSENSE, Germany) was used as described by Zhu et al [9] with a slight modification.

Fifteen ml beer sample were placed in the glass sample bottle. Then the flavor composition of the sample filled the upper headspace and reached the equilibrium state. The headspace gas was absorbed with the electronic nose. Sample determination time interval lasted for 1 s. Preparation time was 3 s and test time was 60 s. After sampling performed, clean air filtered by activated carbon was pumped into the electronic nose, the sensor was cleaned and restored to its initial state with cleaning time of 100 s. the volatile headspace gas was adsorbed through the sensor array, which made the conductivity change. The signal was acquired by the data acquisition system and stored in a computer. Each sample was measured 3 times in parallel at 25 °C, 71 ± 1% relative humidity. All analyses were performed in triplicate.

Electronic nose data was processed as $R(i) = G/G_0$, $i = 1, 2, \ldots, 10$, where the ratio of signals R was the ratio of the conductivity G of the sample gas passing through the sensor to the conductivity G_0 of the standard gas filtered by activated carbon. $R(1)$–$R(10)$ were the number of 10 metal-oxide sensors.

SPME/GC-MS

Volatile compounds were analyzed by solid-phase microextraction (SPME) and gas chromatography coupled to mass spectrometry (GC-MS 5975B/6890N; Agilent Technologies, Santa Clara, CA, USA) following the procedure previously reported by Sanchez et al [10] and Zhou et al [11] with a slight modification. Volatile compounds

adsorbed on the SPME fiber (Qingdao Zhenzheng Industry and Trade Co., Ltd., China) were desorbed at 280 °C for 60 min in the injector port of a GC interfaced with a mass detector (internal ionization source: 70 eV) with a scan range from 35–500 m/z. The ion source temperature was set at 230 °C. Elastic quartz capillary column used was DB-5MS (30 m × 0.32 mm × 0.25 μm). The carrier gas used was high purity helium. The temperature of the GC started at 50 °C, raised to 150 °C at a rate of 5 °C/min for 1 min and then raised to 230 °C at 10 °C/min for 10 min.

Statistical analysis

Principal component analysis is a multivariate statistical analysis technique. By identifying several principal component factors to represent many complex and difficult to discover variables in the original sample, the regularity and difference between samples were then evaluated according to the contribution rate of principal component factors in different samples. PCA highlights the difference in volatile compounds by signal strength [12].

Load is the correlation coefficient between the principal component and the corresponding original index variables. Overload analysis can be used to analyze the closeness between factors and individual variables. The contribution rate and correlation of different sensors to the first and second principal components can be judged [13].

Principal component analysis (PCA) and loading analysis (LA) was carried out by WinMuster 1.6.2.

RESULTS

Electronic nose

Extraction of eigenvalue of sensor data

As shown in Fig. 2, a curve represents a sensor, suggesting that changes of relative conductivity G/G_0 of volatile compounds in beer with time increased when they pass through sensor channel. The response intensity values of some sensors changed quickly at the early stage of detection and then tend to be stable after 56 s. Meanwhile, the response intensity values of the other sensors did not change significantly. Therefore, the original data were analyzed by using the signal values of the curve which changed obviously between 56 s and 58 s.

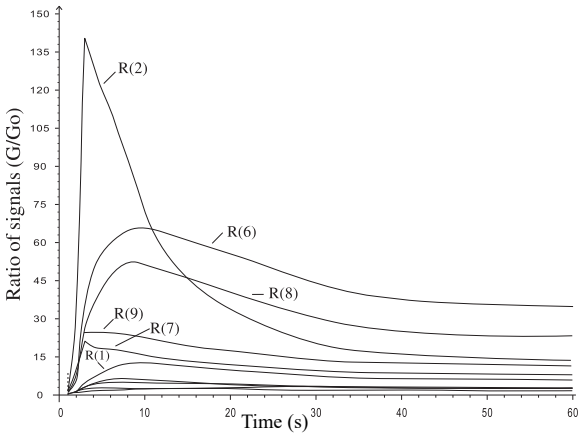

Fig. 2 Response graphs of electronic nose sensors to volatile compounds.

