Olfactory Threshold Concentration of Two Typical Earthy-Musty Odour Compounds in Black Carps and Bighead Carps

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Abstract: 2-methylisoborneol (2-MIB) and geosmin are two typical earthy-musty odour compounds in freshwater. In order to investigate the olfactory thresholds of 2-MIB and geosmin in black carp (Mylopharyngodon piceus) and bighead carp (Hypophthalmichthys nobilis), an improved and optimized pretreatment method of adsorbing the earthy-musty odours combined with the technology of gas chromatography following microwave distillation-headspace solid-phase microextraction was developed. The fish back muscle samples were placed in vials with the 2-MIB/geosmin solution and reached the adsorption equilibrium. Some samples were evaluated by a panel of ten members using Flavour Profile Analysis (FPA) method, the remaining sample was used for instrumental analysis. In fish muscle blocks, it took 90 and 105 min to establish the absorption equilibrium of 2-MIB and geosmin, respectively. The olfactory thresholds of 2-MIB in the bodies of black carps and bighead carps were 0.35 μg/kg and 0.30 μg/kg, respectively, while the geosmin thresholds were 0.59 μg/kg and 0.51 μg/kg, respectively. The concentrations of 2-MIB and geosmin in the bodies of black carps were slightly higher than in bighead carps, which were probably caused by the differences in the body fat content and the volatility of 2-MIB and geosmin.

Key words: fish; olfactory threshold; Flavour Profile Analysis; earthy-musty odour; 2-MIB; geosmin

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0 Introduction

In recent years, odour problems have been among the major causes resulting in complaints regarding freshwater and the aquaculture industry[1]. The earthy-musty flavour was the dominant flavour in 75% of the off-flavour fish[2]. The earthy-musty flavour is usually caused by 2-methylisoborneol (2-MIB) and trans-1,10-dimethyl-trans-9-decalo (geosmin)[3]. 2-MIB and geosmin are monoterpene and sesquiterpene terpenoids, respectively. These compounds are produced by some species of actinomycetes and/or cyanobacteria as secondary metabolites[4-6]. Research on the earthy-musty flavour of aquatic products began in the 1930s. In 1936, the earthy-musty odour in fish in the Scotland River was first reported by Thaysen et al[7]. Since then, this odour has been found in farmed freshwater fish worldwide[1]. These two lipophilic compounds, 2-MIB and geosmin, are mainly taken up through the gills or skin of fish and then accumulate in their lipid tissues[7,8]. The olfactory sense of humans is extremely sensitive to these two substances, and the aquatic products which had this earthy-musty odour were reduced in their quality and sales. In Europe, fish tainted by the earthy-musty odour have been observed in several countries, such as the rainbow trout aquaculture industry in France[9]. In the United States catfish industry, the earthy-musty odour caused losses up to $60 million (at 1998 prices)[10]. A recent confidential survey suggested that up to 20% of UK farmers occasionally suffer from the problem on a seasonal basis[11]. Earthy-musty odours in farmed freshwater fish cost the aquaculture industry millions of pounds in lost revenue per annum[12].
At present, the detection method for the earthy-musty flavour can be divided into sensory analysis and instrument analysis, while the detection of the sensory threshold of aquatic products is primarily based on sensory analysis. Sensory analysis is divided into the Threshold Odour Number (TON)\(^{[13]}\) and Flavour Profile Analysis (FPA)\(^{[14-17]}\). Samples must be diluted with water before detection in the TON method. This treatment may change the particular flavour of the sample. The FPA method, which does not require diluting the sample, is more suitable for the detection of aquatic product odours. An odour’s characteristics and intensity scale will be described by FPA.

When the odour sensory intensity changes from imperceptible to strong, the corresponding rank strength also changes. FPA method has already been used to describe sensory profiles of aroma and flavour of foods\(^{[18]}\) and beverages\(^{[19]}\). It also has evaluated odour qualities and odour thresholds of drink water and fishes\(^{[5,12,20,21]}\).

