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Generation of Olfactory Compounds in Cat Food Attractants: Chicken Liver-Derived Protein Hydrolysates and Their Contribution to Enhancing Palatability

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ABSTRACT: The present study investigated the impact of four chicken liver protein hydrolysate-based cat food attractants on palatability. Aroma compounds were analyzed in these attractants, which were subsequently sprayed onto four different types of cat foods. Results revealed that CF4 exhibited the highest intake ratio and the first choice ratio, followed by CF2 sample. Orthogonal partial least-squares discriminant analysis (OPLS-DA) demonstrated significant differences among 50 volatile compounds identified from the four cat foods. Using variable importance in projection (VIP) values, we selected 17 key flavor compounds responsible for distinguishing between the four cat foods. Peptides with a molecular mass <180 Da showed correlation with nonanoic acid and cedrol, while those >3000 Da correlated with hexanoic acid ethyl ester. Regression coefficients (RCs) calculated from partial least-squares regression (PLSR) results showed positive correlations between compound content and palatability for six compounds, whereas negative correlations were observed for ten compounds. Validation experiments confirmed that nonanal, 2-propylpyridine, and 3-octen-2-one enhanced palatability and correlated with peptides ranging from 180 to 500 Da; conversely, nonanoic acid ethyl ester and 3-methyl-pentanoic acid reduced palatability and correlated with peptides ranging from 1000 to 3000 Da.

KEYWORDS: *cat food, attractant, chicken liver protein, palatability, aroma compounds*

INTRODUCTION

Flavor is an important characteristic of cat food and plays a significant role in determining pet cats' food preferences.[1] While cat food is primarily formulated to provide complete and balanced nutrition, it must also be palatable.[2] The palatability of cat food plays a pivotal role in augmenting the intake rate of cat food. The overall pleasure associated with the hedonic or sensory attributes obtained from ingested food is determined by the product's appearance, aroma, taste, and texture.[3] Although there is no definitive evidence highlighting the relative significance of smell over taste, cats possess a highly evolved olfactory system that enables them to initially select food based on scent. Therefore, investigating the aroma of cat food is a crucial aspect in understanding their food preferences.[1] Therefore, the palatability of cat food can be enhanced through the utilization of spraying techniques to apply attractants with a specific aroma.

Currently, the most prevalent approach employed for the preparation of pet food attractants entails thermal reactions of protein hydrolysates comprising elevated concentrations of short peptides and free amino acids.[4] Yin et al.[5] analyzed dog food attractants prepared from diverse protein sources and subjected to various enzymatic hydrolysis conditions, resulting in the identification of nine compounds associated with enhanced palatability as well as three compounds linked to reduced palatability. Feng et al.[6] focused on preparing different flavors of dog food attractants using chicken liver and mushrooms through enzymatic hydrolysis and Maillard reaction processes while characterizing key volatile flavor compounds related to high intake ratio. Sun et al.[7] prepared cat food attractants with significant differences in volatile flavor compounds by Maillard reaction using grass carp waste enzymatic hydrolysis solution as raw material. However, previous studies on cat food have primarily focused on the formulation of attractants and identification of aromatic compounds. The correlation between volatile compounds and the palatability of cat food has yet to be definitively established. Research findings indicate that the extent of protein hydrolysis significantly influences the formation of fundamental meat aroma.[8] Therefore, further elucidation is required to investigate the impact of varying molecular mass on the content of free amino acids and peptides as well as their influence on flavor compound formation and subsequent contribution to the palatability of cat food. In addition, the validation experiments based on the palatability tests verified the accuracy of the orthogonal partial least-squares discriminant analysis (OPLS-DA) and partial least-squares regression (PLSR) models and provided an experimental basis for the traditional flavor substance evaluation methods concerning the palatability of cat foods (CFs) that lack the subjectivity of pet cats, which is one of the features of this study.

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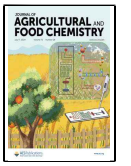

In this study, the cat food attractants were prepared by utilizing the thermal reaction between chicken liver protein hydrolysates with varying degrees of hydrolysis. The distribution of free amino acids and peptide molecular mass in chicken liver protein hydrolysates was determined, and their contribution to the formation of key flavor compounds and the palatability of CFs was explored using headspace solid-phase microextraction and solvent-assisted flavor evaporation combined with gas chromatography−mass spectrometry (HS-SPME/SAFE-GC-MS) and OPLS-DA analysis. The correlation between volatile compounds and the intake ratio of CFs, as well as the first choice ratio, was examined by using PLSR analysis. Relevant validation experiments were conducted to identify pivotal flavor compounds that exert an influence on the palatability of cat food. The findings offer theoretical guidance for the development of highly palatable attractants in cat food production.

MATERIALS AND METHODS

Materials. Neutral cat food made from corn flour and soybean meal was purchased from Xinyuan Co., Ltd. (Shanghai, China). Raw chicken liver (74% moisture content) was purchased from Xinyuan Co., Ltd. (Shanghai, China). Papain (80 U/g) was purchased from Pangbo Biological Engineering Co., Ltd. (Nanning, Guangxi, China). Xylose, cysteine hydrochloride, phosphoric acid (food grade), and chicken fat were obtained from Xinyuan Co., Ltd. (Shanghai, China). Blank control cat food attractant (CFA0) was prepared from animal protein and provided by Xinyuan Co., Ltd. (Shanghai, China).

Chemicals. Dichloromethane (analytical reagent, AR), anhydrous sodium sulfate (AR), sodium chloride (AR), and calcium chloride (AR) were purchased from Shanghai Titan Co., Ltd. (Shanghai, China). 1,2-Dichlorobenzene (internal standard), acetone, and an *n*-alkane mixture (C7−C30) for calculation of retention indices (RIs) were purchased from Sigma-Aldrich Co., Ltd. (St. Louis, MO). The authentic standards for the identification and quantitation experiments were commercially available: propanoic acid (≥99%), 3-methyl-pentanoic acid (98%), butanoic acid (98%), heptanoic acid (≥98%), nonanoic acid (99%), octanoic acid (98%), hexanoic acid (99%), 4-methyl-pentanoic acid (99%), dodecanoic acid, ethyl ester (≥99%), decanoic acid, ethyl ester (97%), triacetin (≥99%), diethyl malonate (≥99%), 7-butyl-2-oxepanone (98%), propanoic acid, 2-methyl-, dodecyl ester (≥98%), butyrolactone (99%), octanoic acid, ethyl ester (98%), *β*-myrcene (≥98%), *β*-pinene (99%), *β*-ocimene (99%), 1-(1*H*-pyrrol-2-yl)-ethanone (≥99%), benzothiazole (≥99%), methylpyrazine (98%), 2,5-dimethylpyrazine (99%), and 2-propylpyridine (99%) were obtained from TCI (Shanghai, China); octadecanoic acid (≥99%), 5-methylhexanoic acid (≥97%), benzaldehyde (99%), nonanal (99%), 2,4-decadienal (≥98%), (*E*,*Z*)-2,4-decadienal (≥99.5), acetic acid, octyl ester (≥99%), nonanoic acid, ethyl ester (98%), hexanoic acid, ethyl ester (99%), pentanoic acid, 4-oxo-, methyl ester (99%), undecanoic acid, ethyl ester (≥98%), butanoic acid, 3-hydroxy-, ethyl ester (≥99.5), butanoic acid, decyl ester (97%), and (*E*,*E*)-2,4-decadienal (≥99%) were obtained from Merck (Darmstadt, Germany); 3-methyl-2-butenal (98%), benzeneacetaldehyde (≥99%), furfural (95%), 3-(2-furanyl)-2-propenal (99%), *α*-ethylidene-benzeneacetaldehyde (99.5%), pentadecanoic acid, ethyl ester (98%), *n*-decanoic acid, ethyl ester (≥98%), 2*H*-pyran-2-one, tetrahydro-6-undecyl- (97%), 2-nonanone (99%), 3-octen-2-one (98%), 2-pentadecanone (>99%), and phenylethyl alcohol (>99%) were obtained from Boer (Shanghai, China); cedrol (99%), 2-ethyl-1-hexanol (98%), 2-methyl-3-buten-2-ol (99%), 3-penten-2-ol (98%), 1-octanol (97%), eucalyptol (98%), hexadecanone (99%), phenol (98%), 2,4-di*tert*-butylphenol (99%), styrene (≥99.5), limonene (99%), D-limonene (98%), *γ*-terpinene (99.5%), 2-pentylpyridine (≥99%), 2-pentyl-furan (99%), 2-ethyl-6-methyl-pyrazine (99%), 2-decylfuran (99%), and 1-octen-3-ol (≥98%) were obtained from Aladdin (Shanghai, China). All chemicals were at least of analytical grade.