Principal component analysis

Principal component analysis (PCA) is a commonly used unsupervised learning method, widely used in data decomposition and visualization. It aims at mapping high dimensional variables in a low dimensional space. When the model is calculated, a straight line is first found to minimize the sum of the residual squares of all samples from the line, and the sum of the vector squares projected in the direction of this number axis is the largest, then the direction of the straight line also reflects the maximum difference between the samples, resulting in the first principal component (PC1); on this basis, the second principal component (PC2) is found along the straight line perpendicular to the previous principal component, and the second principal component is obtained, so repeatedly. In general, principal component analysis can be used when the total contribution rate of PC1 and PC2 is more than 85% [14].

PCA analysis can generally reflect the metabolic differences between the samples in each group and the variation between the samples in the group. The aggregation and dispersion of the samples can be observed from the PCA score diagram; the closer the sample distribution point, the closer the composition and concentration of the variables/molecules contained in these samples. On the contrary, the farther away the sample point, the greater the difference.

As shown in Fig. 3, the blue and green ellipse represents CMB and NB, respectively. The contribution rates of PC1 and PC2 were 99.93% and 0.03%, respectively. The total contribution rate was

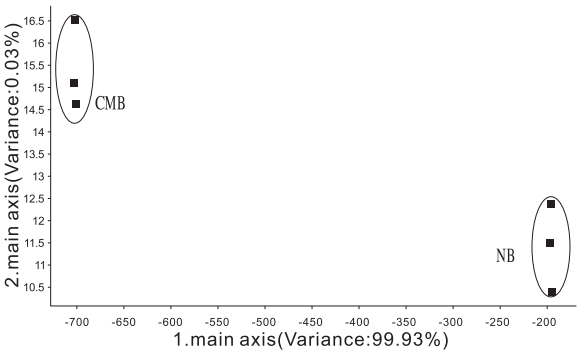

Fig. 3 PCA of volatile components in beer.

99.96%, which is more than 85%, suggesting that both principal component could reflect the information of the original high dimensional matrix without any important information missing. The elliptical region in which the data acquisition points were located had a specific distribution area and did not overlap, which indicates that PCA can effectively distinguish the difference between the two kinds of beer. In terms of the distribution of ellipse, CMB was in the PC1 negative axis of NB, indicating that the contribution rate of PC1 in CMB was smaller than that in NB and the contribution rate of PC2 was higher than that of NB. In terms of distance, CMB and NB were localized far away from each other, indicating that the odor components were significantly different. Therefore, GC-MS was needed to analyze volatile flavor components.

Loading analysis

Load analysis is used to judge the contribution rate of the sensor to volatile odor. The closer the response value of the sensor is to zero, the smaller its contribution to the overall fingerprint information and the smaller the recognition effect. In contrast, the greater the deviation from zero, the stronger the recognition ability. This e-nose consisted of 10 gas sensors and its main applications are presented in Table 1. The importance of volatile compounds could be differentiated by LA according to response value.

As shown in Fig. 4, the contribution rates of the first and second principal component were 99.65% and 0.26%, respectively. Among them, W5S and W1C sensors had a great impact on the first principal component. Additionally, W2S sensor showed a large influence on the second principal component. It is inferred that electronic nose gave strong response to nitroxides, aromatic compounds and ethanol, implying that those are main volatile components.

Although ester content of CMB was less than that of NB but alcohol content was higher, the contribution rate of ester was more effective than alcohol counterpart. This result may be caused by the higher threshold value of alcohols than esters. Therefore, it is still a beer dominated by ester flavor.

Table 1 Performance of 10 sensors for PEN3 portable electronic nose.