The aquatic products grown in freshwater have an earthy-musty flavour\(^{[22]}\). The sensory thresholds of 2-MIB and geosmin in different species of fish are summarized in Table 1. The sensory detection thresholds of 2-MIB and geosmin in water were 9 ng/L and 4 ng/L, respectively\(^{[21]}\). As seen from Table 1, the sensory detection thresholds for 2-MIB and geosmin in fish were 0.1-0.7 and 0.25-10 \(\mu\)g/kg, respectively.

| Fish species          | Odour sources in fish samples | Evaluation method | 2-MIB / \(\mu\)g kg\(^{-1}\) | Geosmin / \(\mu\)g kg\(^{-1}\) | Ref.   |
|-----------------------|------------------------------|-------------------|-----------------------------|--------------------------------|-------|
| Rainbow trout/        | Cultured in off-flavour water| T*                | —                           | 6                              | [23]  |
| Oncorhynchusmykiss    | Cultured in off-flavour water| T*                | —                           | 0.9                            | [12]  |
| Bream/                | Added to minced flesh        | S**               | 0.55                        | 6.5                            | [25]  |
| Abramisbrama          |                              |                   |                             |                                |       |
| Pike/Esoxluclus       | Added to minced flesh        | S**               | 0.095                       | 0.9                            | [25]  |
| Pike perch/           | Added to minced flesh        | S**               | 0.085                       | 0.59                           | [25]  |
| Sander lucioperca     |                              |                   |                             |                                |       |
| Channel catfish/      | Cultured in off-flavour water| T*                | —                           | 8.4                            | [26]  |
| Ictaluruspunctatus    | Cultured in off-flavour water| S** & T*          | 0.1-0.2                     | 0.25-0.5                       |       |
| Common carp/Cyprinuscarpio | Added to minced flesh    | T*                | —                           | 0.5                            | [27]  |

T*: Taste; S**: Sniff

As shown above, different species of fish have different sensory threshold concentrations. The sensory threshold concentration of these two substances can be influenced by the species and size, as well as the fat content, of the fish. The main reason is that different species of fish with different living environments and feeding habits make different types and amounts of volatile flavours. The different odours masked the earthy-musty odour attributes, such as the carbonyl compounds and alcohols, which contribute greatly to a fish’s flavour and will obstruct the smell of 2-MIB and geosmin\(^{[28]}\). The fat content varies with the size of the fish. The greater the proportion of lipid has, the higher the value of the threshold is, that is, more of the chemical needs to be present in the flesh to reach the threshold value\(^{[8]}\). In addition, the cultural backgrounds of the panelists also have an effect on sensory detection\(^{[5]}\). However, there are also some defects in sensory analysis, such as sensitivity differences among panel members that cannot be accurately quantified\(^{[29,30]}\).

The total output of China’s freshwater aquatic products accounted for 31.9 million tons, and the total output of freshwater aquaculture fish products accounted for 27.1 million tons in 2019. The four major Chinese carps (grass carp, silver carp, bighead carp, and black carp) are important food resources, being the primary products of freshwater aquaculture in China. The carps’ habitat distributions are in the lower water column, and
upper, middle and upper layers, and their food sources are molluscs, aquatic plants, zooplankton and phytoplankton, respectively. This study selected two representative freshwater fishes as experimental subjects: one is the bighead carp dwelling in the middle-upper water and feeding on plankton, and the other is black carp dwelling in the lower layer of the water column and feeding on zooplankton. On the basis of previous reports, an improved and optimized sensory analysis is combined with gas chromatography following microwave distillation-headspace solid-phase microextraction to detect geosmin and 2-MIB in flesh. From this determination, the olfactory threshold concentrations of earthy-musty odour compounds in two species of fishes were obtained. Thus, the selected pre-treatment methods of absorbing earthy-musty flavours and the theoretical support for off-flavours in the freshwater aquaculture industry were provided.