Preparation of Enzymatic Hydrolysates. The enzymatic reaction conditions were optimized based on the findings of a previous study.[6] The chicken livers were homogenized with deionized water to obtain four different dry matter concentrations (10, 15, 20, 25%) of the resulting homogenate. The four homogenates were subjected to heat treatment at 95 °C for 10 min to induce endogenous enzyme inactivation, followed by cooling to room temperature. The homogenates were treated and adjusted to pH 6 by using food-grade phosphoric acid (85%). The addition of papain (80 U/g) at a concentration of 0.6% (w/w) was performed subsequently. The enzymatic hydrolysis was conducted at a temperature of 60 °C for a duration of 3 h. After hydrolysis, the enzymatic hydrolysates were subjected to thermal treatment at 90 °C for 10 min to inactivate the papain. The hydrolysates were centrifuged at a relative centrifugal force rcf = (8019*g*) at 23 °C for about 10 min. The supernatant was subsequently analyzed for the determination of the degree of hydrolysis (DH/%). DH is calculated according to the following formula: $\mathrm{DH}\ (\%) = \frac{\text{free amino nitrogen content (g / mL)}}{\text{total nitrogen content (g / mL)}} \times 100\%$. Free amino nitrogen was determined by formaldehyde titration, and total nitrogen content was determined by Kjeldahl apparatus according to the methods of Feng.[6] The four chicken liver protein hydrolysates obtained are designated as CLPH1, CLPH2, CLPH3, and CLPH4, respectively.

Determination of Free Amino Acids. Free amino acids were determined according to Wang's method with appropriate modifications.[9] The four chicken liver protein hydrolysates (CLPH1, CLPH2, CLPH3, and CLPH4) were diluted 3-fold with trichloroacetic acid (10 g/dL), followed by sonication for 20 min at room temperature. Subsequently, centrifugation was performed at a relative centrifugal force of rcf = (111*g*) at 23 °C for approximately 10 min to obtain the supernatant. The supernatant was injected for detection using a volume of 400 *μ*L. The chromatographic conditions were as follows: ODS Hypersil (250 × 4.6 × 5 μm^3), 40 °C; mobile phase: 0.6 mmol/L sodium acetate in phase A, 0.15 mmol/L sodium acetate, methanol, and acetonitrile in phase B (1:2:2, v/v/v); flow rate: 1.0 mL/min.

Determination of Peptide Molecular Mass Distribution. The molecular weight of the samples was determined according to Liu's method with slight modifications.[10] The molecular mass distribution was determined by using the gel permeation chromatography (GPC) method. Individual 10 mL vials were filled with 100 mg of chicken liver protein hydrolysates, and the mobile phase was added to achieve a final volume of 10 mL. After volumetric adjustment, the samples were centrifuged at a relative centrifugal force rcf = (11,100*g*) at 23 °C for 15 min. Subsequently, they were subjected to filtration and injection through a microporous membrane. The data were analyzed with GPC software. The relative molecular mass of the sample and its distribution were determined by employing a standard curve equation. Chromatographic conditions were as follows: TSKgel 2000SWXL (300 mm × 7.8 mm). Mobile phase V (acetonitrile)/V(water)/V(trifluoroacetic acid) was 40:60:0.1. The UV detection wavelength was set to be 220 nm, and the flow rate and column temperature were 0.5 mL/min and 30 °C, respectively.

Preparation of Attractants by Maillard Reaction. The optimization of Maillard reaction conditions was conducted based on previous studies.[11] The pH of the above chicken liver protein hydrolysates (CLPH1, CLPH2, CLPH3, and CLPH4) was adjusted to 6.5 with food grade phosphoric acid (85%). Chicken liver protein hydrolysates (50.00 g), cysteine (1.20 g), and xylose (3.00 g) were dissolved in water at 25 °C. The pH of the solution was adjusted to 7.0 with 3.0 M NaOH, followed by transferring it into a high-pressure reactor with a volume of 100 mL. The Maillard reaction was conducted in an oil bath at a temperature of 120 °C. Following the reaction, the Maillard reaction products (MRPs) were promptly cooled using an ice bath. The pH was adjusted to 3 using phosphoric acid (85%) in order to obtain four distinct cat food attractants, designated as CFA1, CFA2, CFA3, and CFA4, respectively.

Preparation of Five Types of Cat Food. To assess the palatability of the aforementioned cat food attractant, a two-bowl test

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was conducted to establish a control sample. Therefore, a commercially available cat food attractant with comparable production costs (hereafter termed CFA0) was selected as the control in the experiment. In the preparation process of cat foods (CFs), the chicken fat was coated onto the odorless base CF at a weight ratio of 1:12 (w/w) under controlled conditions at 40 °C. Then five different cat food attractants (CFA0, CFA1, CFA2, CFA3, and CFA4) were sprayed in a 1:6 (w/w) overlay onto the base CF and mixed thoroughly to obtain five types of cat food (hereafter termed CF0, CF1, CF2, CF3, and CF4). An ZJB-100 vacuum sprayer, manufactured by Chongqing Tongrui Filter Equipment Manufacturing Co., Ltd., was employed for the spraying process. The specific procedure involved the following steps: Initially, half of the total cat food attractant was sprayed in a straight line while maintaining a spraying duration of 5 min. The pressure rise time was precisely controlled to be 10 min, and once the chamber pressure reached 0.030 MPa, revacuuming was performed until it reached 0.060 MPa before proceeding to spray the remaining half of the cat food attractant.

Preference Test of Four CFs. The preference test was performed using a two-bowl test with reference to Ilias et al.[12] Ten adult cats (five males and five females, Blue cat, Persian cat, Dragon li, Chinese pastoral cat, and Short haired cat) with an average body weight of 4 ± 1.5 kg and an age of 12−18 months were used in this experiment. The experimental cats were from the School of Zoology, Shanghai Jiao Tong University. Before the experiment, the cats lived in groups, the ambient temperature was controlled at 24 ± 2 °C, and the cats had sufficient water and food. Then, they were fasted for a night to ensure that they are hungry during the test, which increased their sensitivity to food. The palatability testing received ethical approval from the Animal Research Ethics Committee of Shanghai Jiao Tong University. The experimental cats were required to be in optimal health and to exhibit no signs of emesis or gastrointestinal distress during the feeding process. Besides, the experimental cats had not received antibiotic treatment for at least six months before the samples were collected.