Sensor		General description	Reference
R1	W1C	Aromatic compounds	Toluene, 10 ml/m^3
R2	W5S	Very sensitive, broad range sensitivity, react on nitrogen oxides	NO_2, 1 ml/m^3
R3	W3C	Ammonia, used as sensor for aromatic compounds	Benzene, 10 ml/m^3
R4	W6S	Mainly hydrogen, selectively, (breath gases)	H_2, 100 ml/m^3
R5	W5C	Aliphatic hydrocarbons, aromatic compounds	Propane, 1 ml/m^3
R6	W1S	Aromatic compounds	CH_4, 100 ml/m^3
R7	W1W	Sulphur organic compounds	H_2S, 1 ml/m^3
R8	W2S	Alcohol	NO, 100 ml/m^3
R9	W2W	Aromatic compounds, sulphur organic compounds	H_2S, 1 ml/m^3
R10	W3S	Hydrocarbons	CH_4, 100 ml/m^3

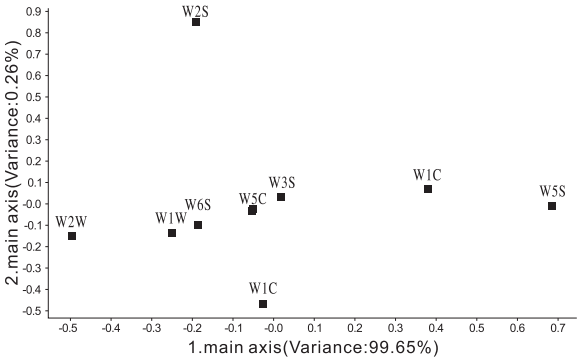

Fig. 4 Loading analysis of the volatile components in beer.

GC-MS

As shown in Table 2 and Fig. 5, a total of 27 volatile compounds including 11 common volatile components were identified by SPME/GC-MS from CMB and NB. Among them, 19 volatile compounds in CMB, accounting for 80.28% of total compounds, mainly included ethanol (23.24%), isoamyl alcohol (7.96%), isoamyl acetate (11.73%), ethyl hex-

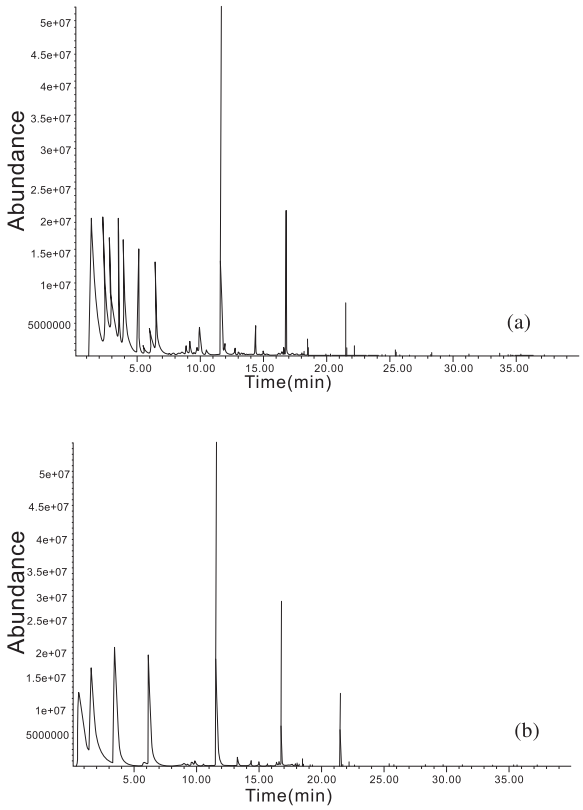

Fig. 5 GC-MS total ionic chromatogram of volatile compounds in (a) NB and (b) CMB.

Table 2 GC-MS analysis of volatile flavor compounds in beers.