1 Materials and Methods

1.1 Materials and Instruments

Test materials: black carp and bighead carp (1 250 ± 150 g) were obtained from the fish ponds of the Dazhanpo farm in Yidu, Hubei Province, China. The standard preparations of 2-MIB (100 mg/mL) and geosmin (95.8 mg/mL) were both purchased from Supelco (Sigma-Aldrich Company, USA).

Instruments: gas chromatography FL9790Plus (Fuli Company, China); heating circulator (IKA, Germany); magnetic stirrer (Changzhou Guohua, China), electric thermostatic water bath DK-S11 (Sensin Company, China); microwave reactor EM7KCG4-NR (Midea Company, China, power 700W); hand-held high-speed homogenizer F6-10 (Shanghai Jingxin Company, China); 40 mL Teflon brown vials with cap.

1.2 Experimental Methods

1.2.1 Sample preparation

The experiments were carried out under the approval of the Animal Care Committee of China Three Gorges University, in accordance with the Animal management regulations of China. Before sample preparation, the fish were placed in clean water for 2 weeks to eliminate the odour, during which the water was changed daily. The back muscles of the fish were taken as the experimental materials, and the skin and the red-coloured muscle were removed and cut into blocks 3.0 (± 0.5) cm × 1.0 (± 0.3) cm × 1.0 (± 0.3) cm, with a mass of 10.0 ± 1.0 g. The prepared samples were stored in a refrigerator at -40 °C. Before the experiments the fish flesh was thawed at 4 °C for 12 h. All fish flesh samples were stored in the refrigerator for less than two weeks.

1.2.2 The selected pre-treatment method of adsorbing earthy-musty flavour by fish flesh

According to the reports, there are two primary methods to obtain the earthy-musty fish flavour as the experimental material. The first method to let fish obtain this off-flavour is by catching fish living in eutrophic freshwater by Robertson and Lawton. For the other step, the minced flesh of fish that had been eliminated off-flavours was directly added to 2-MIB/geosmin before the evaluation. For the first method, the fish which developed earthy-musty odour in natural freshwater were taken directly as the experimental materials for the sensory evaluation. Robertson and Lawton indicated that there was no pollution of 2-MIB in the selected region; therefore, the process of removing the off-flavour and re-adsorption experiment could be ignored prior to the sensory evaluation. However, most of the freshwater in China has reported the concurrence of these two types of odour substances, and it is not suitable to take naturally captured fish for the evaluation of a single olfactory threshold.

For the second method, it was reported that the fish flesh was minced, and the earthy-musty flavour was later added to it directly. However, it was not revealed whether the odour substances had complete access to the flesh or remained on the surface of the flesh. If these odour substances were only kept on the surface of flesh, it would affect the experimental results. Therefore, this method in the study was improved. On the basis of the treatment methods for sensory analysis of solid samples in international standard, the samples in our experiments were prepared in a closed container and the accurately segmented back muscles of the fish were added to the 2-MIB/geosmin water. After a period of time as these two odour substances reached adsorption equilibrium, the fish flesh was taken out and used in the sensory evaluation.

The pre-treatment method of this experiment was to cut the back muscles of the fish into blocks (approximately 10.0±1.0 g) and later soaking them in different concentrations of 2-MIB/geosmin water solution in a closed container (100 mL) with no air phase to give the flesh full contact with the odour substances until the concentrations of 2-MIB/geosmin in the flesh reached equilibrium. Next, the residual contaminants on the sur-
face were washed with distilled water and the fish flesh was crushed by a hand-held high-speed homogenizer. The samples obtained were used in the gas chromatography following microwave distillation-headspace solid-phase microextraction and FPA analyses.

1.2.3 Heating method in olfactory sensory evaluation

The method of heating fish in most of the reports is by cooking or microwave\cite{12, 25}. In the process of cooking the heated steam will take away part of the odorous substances\cite{23}. The sample was kept in a closed brown PTFE vial for microwave heating, and the vial was later opened to conduct the sensory evaluation. Microwave heating have the advantages of heating rapidly, high efficiency and convenient use, so microwave heating used an effective method of the extraction of off-flavours from fish tissues\cite{32, 37}. The earlier results were confirmed that lower stat microwave heating could not bring about a decrease in the amount of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on fish by Kola-kowska and Bienkiewicz\cite{38} and Hearn et al\cite{39}. Ågren and Hänninen\cite{40} also reported that the total fatty acid contents were slightly increased without great differences between individual fatty acids in lean pike in conventional and microwave baking methods.