The experimental cats were grouped for palatability testing, and each one underwent individual testing. Each cat was kept in a clean room (1.5 m × 3.5 m) at a temperature of (24 ± 2) °C under normal health conditions. Specifically, the palatability testing is categorized into four groups, wherein each experimental group comprises 10 cats that are fed continuously for two consecutive days. There is a 1 week rest period between each experimental group. The four experimental groups were selected as CF1, CF2, CF3, and CF4, respectively, as controls against CF0. The first group, comprising 10 cats, was fed once daily at 13:00 pm, and the remaining food was removed at 9:00 am the following day, resulting in a feeding period of 20 h. Additionally, it was imperative that the dry food following each meal was discarded and not reused. On the second day of feeding, it was necessary to interchange the right and left containers to minimize the potential error caused by the animals' directional preference. During palatability testing, the experimental cats had free access to water between 9:00 am and 13:00 pm but were not fed other kinds of experimental food. CF0 and CF1 were both accurately weighed at 100 g each, and the preferred food for each group was recorded. The subsequent palatability tests for CF0 and CF2, CF0 and CF3, and CF0 and CF4 were carried out in the same sequence as that described above. The first choice (the experimental cat eats the first ration as its first choice) and the amount of each type of food left were carefully recorded.[13] The intake ratio of the sample was calculated by dividing the grams consumed by the number of grams provided.[14] Following the tests, the experimental cats were collectively housed and given free movement.

HS-SPME. The SPME procedures have been optimized and modified based on a previously reported method.[15] 5.0 g of CF and 5 μL of 1,2-dichlorobenzene (dissolved in acetone with a concentration of 100 mg/kg) were placed in a 15 mL headspace vial. The headspace vial was placed in a 55 °C water bath for 20 min of equilibration. A flex fiber coated with a 50:30 μm layer of DVB/CAR/PDMS (Supelco, Bellefonte, PA) was inserted into the headspace bottle to extract the volatiles of the CFs at 55 °C for 40 min. The extracted fibers were inserted into the injector of a gas chromatograph and maintained at a desorption temperature of 250 °C for 5 min.

SAFE. The volatile compound extraction was performed according to a previous report with minor modifications.[16] The CFs were crushed into powder with a grinder (800A, Yongkang Red Sun Electromechanical Co., Ltd., Yongkang, Zhejiang, China). Then, an aliquot of the powdered CFs (30 g) and 300 μL of 1,2-dichlorobenzene (100 mg/L, solvent: acetone) were mixed. The mixture was extracted three times with 300 mL of dichloromethane as the solvent at room temperature for 1 h using a magnetic stirrer (85−1, Shanghai Meiyingpu Instrument Manufacturing Co., Ltd., Shanghai, China). After filtration, organic phase extracts were obtained. Afterward, the organic extracts were concentrated to 200 mL using a rotary evaporator (RE 52−86A, Shensheng Co., Ltd., Shanghai, China). The concentrates were subjected to high vacuum distillation using the SAFE technique under a relatively low temperature (40 °C) and high vacuum (5×10^{-5} mbar) to separate the volatiles. The extract was dried with anhydrous sodium sulfate before filtration and concentration to 5 mL by a rotary evaporator at 40 °C.[17,18] The above extraction solution was further concentrated to 1 mL with a nitrogen stream. The concentrate was stored at −20 °C before analysis.

GC-MS Analysis. The analysis of volatile compounds in CFs was performed using an Agilent 6890 gas chromatograph equipped with a 5975 mass selective detector (Agilent Technologies, Santa Clara, CA), with separation carried out on two different polar columns of DB-5 analytical fused silica capillary column and HP-INNOWax analytical fused silica capillary column (both 60 m × 0.25 mm i.d. × 0.25 μm film thickness; Agilent Technologies). For mass spectrometry (MS), the electron impact (EI) ionization energy was 70 eV with a mass range of 30−400 m/z in a full-scan mode, and the temperature of the ion source and quadrupole temperature were set to 230 and 150 °C, respectively. The GC oven temperature was set to 40 °C and maintained for 3 min, then increased to 100 °C at a rate of 3 °C/min, heated to 180 °C at a rate of 4 °C/min, ramped at a rate of 5 °C/min to 230 °C, and finally held at 230 °C for 10 min. The flow rate of helium (99.999%) as a carrier gas was 1.2 mL/min in a splitless mode.[19]

Identification and Quantification of Volatile Compounds. The volatile compounds in CFs were qualitatively identified by the NIST Mass Spectral Library (2020 version) and comparison of their linear retention indices (RIx) with reference values.[20] The calculation of linear RIx is shown below

$$\mathrm{RI}_x = \left(\frac{\lg(t_x) - \lg(t_z)}{\lg(t_{z+1}) - \lg(t_z)} + z \right) \times 100$$

where z is the carbon number of n-alkane, which appears in front of the identified compound under the same GC conditions, $t(x)$ is the retention time of volatile compounds, and $t(z)$ is the retention time of n-alkanes before and after the retention time of the compound (x).

The volatile compounds in CFs were semiquantified according to the following formula

$$c = c_s \times v_s \times A_i / m_0 \times A_s$$

where c is the concentration of volatile compounds (μg/g), c_s is the concentration of the internal standard (mg/L), v_s is the volume of the internal standard (μL), m_0 is the quantity of the CF samples (g), and A_i and A_s are the peak area of volatiles and the internal standard, respectively.

The quantitative methodology was slightly adapted based on the work of Huang et al.[17] To obtain precise quantitative data on aroma-active compounds, base CF was ground and extracted with dichloromethane until no aroma compound could be detected from base CF. 10 mg portion of each standard compound and 300 μL of the internal standard (1,2-dichlorobenzene) were added into 10 mL of dichloromethane. The resulting mixture of standards served as the initial solution. Then, the original solution was diluted to five levels (1:5, 1:10, 1:50, 1:100, and 1:200) for calibration. The original

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Table 1. Free Amino Acid Content of Four Chicken Liver Protein Hydrolysates[b]

amino acid	concentration[a] (mg/100 mL)			
	CLPH1	CLPH2	CLPH3	CLPH4
Asp	26.87 ± 0.2^{d}	30.29 ± 0.1^{c}	33.89 ± 0.5^{b}	35.2 ± 0.45^{a}
Glu	46.77 ± 0.05^{c}	41.2 ± 0.45^{d}	48.49 ± 0.05^{a}	47.27 ± 0.15^{b}
Ser	12.05 ± 0.35^{c}	14.08 ± 0.2^{b}	16.28 ± 0.25^{a}	14.32 ± 0.15^{b}
Gly	23.56 ± 0^{d}	28.55 ± 0.1^{c}	35.1 ± 0.05^{b}	42.79 ± 0.4^{a}
Thr	19.37 ± 0.3^{d}	26.56 ± 0.2^{c}	28.5 ± 0.65^{a}	27.56 ± 0.2^{b}
Ala	25.04 ± 0.3^{d}	30.83 ± 0.31^{c}	36.79 ± 0.1^{a}	35.4 ± 0.2^{b}
Pro	21.71 ± 0^{a}	20.83 ± 0.05^{b}	19.67 ± 0.45^{c}	17.77 ± 0.05^{d}
His	9.27 ± 0.2^{c}	13.88 ± 0.35^{a}	4.22 ± 0.1^{d}	12.42 ± 0.35^{b}
Arg	30.41 ± 0.1^{d}	38.89 ± 0.15^{c}	45.84 ± 1.7^{b}	48.56 ± 0^{a}
Val	21.89 ± 0.4^{bc}	22.41 ± 0.4^{b}	23.11 ± 0^{a}	21.69 ± 0.3^{c}
Met	9.16 ± 0.15^{c}	11.09 ± 0.15^{b}	18.31 ± 0.3^{a}	18.16 ± 0.3^{a}
Phe	18.49 ± 0.7^{c}	23.51 ± 0.45^{b}	25.45 ± 0.2^{a}	24.05 ± 0.65^{b}
Ile	25.26 ± 0.8^{a}	25.41 ± 0.25^{a}	23.21 ± 0.1^{b}	21.45 ± 0.1^{c}
Leu	33.36 ± 0.15^{d}	49.8 ± 0.25^{c}	57.97 ± 0.15^{a}	54.8 ± 0.45^{b}
Tyr	12.12 ± 0.2^{d}	16.85 ± 0.45^{c}	18.68 ± 0.1^{b}	20.69 ± 0.68^{a}
Cys-s	2.31 ± 0.5^{a}	2.53 ± 0.65^{a}	2.4 ± 0.08^{a}	3.02 ± 0.15^{a}
Lys	34.26 ± 0.35^{b}	36.54 ± 0.05^{a}	29.31 ± 0.15^{c}	26.73 ± 0.1^{d}
umami	73.64 ± 0.27^{b}	71.49 ± 0.53^{b}	82.38 ± 0.57^{a}	82.47 ± 0.62^{a}
sweetness	92.59 ± 1.25^{b}	104.61 ± 1.33^{a}	92.39 ± 0.82^{b}	95.34 ± 0.71^{b}
bitterness	74.16 ± 1.32^{c}	87.81 ± 0.79^{b}	94.5 ± 1.50^{a}	94.06 ± 0.77^{a}
other	131.51 ± 1.49^{c}	169.34 ± 1.6 ^{b}	197.95 ± 1.38^{a}	200.01 ± 1.83^{a}
total	371.9 ± 3.88^{c}	433.27 ± 3.72^{b}	467.24 ± 4.02^{a}	471.87 ± 3.82^{a}