Class	Identification	Peak area %		Aroma description
		CMB	NB	
Acid	Total	1.12	–	
	Butenoic acid	0.56	–	–
	Octanoic acid	0.56	–	rancid, butter, astringent
Alcohol	Total	35.54	30.86	
	Ethanol	23.24	21.30	–
	Isoamyl alcohol	7.96	9.36	fruity
	Phenethyl alcohol	0.21	0.16	rose
	2-Butanediol	4.13	–	
	Coriandrol	–	0.08	flower, fruity
Ester	Total	43.48	58.96	
	Isoamyl acetate	11.73	19.86	banana
	Ethyl hexanoate	7.56	12.91	fruity
	Ethyl caprylate	12.47	15.78	pear, flower, pineapple
	Ethyl laurate	0.89	1.68	flower, fruity, soap
	Ethyl caprate	3.57	4.33	apple, soap
	Ethyl pelargonate	0.63	0.16	garnetberry, pawpaw, fruity
	Methyl folate	0.78	–	–
	Ethyl 4-decenoic acid	0.65	–	–
	N-butyl octanoate	4.59	–	–
	Ethyl palmitate	0.33	–	wax, butter
	Phenylethyl acetate	–	2.50	honey, apple
	Cognac oil	–	0.56	jackfruit
	Trans-4-decenoic acid ethyl ester	–	0.13	–
	Ethyl 9-decenoate	–	0.16	–
	3-Methyl butyl sebacate	–	0.17	–
	Methyl arachidonate	–	0.18	–
	Octanoic acid 3-methyl butyl ester	0.30	0.58	fruity
	Ethyl myristate	–	0.03	–
Terpene	Total	0.14	0.06	
	Caryophyllene	0.09	0.06	clove
	1-Caryophyllene	0.05	–	slightly clove

anoate (7.56%) and ethyl octanoate (12.47%). Similarly, 19 volatile compounds in NB, accounting for 89.89% of total compounds, mainly included ethanol (21.3%), isoamyl alcohol (9.36%), isoamyl acetate (19.86%), ethyl hexanoate (12.91%) and ethyl octanoate (15.78%). Main components were the same between CMB and NB and they produced some aroma such as fruity, flower and rose, suggesting both taste were similar. Meanwhile, compared with NB, ethyl caprylate was the ester compound with the highest amount instead of isoamyl acetate. And ethyl caprylate is the important flavor in rich fragrance white wine, contributing to aromatic fragrance [15]. Although acids in beer are difficult to volatilize and are not aroma component, they are main flavoring materials and could give debonaire taste [16, 17]. Additionally, 8 kinds of substances such as butenate, 3-butanediol, methyl folate 1-caryophyllene were detected only in CMB, accounting for 15% of total volatile compounds, and establish CMB with unique taste.

DISCUSSION

Nowadays, the main production process of health benefit beer uses the additive method. For example, the active component extract in Chinese medicine herbs can be added directly in the saccharification process of beer production or in the brewing process or after fermentation [18]. In the present study, CME was added in the brewing process and then the volatile flavor compounds in CMB were analyzed by SPME/GC-MS and electronic nose compared with NB as control. Obtained results showed that 8 kinds of compounds were detected only in CMB, which is probably attributed to microorganism fermentation. The microorganism fermentation theory in Chinese herbal medicine suggests that microorganism could secrete some enzymes, break the cell structure and increase the content of bioactive substances [19]. Meanwhile, some macromolecular even toxic substances could be degraded to simple compounds

that could be absorbed directly or transformed to new compounds [20]. With respect to the other 8 compounds detected only in NB, combined with conclusion of electronic nose analysis, exactly ester content of CMB was less than that of NB while alcohol content was higher. The reason may be that CME possessing antioxidant activity promoted the production of reducing agents such as glutathione in yeast cells under oxidative stress [21] and thus inhibited formation of esteryl coenzyme A in fatty acid oxidation. It is known that condensation reaction of alcohols with esteryl coenzyme A in yeast is the main pattern of ester production [22].

Furthermore, it is reported that extracts of herbal medicines possess antimicrobial activity [23–26]. Thus, it may influence fermentation by inhibiting the metabolism of microorganisms such as yeast. However, whether antioxidant or antimicrobial activity of CME influenced the formation of flavor of CMB, further study is needed to investigate the mechanism of changes of flavor compounds.

Overall, addition of CME changed the composition of volatile components, but did not reduce their types. It introduces CMB with unique taste and health benefit.

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