1.2.4 Screening of Flavour Profile Analysis (FPA) and the training of panellists

At present, the sensory analysis commonly used includes olfactory and taste evaluation. Comparing the two methods, the olfactory evaluation turned out to be more sensitive than taste\cite{41}. The two substances are thought to be lipophilic, which means that they adhere to the fat of cell membranes. Water rinses are ineffective for clearing the odour out of the mouth; therefore, subsequent evaluations are compromised\cite{42}. Additionally, olfactory evaluation is more acceptable; therefore, olfactory evaluation was adopted as a sensory evaluation tool in our experiment. The training of the panellists was based on the literature and was improved according to the actual situation\cite{17, 29}. The quality of the sensory profile depends on the quality of the panel. Assessors were correctly trained and coached before selection required. At last, ten panellists pass the train. They are 6 females and 4 males, and the ages range from 20 to 50. The sensory profile descriptors use 0-12 to express intensity. The larger the number is, the stronger the strength grade will be.

1.2.5 Evaluation and analysis of the off-flavour

The back muscle samples from the fish were thawed and placed in a closed brown vials with full of the same concentration of 2-MIB/geosmin solution and no air space. The vials then placed into a water bath at 25.0±0.5 °C. When the adsorption equilibrium was reached, the flesh was taken out and the residual 2-MIB/geosmin on the surface of flesh was removed with 2 mL of distilled water. After removing the fish, several were evaluated by sensory analysis, and the remainder of the fish samples was used for instrumental analysis.

The samples for sensory evaluation were put in a microwave and heated for 1 min. A one-minute break was taken to equilibrate the air pressure of the sample. Each sample was only sensed by one panel member to prevent a perception difference of 2-MIB/geosmin after one panellist had evaluated it.

The samples for instrumental analysis were subjected to gas chromatography following microwave distillation-headspace solid-phase microextraction\cite{9, 43, 44}.

1.2.6 Determination of fat content

It is suitable to extract the fat with chloroform-methanol method as the freshwater fish flesh contains unsaturated fatty acids and phospholipids. The back muscle of black carps and bighead carps was dried at 70 °C for 24 h and later smashed and sifted through a 60-mesh sieve. The powder samples obtained were reserved for fatcontent determination. After that step, 0.5 g of fish powder was soaked in 20 mL of chloroform-methanol (2:1, V/V) to extract for 12 h. The chloroform-methanol solution was increased to 40 mL and the supernatant was filtered. Next, another 20 mL of chloroform-methanol solution was added to the residue and shocked and later filtered. The liquid obtained from these two filtrations was collected in a round bottom flask for distillation recovery, and the flask was then placed into a 70 °C oven and dried to a constant weight.

2 Results

2.1 Determining the Balance Time of the Off-Flavour in Fish Flesh

The adsorption by black carp in different concentrations of 2-MIB solution is shown in Fig. 1. At the initial stage of adsorption (0-30 min), the adsorption rate increased with 2-MIB concentration increasing. At 60 min, the adsorption reached equilibrium at the concentrations of 10.00 μg/L and 13.33 μg/L and the adsorption capacities were 3.22 μg/kg and 3.89 μg/kg, respectively. With an extension of time, the 2-MIB content in fish flesh showed no significant change. In the low concentrations (3.33 and 6.67 μg/L), the adsorption reached equilibrium at 90 min, and the adsorption capacities were 1.703 and
3.01 μg/kg, respectively.