[a]Results are expressed as "Mean ± SD" (n = 3). [b]Values bearing different letters (a, b, c, and d) are significantly different ($p \leq 0.05$).

Table 2. Molecular Mass Distribution of Peptides in Four Chicken Liver Protein Hydrolysates[b]

peptide molecular mass (Da)	peptide components[a]/%			
	CLPH1	CLPH2	CLPH3	CLPH4
<180	63.86 ± 1.05^{b}	64.15 ± 0.58^{b}	63.76 ± 0.87^{b}	66.44 ± 0.57^{a}
180−500	25.12 ± 0.42^{b}	27.16 ± 0.36^{a}	26.5 ± 0.77^{a}	24.22 ± 0.41^{b}
500−1000	7.05 ± 0.2^{a}	5.72 ± 0.43^{c}	6.46 ± 0.38^{ab}	6 ± 0.07^{bc}
1000−3000	3.36 ± 0.09^{a}	2.43 ± 0.19^{b}	2.72 ± 0.09^{b}	2.67 ± 0.29^{b}
>3000	0.61 ± 0.05^{a}	0.55 ± 0.03^{b}	0.55 ± 0.02^{b}	0.66 ± 0.07^{a}

[a]Results are expressed as "Mean ± SD" (n = 3). [b]Values bearing different letters (a, b and c) are significantly different ($p \leq 0.05$).

solution was mixed with the odorless base CF. The pretreatment was conducted using the same method as previously described in the SAFE method. The calibration curves were constructed by plotting the concentration ratio of the standard compound to 1,2-dichlorobenzene against the corresponding peak area ratio.

Validation Experiment. The reliability of the PLSR model for predicting key aroma compounds was further investigated through validation experiments. Before the validation experiment, a control group was established by selecting a base food without the application of cat food attractants spray. Then, three compounds with significant positive correlation, two compounds with significant negative correlation, and three compounds with no significant correlation were selected and sprayed on the base food. Preference tests were performed separately with the control group. Each of the volatile compounds was added to the base food concentration to determine the maximum concentration of the four kinds of CFs. The preference test was chosen using the same method employed to measure palatability as the two-bowl method described earlier.

Statistical Analysis. Statistical data were analyzed with Microsoft Excel 2019 (Microsoft, Redmond, WA). Data from GC-MS were evaluated by analysis of variance (one-way ANOVA) of SPSS 21 (SPSS Inc., Chicago, IL). Nonparametric testing methods, specifically the Kruskal−Wallis H test and the Mann−Whitney U test, were employed to evaluate the differences in intake ratio and first choice ratio among different cat food types. The heat map and histogram of aroma compounds in CFs were created using Origin 2023 software (OriginLab Corporation, Northampton, MA). The OPLS-DA was carried out using SIMCA 14.1 (Umetrics, Sweden). The volatile compounds data set was assigned as X-variables, and the cat sensory results of the preference test were designated as Y-variables in PLSR analysis by Unscrambler 10.4 (CAMOAnalytics, Montclair, NJ).

RESULTS AND DISCUSSION

Free Amino Acid and Peptide Molecular Mass Distribution of Four Chicken Liver Protein Hydrolysates. The degree of hydrolysis (DH) of protein hydrolysates from four chicken livers, at dry matter concentrations of 10, 15, 20, and 25%, was found to be 18.22, 22.57, 25.30, and 25.19%, respectively. The results presented in Tables 1 and 2 demonstrate significant variations in the molecular mass of free amino acids and peptides among the four chicken liver protein hydrolysates. From the Table 1 data, it can be observed that there are significant differences in the concentrations of free amino acids under different degrees of hydrolysis (CLPH1, CLPH2, CLPH3, CLPH4), ranging from 371.90 to 471.87 mg/100 mL. This variance indicates that the degree of hydrolysis is a crucial factor affecting amino acid release, which could further impact the flavor formation of Maillard reaction-induced food attractants. For instance, the concentration of Asp is lower under lower degrees of hydrolysis (CLPH1 and

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CLPH2) and higher under higher degrees of hydrolysis (CLPH3 and CLPH4). Conversely, Leu exhibits a higher concentration in CLPH1 and CLPH2 but a lower concentration in CLPH3 and CLPH4. Different amino acids exhibit varying behaviors under different degrees of hydrolysis, which may be related to their positions within the protein.

Based on cats' perception of amino acid taste, we can categorize these amino acids into umami, bitter, and sweet types.[1,21] Studies have shown that cats' sensitivity to umami taste is far greater than that of humans, and they can bind to at least six amino acids such as Gly and Ala with their umami receptors. Changes in the concentrations of these amino acids may affect cats' preferences and appetite for food. Although cats cannot taste sweetness, they may respond to other characteristics of sweet amino acids such as texture and aroma. However, cats tend to exhibit aversion toward bitter amino acids. As the focus of this study is to observe the effect of hydrolysis degree on the flavor produced by the Maillard reaction, the specific impact of these changes on the palatability of cats will be elaborated in future research.

During enzymolysis, the relative molecular weight distribution of the peptides undergoes potential alterations. As evident from Table 2, the peptides resulting from enzymatic hydrolysis predominantly have a molecular weight below 3 kDa. Specifically, the proportion of peptide molecules with a molecular weight less than 180 Da in CLPH4 is conspicuously higher compared to those in the other three chicken liver protease hydrolysates. While the percentages of peptide molecules ranging from 180 to 500 Da in CLPH2 and CLPH3 remain similar, they are notably higher than those in CLPH1 and CLPH4. Interestingly, the proportion of peptide molecules within the 1000−3000 Da range in CLPH1 is significantly greater than that in the other three chicken liver protease hydrolysates. Moreover, CLPH1 and CLPH4 exhibit no significant difference in the percentage of peptide molecules exceeding 3000 Da, but these values are significantly higher than those in CLPH2 and CLPH3. Chen et al.[22] discovered that flavor peptides are usually small molecular mass peptides with a molecular mass of less than 1 kDa, and peptides with smaller relative molecular mass are easier to form more volatile compounds as precursors of flavor substances in meat.

Preference Test of Cat Food Attractants. The preference test is a key method to evaluate the preference of animals for pet foods with different flavors. The preference of the experimental cats for cat food coated with four different attractants was assessed. As depicted in Figure 1, the intake ratio and first choice ratio were evaluated for the four types of cat foods. Significant variations in intake ratio were observed between CF1 and CF2, as well as CF4, whereas CF1 and CF3 exhibited no such significant difference. Among CF2, CF3, and CF4, there were no significant differences in the intake ratio. Regarding the first choice ratio, a distinct difference was evident in all pairwise combinations, except between CF1 and CF2. The descending order of the first choice ratio is as follows: CF4 (70.00%), CF1 (60.00%), CF2 (60.00%), and CF3 (55.00%). These differences in palatability were mainly attributed to the DH of the chicken liver enzymatic hydrolysates. The different degrees of hydrolysis can result in different levels of the free amino acid content and peptide molecular mass distribution in the hydrolysates of chicken liver enzymatic hydrolysis. Free amino acids and peptides are important flavor precursors that undergo Maillard reactions with reducing sugars to enhance aroma.[23] A previous study has

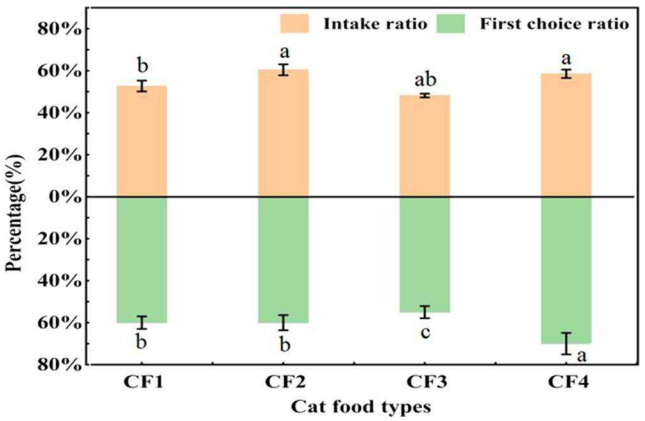

Figure 1. Results of intake ratio and first choice ratio (two-bowl method). Note: 10 experimental cats were fed a set of test diets (CF1/CF2/CF3/CF4) daily for 2 days. The preferred feed was used to calculate the preference rate and the remaining feed in the tray was used to calculate the intake rate. Different letters indicate significant differences ($p \leq 0.05$).

reported that chicken liver protein polypeptides, with a relative molecular mass exceeding 5000 Da, exhibit inhibitory effects on flavor formation in the Maillard reaction.[24] Another research suggested that in MRPs of chicken enzymatic hydrolysate, a peptide fraction <500 Da contributed to the roasted scent of chicken, and a peptide fraction >3 kDa imparted a bitter taste.[25] The length of the peptide chain can significantly impact the flavor profile characteristics of MRPs. Therefore, the significantly higher intake ratio of CF2 was attributed to the higher content of peptide molecules <500 Da. Besides, a higher amount of free amino acid can facilitate the generation of key flavor compounds in subsequent Maillard reaction, such as aldehydes, ketones, furans, thiophenes, pyrazines, pyrrols, and so forth.[26] CF4 contains the highest amount of free amino acids, which contributed to the higher first choice rate of CF4 than other CFs.

Identification and Quantitation of Aroma Compounds in Different Cat Foods. Based on the analytical results obtained from the HS-SPME/SAFE-GC-MS technique, a total of 50 volatile aromatic compounds were detected and identified. The results revealed the presence of 9 acids, 5 aldehydes, 7 alcohols, 12 esters, 3 ketones, 1 phenols, 7 terpenes, and 6 heterocyclic compounds (Table 3). The quantitative ion fragments and calibration curves (m/z) of the scanned aromatic compounds are presented in Table 4. The aromatic compounds in the cat food exhibited excellent linearity ($R^2 > 0.99$).

To more intuitively represent the content of volatile compounds in four types of cat food, a heat map analysis was performed based on the content of volatile compounds in Table 4 (Figure 2A). The 50 odor compounds were clustered using hierarchical cluster analysis and visualized through heat maps. The utilization of distinct hues was employed to discern the variations in the content among diverse compounds. The colors ranging from green to yellow correspond to varying levels of content, with green indicating high content and yellow indicating low content. The four cat foods can be classified into two primary groups: CF1 and CF2, and CF3 and CF4. The 50 flavor compounds are categorized into two primary groups: Group I (A1−D1) and Group II (A2−G7) (Figure 2A). As can be seen from Figure 2A, a relatively high content of

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Table 3. Identification of Aroma Compounds in Four CFs

no.[a]	name	RI[b] DB-5	RI[b] HP-INNOWax	identification[c]	odor description[d]
	acids				
A1	propanoic acid	710	1526	MS,RI,Std	sour
A2	3-methyl-pentanoic acid	941	1783	MS,RI,Std	sour, fruity
A3	butanoic acid	824	1639	MS,RI,Std	rancid, buttery
A4	heptanoic acid	1076	1958	MS,RI,Std	rancid, sour
A5	nonanoic acid	1272	2192	MS,RI,Std	rancid, cheese
A6	octanoic acid	1182	2039	MS,RI,Std	fatty, sweaty
A7	hexanoic acid	990	1838	MS,RI,Std	sweaty, sour
A8	4-methyl-pentanoic acid	949	1813	MS,RI,Std	cheese
A9	octadecanoic acid	2162	3136	MS,RI,Std	fatty
	aldehydes				
B1	benzaldehyde	964	1530	MS,RI,Std	almond
B2	nonanal	1102	1396	MS,RI,Std	fatty, fresh
B3	(*E*,*Z*)-2,4-decadienal	1293	1779	MS,RI,Std	fried fatty
B4	(*E*,*E*)-2,4-decadienal	1314	1819	MS,RI,Std	fatty, meat
B5	*α*-ethylidene-benzeneacetaldehyde	1273	1896	MS,RI,Std	sweet, honey
	alcohols				
C1	phenylethyl alcohol	1120	1872	MS,RI,Std	rose-like
C2	cedrol	1601	2149	MS,RI,Std	sweet
C3	2-ethyl-1-hexanol	1030	1490	MS,RI,Std	floral
C4	3-penten-2-ol	774	1177	MS,RI,Std	green
C5	1-octanol	1078	1562	MS,RI,Std	citrus
C6	eucalyptol	1020	1216	MS,RI,Std	herbal
C7	1-octen-3-ol	1002	1451	MS,RI,Std	mushroom
	esters				
D1	dodecanoic acid, ethyl ester	1581	1835	MS,RI,Std	sweet, floral
D2	decanoic acid, ethyl ester	1381	1647	MS,RI,Std	fruity
D3	triacetin	1306	2077	MS,RI,Std	tropical fruity
D4	diethyl malonate	1069	1572	MS,RI,Std	sweet
D5	7-butyl-2-oxepanone	1537[e]	2013[e]	MS,RI,Std	creamy
D6	propanoic acid, 2-methyl-, dodecyl ester	1796[e]	2000[e]	MS,RI,Std	fatty
D7	butyrolactone	908	1635	MS,RI,Std	caramel aromatic
D8	octanoic acid, ethyl ester	1184	1435	MS,RI,Std	wine, sweet
D9	nonanoic acid, ethyl ester	1319	1568	MS,RI,Std	tropical, fruity
D10	hexanoic acid, ethyl ester	999	1223	MS,RI,Std	sweet, fruity
D11	pentanoic acid, 4-oxo-, methyl ester	946	1560	MS,RI,Std	caramellic
D12	*n*-decanoic acid, ethyl ester	1382	2265	MS,RI,Std	sour, fatty
	ketones				
E1	2-nonanone	1091	1398	MS,RI,Std	floral
E2	3-octen-2-one	1016	1416	MS,RI,Std	herbal, mushroom
E3	2-pentadecanone	1800	2023	MS,RI,Std	fatty, sweet
	phenols				
F1	phenol	978	1962	MS,RI,Std	rubber
	terpenes				
G1	styrene	915	1242	MS,RI,Std	balsamic, gasoline
G2	limonene	1033	1203	MS,RI,Std	citrus, herbal
G3	D-limonene	1523[e]	1810[e]	MS,RI,Std	sweet, orange
G4	*γ*-terpinene	1062	1255	MS,RI,Std	oily, herbal
G5	*β*-myrcene	992	1173	MS,RI,Std	peppery, spicy
G6	*β*-pinene	980	1136	MS,RI,Std	hay
G7	*β*-ocimene	1023	1251	MS,RI,Std	tropical
	heterocyclics				
H1	1-(1*H*-pyrrol-2-yl)-ethanone	1072	1980	MS,RI,Std	nut, coumarinic
H2	methylpyrazine	827	1257	MS,RI,Std	nutty, chocolate
H3	2,5-dimethylpyrazine	908	1328	MS,RI,Std	roasted, beans-like
H4	2-propylpyridine	980		MS,RI,Std	fatty, roasted
H5	2-pentyl-furan	1001	1231	MS,RI,Std	fruity, metallic
H6	2-ethyl-6-methyl-pyrazine	986	1389	MS,RI,Std	nutty