Figure 1 shows that the absorption of 2-MIB also increases with the increase of 2-MIB concentration. Flesh reached adsorption equilibrium in the low-concentration solutions of 2-MIB (3.33 and 6.67 μg/L) in 90 min and in high-concentration 2-MIB solutions (10.00 and 13.33 μg/L) reached equilibrium in 60 min. These results might be due to the adsorption rate of the fish’s flesh at the concentrations of 10.00 and 13.33 μg/L 2-MIB aqueous solutions being higher than the lower concentrations of 3.33 and 6.67 μg/L 2-MIB in the initial stage of adsorption (0-30 min); therefore, the balance time in high-concentration 2-MIB solution is shorter than that in 3.33 and 6.67 μg/L 2-MIB solution.

Fig. 1 Relationship between the concentration of the 2-MIB solution and adsorption by the fish flesh of black carp

The adsorption process of flesh for geosmin was similar to that of 2-MIB, but the time to balance was slightly longer than that for 2-MIB, and the geosmin content in fish muscle was higher than 2-MIB. In the 3.33, 6.67, 10.00 and 13.33 μg/L aqueous solutions of geosmin, the adsorption of geosmin increased by 0.11, 0.31, 0.48 and 0.53 μg/kg compared with 2-MIB, and the equilibrium times were 15 minutes longer. This phenomenon may be because, compared with geosmin, the 2-MIB logKow is less (the logKow values of 2-MIB and geosmin are 3.31 and 3.51, respectively) [8]. The greater the hydrophilicity, the higher the solubility in water, and the lower the solubility in fish. The difference in the partition coefficients of octanol makes their bioconcentration coefficients different such that for the same amount of fish, the two types of odour substances will also be different, which impacts their accumulation in fish.

In accordance with experimental method in Section 1.2.6, the fat contents of back muscle of black carp and bighead carp were approximately 3.99% and 2.47%, respectively. Black carp and bighead carp are among the “Four Major Chinese Carps”. The fat content of bighead carp was slightly lower than that of black carp [45], and its adsorption process was similar. Therefore, the concentration of the earthy-musty odour compounds used for sensory analysis in this experiment are within the range of 0 to 13.33 μg/L, and the equilibrium time of 2-MIB and geosmin reaching equilibrium was 90 and 105 min.

2.2 Relationship between the Intensity and Odour Substance Contents in Two Carps

For different concentrations of 2-MIB/geosmin aqueous solutions in the container, and a given quantity of fish muscle (10.0±1.0 g), the best adsorption time, the odour assessment of different intensity (0-12) and the relationship between the content of 2-MIB/geosmin in fish are shown in Figs. 2 and 3.

Figures 2 and 3 show a positive linear correlation between the perceived intensity and actual chemical concentration in the flesh between the content and the intensity of 2-MIB in black carp: \( y = 0.326 + 0.149x \), \( R^2 = 0.693 \); the relationship between the content and the intensity of 2-MIB in bighead carp: \( y = 0.317 + 0.130x \), \( R^2 =... \)
0.751; the relationship between the content and the intensity of geosmin in black carp: \( y = 0.591 + 0.146x \), \( R^2 = 0.790 \); and the relationship between the content and the intensity of geosmin in bighead carp: \( y = 0.514 + 0.150x \), \( R^2 = 0.744 \).

In ISO13301:2018\(^{[29]}\), there are 2 definitions of threshold. One definition is the traditional notion of threshold: a stimulus concentration above which the stimulus can be detected, and below which it cannot. Another definition is the probabilistic nature of threshold: the stimulus concentration for which the probability of detection is 0.5. According to the traditional definition of threshold, the intensity of 0 in Figs.2 and 3 is the sensory evaluation of pollution-free points. That is, a panellist’s sense of smell cannot perceive these two kinds of flavour, and means the traditional notion of the olfactory threshold of 2-MIB/geosmin in black carp/bighead carp. Robertson et al\(^{[12]}\) have reported that the results omitted the intensity of 0 points, which were evaluated by a panel, fitting the relationship between the content of geosmin in rainbow trout and intensity of the geosmin; the intercept of the \( y \) axis at \( x = 0 \) was defined as the sensory evaluation threshold of geosmin. Yamprayoon and Noomhorm\(^{[46]}\) also defined an intensity of 0 for sensory evaluation as a sensory threshold. In actual situations, the probability nature of threshold is more widely used and more reliable, as in previous reports\(^{[5,17]}\). Therefore, in accordance with the definition of the probability nature, the threshold-based results are as follows: the olfactory threshold of 2-MIB in black carp and bighead carp were 0.35 \( \mu \)g/kg and 0.30 \( \mu \)g/kg, respectively, and the olfactory threshold of geosmin in black carp and bighead carp were 0.59 \( \mu \)g/kg and 0.51 \( \mu \)g/kg, respectively.