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Table 3. continued

[a]No.: A/B/C/D/E/F/G/H represent the types of compounds including acids, aldehydes, alcohols, esters, ketones, phenols, terpenes, and heterocyclics. [b]Retention index of compounds on DB-5 and HP-INNOWax columns. [c]MS means identification by comparison with the NIST 20 mass spectra database; RI means confirmed by comparison of the retention index with reference standards (https://webbook.nist.gov/); and S means identified by authentic aroma standards. [d]Odor descriptors from the database (http://www.thegoodscentscompany.com). [e]The retention index is calculated.

compounds from region I was detected in CF2. Among them, the compounds with higher content included 3-octen-2-one (E2), which provides a mushroom aroma, and 2-propelpyridine (H4) and nonanal (B2) with a fatty aroma. The levels of compounds A2−G7 were significantly elevated in CF3 and CF4, with high concentrations of H2 (methylpyrazine), D5 (7-butyl-2-oxepanone), and D6 (propanoic acid, 2-methyl-, dodecyl ester) observed specifically in CF3. These compounds were reported in the literature to exhibit nutty, creamy, fatty, and burnt flavor.[27] B4 ((*E*,*E*)-2,4-decadienal), D10 (hexanoic acid and ethyl ester), and G1 (styrene) are found in high amounts in CF4, providing a meaty, fatty, and fruity flavor. It can be observed (Figure 2B) that CF1 and CF2 exhibited relatively higher levels of compounds including alcohols and acids. For instance, a set of four commonly encountered acids are comprised of propanoic acid, butanoic acid, octanoic acid, and hexanoic acid. Seven compounds were common to both CF3 and CF4, including one aldehyde, one ester, three terpenes, and two heterocyclic compounds. Aldehyde compounds often exhibit barbecue, meaty, and fatty aromas. Their typical production pathway involves the oxidation of fats to generate hydrogen peroxide, which subsequently undergoes subsequent reactions.[28] Aldehyde compounds generally have low odor thresholds, thus presenting a significant impact on the aroma of products.[29] Acidic compounds are the final products of the oxidation of hydrocarbon organic compounds. In comparison to aldehydes, their odor thresholds are generally higher. Alcohol compounds exhibit low odor thresholds and are primarily formed through the oxidation and degradation processes of lipids.[30] Among the contributions of unsaturated acids to flavor, the main ones are cheese and fat aroma.[31] Pyrazine compounds have been reported multiple times in the Maillard system of low molecular mass peptides.[32] High content of pyrazine compounds was identified in CF2, which was speculated to be significantly related to the low molecular mass peptide content in CF2. The number of compounds in CF1 and CF2 is lower compared to that observed in the CF3 and CF4 compounds (Figure 2C). Figure 2B,C is consistent with the results of the clustering heat map of flavor compounds. The above results confirmed that the free amino acid content and peptide molecular mass distribution affected the process of the Maillard reaction and further conduced to different flavor characteristics of the food attractants.

OPLS-DA Analysis of Different Cat Foods. OPLS-DA is a widely employed statistical method for discriminating between two or more distinct groups. It is a statistical model based on the partial least squares discriminant analysis method that can model and classify multiple groups. In OPLS-DA, the score plot is an intuitive visualization tool used to show the distribution of samples in principal component space and reveal differences between categories. The aromatic compound content of four CFs was subjected to OPLS-DA analysis in order to elucidate the discrepancies among them. The obtained results showed a satisfactory fitting of the independent variables (R^2 X = 0.925), dependent variables (R^2 Y = 0.988), and model prediction (Q^2 = 0.976), all of which exceeded the threshold value of 0.5 (Figure 3A). These results indicated that the model fitting outcomes were reliable. After 200 permutations, the intersection of the Q^2 regression line with the vertical axis was less than 0, indicating that the model was not overfitted and the model validation was valid. The findings further indicate that effective differentiation of the four cat food samples was achieved (Figure 3B). As can be seen from Figure 3A, CF3 and CF4 are clustered in one area, with CF1 and CF2 dispersed on both sides of the y-axis, suggesting that the different groups of cat food samples can be clearly distinguished from each other. Among the four cat foods, CF3 and CF4 were less different in closer proximity, which is consistent with the previous results of the free amino acid and peptide molecular mass distribution. In order to investigate the key aroma compounds contributing to the classification of the OPLS-DA model described above, VIP scores were employed as criteria for screening differential flavor substances.

VIP values reflect the contribution of flavor substances to the model classification, calculated from the volatile compound composition of the four CFs (Figure 4A). Among the 50 compounds, 17 aromatic compounds had VIP scores higher than 1, including 2 acids, 2 aldehydes, 2 alcohols, 5 esters, 1 ketone, 1 phenol, 2 terpenes, and 2 heterocyclics. The PLSR model was developed using the amino acid content and peptide molecular distribution of the four chicken liver protein hydrolysates (Tables 1 and 2) as the dependent variable (X) and the content of 17 key differential flavor compounds (Table 4) as the independent variable (Y) (Figure 4B). The proximity of samples within the same quadrant positively correlates with their degree of correlation. The inner ellipse in the figure represents R^2 = 0.5, and the outer ellipse represents R^2 = 1. The majority of flavor compounds are situated within the inner and outer ellipses, indicating that the model exhibits a certain degree of predictive capability. As can be seen in Figure 4B, 3-octen-2-one (E2), 2-propylpyridine (H4), and nonanal (B2) correlate most strongly with tasteless amino acids and 180−500 Da peptide molecules. In accordance with the analysis results in Figure 2A, the content of 3-octen-2-one (E2) and 2-propylpyridine (H4) was the most significant in CF2. They provided CF2 with mushroom, barbecue, and fat flavor. Cats are strict carnivores. The results indicated that CLPH2, characterized by the highest content of low molecular mass peptides, can effectively generate meat-flavor-related compounds through the Maillard reaction, thereby significantly enhancing the palatability of CF2. As shown in Figure 4, the closer the distance between the samples, the stronger the correlation. Propanoic acid, 2-methyl-, dodecyl ester (D6), and styrene (G1) had the strongest correlation with umami amino acids. Peptides with molecular mass <180 Da are associated with nonanoic acid (A5) and cedrol (C2). Peptides with molecular mass >3000 Da correlated with hexanoic acid, ethyl ester (D10). Nonanoic acid, cedrol, hexanoic acid, and ethyl ester are flavor compounds that exhibit specificity toward CF4. The above results indicated that the differences in amino acid

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Table 4. External Standard Quantification of Aroma-Active Compounds in Four CFs

no.[a]	compounds	ions (m/z)[b]	calibration equations[c]	R^2	concentration (mg/kg)[d] CF1	CF2	CF3	CF4
	acids							
A1	propanoic acid	74, 28, 45	$y = 1.0052x - 0.0137$	0.9908	0.63 ± 0.101[a]	0.648 ± 0.097[a]	0.43 ± 0.015[b]	0.442 ± 0.027[b]
A2	3-methyl-pentanoic acid	60, 41, 57	$y = 0.0073x - 0.0601$	0.9963	0.63 ± 0.107[a]		0.176 ± 0.06[b]	
A3	butanoic acid	60, 73, 41	$y = 1.0506x + 0.0283$	0.9927	0.402 ± 0.024[b]	0.56 ± 0.052[a]	0.21 ± 0.016[d]	0.308 ± 0.005[c]
A4	heptanoic acid	60, 73, 41	$y = 0.1739x - 0.0013$	0.9960	0.75 ± 0.09[b]	1.355 ± 0.115[a]	0.228 ± 0.021[c]	
A5	nonanoic acid	60, 73,57	$y = 0.062x + 0.0019$	0.9993				0.041 ± 0.005[b]
A6	octanoic acid	60, 73, 43	$y = 0.0036x - 0.0526$	0.9948	0.397 ± 0.024[a]	0.419 ± 0.073[a]	0.111 ± 0.014[b]	0.076 ± 0.007[b]
A7	hexanoic acid	60, 73, 41	$y = 0.0029x - 0.0228$	0.9995	0.863 ± 0.048[b]	0.886 ± 0.152[b]	0.231 ± 0.042[c]	0.378 ± 0.021[c]
A8	4-methyl-pentanoic acid	57, 74, 43	$y = 0.0482x + 0.0085$	0.9928		0.485 ± 0.054[a]		
A9	octadecanoic acid	60, 73, 43	$y = 0.0195x - 0.0012$	0.9998	0.796 ± 0.166[b]	1.38 ± 0.061[a]		
	aldehydes							
B1	benzaldehyde	106, 77, 51	$y = 0.0006x + 0.0042$	0.9910			0.171 ± 0.009[b]	0.164 ± 0.016[b]
B2	nonanal	57, 41, 43	$y = 0.0142x - 0.0037$	0.9839		0.448 ± 0.063[a]	0.194 ± 0.02[b]	0.247 ± 0.03[b]
B3	(*E*,*Z*)-2,4-decadienal	81, 67, 95	$y = 0.0977x - 0.0639$	0.9995			0.09 ± 0.011[b]	0.098 ± 0.007[b]
B4	(*E*,*E*)-2,4-decadienal	81, 41, 67	$y = 1.3025x - 0.0573$	0.9987			0.072 ± 0.009[b]	0.108 ± 0.003[a]
B5	α-ethylidene-benzeneacetaldehyde	117,146,115	$y = 0.0496x - 0.0038$	0.9995	0.092 ± 0.01[b]		0.327 ± 0.046[a]	
	alcohols							
C1	phenylethyl alcohol	91, 92, 65	$y = 0.0430x - 0.0051$	0.9940			0.055 ± 0.006[b]	0.061 ± 0.012[b]
C2	cedrol	95,150,151	$y = 0.0627x - 0.0126$	0.9920				0.053 ± 0.001[b]
C3	2-ethyl-1-hexanol	57, 41, 43	$y = 0.0349x - 0.0109$	0.9975	0.552 ± 0.014[b]	0.622 ± 0.017[a]		
C4	3-penten-2-ol	71, 43, 41	$y = 0.0168x + 0.0036$	0.9997			0.294 ± 0.001[b]	
C5	1-octanol	56, 55, 41	$y = 0.0563x - 0.0073$	0.9997		0.299 ± 0.039		
C6	eucalyptol	41, 81, 108	$y = 0.0247x - 0.0060$	0.9979		0.201 ± 0.026		
C7	1-octen-3-ol	57, 43, 72	$y = 0.0722x - 0.0076$	0.9989	0.237 ± 0.026[b]	0.472 ± 0.061[a]	0.129 ± 0.018[c]	0.095 ± 0.009[c]
	esters							
D1	dodecanoic acid, ethyl ester	88, 101, 43	$y = 0.0451x - 0.0036$	0.9941		0.169 ± 0.033[b]	0.154 ± 0.099[b]	0.087 ± 0.002[b]
D2	decanoic acid, ethyl ester	88, 101, 43	$y = 0.0011x + 0.0015$	0.9986			0.287 ± 0.015[b]	0.375 ± 0.022[a]
D3	triacetin	43, 103, 145	$y = 0.0782x - 0.0007$	0.9838			0.038 ± 0.004[b]	
D4	diethyl malonate	43, 115, 133	$y = 0.3594x - 0.0943$	0.9998	0.426 ± 0.09[b]		0.337 ± 0.008[b]	0.434 ± 0.034[b]
D5	7-butyl-2-oxepanone	85, 84, 57	$y = 1.4624 - 0.0140$	0.9999			0.039 ± 0.002	
D6	2-methylpropionic acid dodecyl ester	88, 73, 57	$y = 0.0136x + 0.0034$	0.9969			0.051 ± 0.005	
D7	butyrolactone	42, 41, 86	$y = 0.1768x - 0.0038$	0.9993	0.046 ± 0.005[b]	0.111 ± 0.014[a]		
D8	octanoic acid, ethyl ester	88, 101, 57	$y = 0.0039x - 0.0003$	0.9988		0.272 ± 0.035		
D9	nonanoic acid, ethyl ester	88, 101, 60	$y = 0.0576x + 0.0012$	0.9993	0.152 ± 0.017[b]			
D10	hexanoic acid, ethyl ester	88, 43, 99	$y = 0.0176x - 0.0162$	0.9996				0.121 ± 0.011
D11	pentanoic acid, 4-oxo-, methyl ester	43, 99, 115	$y = 1.8524x - 0.0063$	0.9965		0.105 ± 0.014		
D12	*n*-decanoic acid, ethyl ester	60, 73, 41	$y = 0.1257x - 0.0059$	0.9991		0.302 ± 0.183[a]		0.046 ± 0.016[b]
	ketones							
E1	2-nonanone	43, 58, 41	$y = 0.6762x + 4.265 \times 10^{-04}$	0.9960		0.058 ± 0.008		
E2	3-octen-2-one	55, 43, 111	$y = 0.0816x + 0.0021$	0.9999		0.087 ± 0.011[a]	0.051 ± 0.007[b]	
E3	2-pentadecanone	58, 43,59	$y = 0.5231x + 0.0089$	0.9990			0.591 ± 0.083[c]	0.76 ± 0.068[b]
	phenols							