3 Discussion

In this experiment, the olfactory threshold of 2-MIB in these two fish species was lower than that of geosmin. This finding indicates that people’s sense of smell is more sensitive to 2-MIB. Comparing the olfactory threshold of black carp with that of bighead carp, the thresholds of 2-MIB and geosmin in black carp were slightly higher than those in bighead carp. This finding may be due to the higher fat content of black carp. The higher the fat has, the greater the olfactory sensory threshold of 2-MIB/geosmin in fish. Fish meat contains a variety of volatile components and minerals, which produce flavour in fish. By microwave heating the sample, fat oxidation produced other volatile gases, and some volatile components in black carp more easily cover the earthy-musty odour. The composition of volatile components in fish is complex and changeable, and the growth environment and diet have an influence on the olfactory sensory threshold; thus, further research is needed.

To compare the odour threshold concentrations of two earthy-musty compounds in the two species of fish with different sensory thresholds (taste threshold and olfactory threshold), this experiment selected sniffing as the sensory evaluation method. The thresholds obtained are generally lower than the taste thresholds in Table 1. The reason for this result is that smell is more sensitive than taste\(^{[41]}\). From the comparison of the 2-MIB and geosmin olfactory thresholds in fish, 2-MIB is considerably lower than geosmin. The olfactory threshold range of these two off-flavours of different kinds of fish in
Table 1 is 0.095-6.5 μg/kg, and the experimental results are in this range.

Muscle is the main part of fish consumed, including the back and abdomen. This experiment chose back muscle as the material, since the fat content of back muscle is lower compared with that of abdomen. The greater the proportion of lipid, the higher the value of the olfactory threshold; therefore, the olfactory thresholds of earthy-musty odours in back muscle are relatively lower compared with those of the edible parts of the fish. If the content of earthy-musty odour compound in the edible part are lower than the olfactory threshold in the fish’s back, people will not smell it out. In this paper, the quantitative relationship between the content of earthy-musty odorous and the panellists sense of smell was established, and the concentration of the off-flavour in fish flesh can be estimated according to the intensity of flavor. The change in flavor intensity will indicate whether the odor problem is reduced or aggravated, and this information may affect the choice of odor processing methods.

4 Conclusion

Earthy-musty odours in freshwater fish seriously affect the economic aspects of the fish farming industry. This study established a complete method for the olfactory sensory threshold of 2-MIB and geosmin in fish to improve the quality of freshwater fish farming and provided suggestions for containing the earthy-musty odour in freshwater fish sales. Compared with previous studies, this study, based on the analysis of the experimental method, improves the way of off-flavour entered fish flesh. After the flesh accumulates the earthy-musty odour to a steady state, the analysis combines FPA analysis with gas chromatography following microwave distillation-headspace solid-phase microextraction, which combines qualitative and quantitative studies to obtain results. The data show that people’s sense of smell is more sensitive to 2-MIB. The olfactory thresholds of 2-MIB in black carp and bighhead carp were 0.35 and 0.30 μg/kg; the olfactory thresholds of geosmin in black carp and bighhead carp were 0.59 and 0.51 μg/kg. In both species of fish, the olfactory threshold of 2-MIB is much lower than that of geosmin.

Conflicts of Interest
The authors declare that there are no conflicts of interest regarding the publication of this paper.

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