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Table 4. continued

no.[a]	compounds	ions (m/z)[b]	calibration equations[c]	R^2	concentration (mg/kg)[d]			
					CF1	CF2	CF3	CF4
F1	phenol	94, 66, 65	$y = 0.0756x - 0.0140$	0.9982			0.05 ± 0.007[b]	
	terpenes							
G1	styrene	104, 103, 78	$y = 0.0108x + 0.0003$	0.9998				0.103 ± 0.017
G2	limonene	68, 93, 67	$y = 0.0459x - 0.0321$	0.9999		0.168 ± 0.006		
G3	D-limonene	68, 93, 67	$y = 0.0386x + 0.0076$	0.9958		0.188 ± 0.015[c]	22.474 ± 0.342[b]	23.83 ± 0.218[a]
G4	γ-terpinene	93, 91, 136	$y = 0.0159x - 0.0088$	0.9949			8.414 ± 0.1[b]	8.649 ± 0.052[a]
G5	β-myrcene	41, 93, 69	$y = 0.0159x - 0.0085$	0.9988			0.814 ± 0.066[a]	0.877 ± 0.054[a]
G6	β-pinene	93, 41, 69	$y = 1.2431x - 0.0047$	0.9946			0.826 ± 0.038[b]	0.87 ± 0.024[a]
G7	β-ocimene	93, 91, 79	$y = 0.0261x - 0.0015$	0.9997			1.869 ± 0.021	
	heterocyclics							
H1	1-(1*H*-pyrrol-2-yl)-ethanone	94, 109, 66	$y = 1.0198x + 0.0081$	0.9931			0.063 ± 0.014[a]	0.05 ± 0.013[a]
H2	methylpyrazine	94, 67, 40	$y = 0.0971x - 0.0017$	0.9828			0.036 ± 0.005[a]	0.018 ± 0.002[b]
H3	2,5-dimethylpyrazine	42, 108, 81	$y = 1.0541x - 0.0111$	0.9988		0.216 ± 0.028[a]		0.049 ± 0.004[c]
H4	2-propylpyridine	93, 106, 120	$y = 1.6183x - 0.0059$	0.9999		0.211 ± 0.027[a]	0.065 ± 0.009[b]	
H5	2-pentyl-furan	81, 82, 138	$y = 0.4013x + 0.0132$	0.9987	0.126 ± 0.014[b]	0.203 ± 0.026[a]	0.072 ± 0.01[c]	0.062 ± 0.006[c]
H6	2-ethyl-6-methyl-pyrazine	121, 122, 39	$y = 1.9326x - 0.0239$	0.9959		0.069 ± 0.009		

[a]No.: A/B/C/D/E/F/G/H represent the types of compounds including acids, aldehydes, alcohols, esters, ketones, phenols, terpenes, and heterocyclics. [b]Scanned ions used for quantitation in selected ion monitoring (SIM) mode. [c]Variables: x is the peak area relative to that of the internal standard, and y is the concentration (mg/kg) in the sample relative to that of the internal standard. [d]Results are expressed as "Mean ± SD" ($n = 3$). Values bearing different letters (a, b, c, and d) are significantly different ($p \leq 0.05$).

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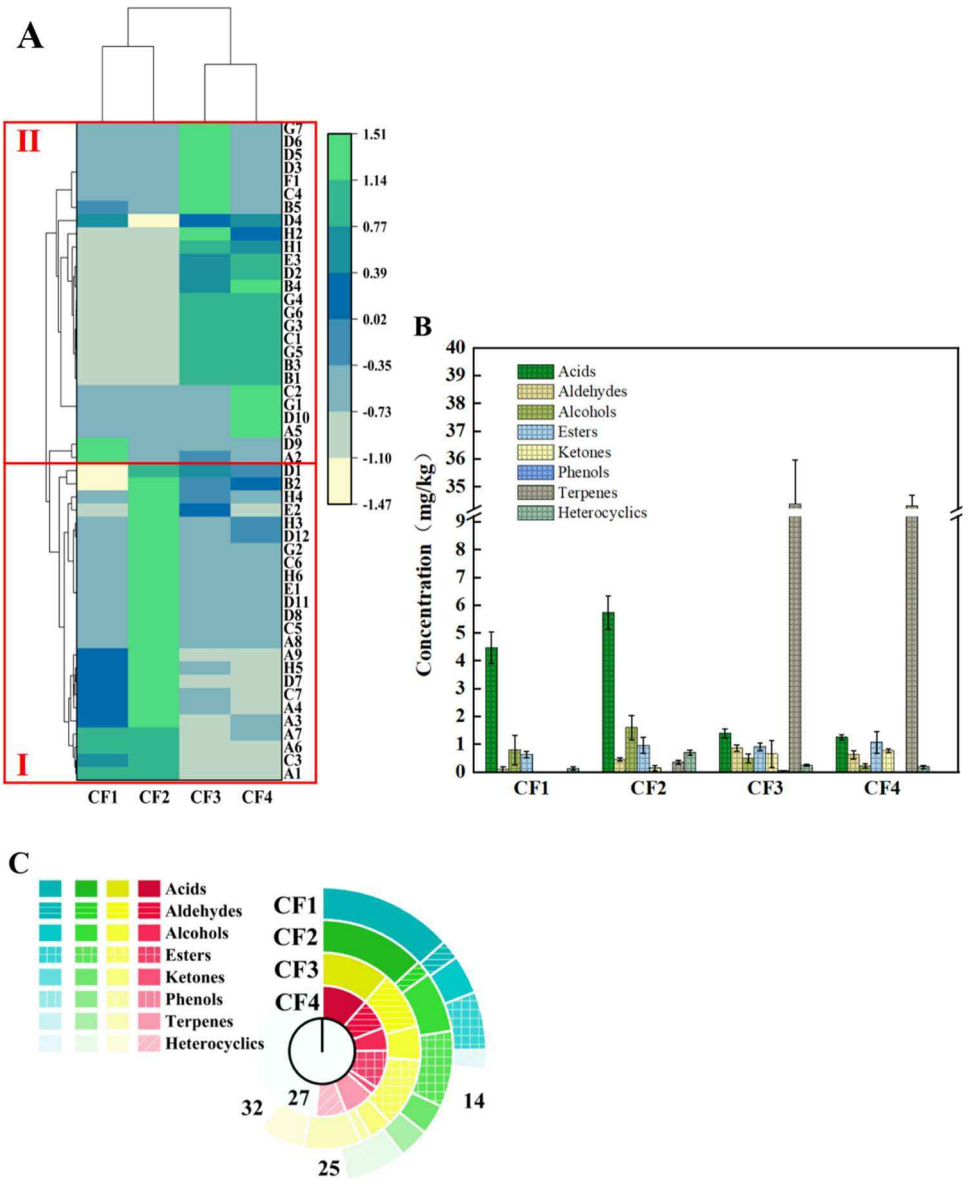

Figure 2. (A) Heat map of the content of volatile aroma compounds in four cat foods. (B) Histogram of the content of different types of aromatic compounds in four cat foods. (C) Circular histogram of the amount of different types of aromatic compounds in four cat foods.

and peptide molecular mass of chicken liver protein hydrolysates caused the diversity of the aroma compounds. Therefore, the palatability might be improved by the changes in free amino acids and peptide length of protein hydrolysates.

Screening of Key Aromatic Compounds by Correlation Analysis. The PLSR model was developed using the content of 50 volatile compounds (Table 4) as the independent variable (X) and the intake ratio and first choice ratio as the dependent variable (Y) (Figure 5A). Most of the volatile compounds lie between the inner and outer ellipses, indicating that the model had some predictive power. Regression coefficient (RC) is an important parameter reflecting the importance of predictive variables to dependent variables in PLSR modeling.[33] To visualize the effect of different flavor compounds on palatability in the PLSR model, RCs were calculated (Figure 5B). As can be seen from Figure 5A, the content of 3-octen-2-one (E2), 2-propylpyridine (H4), 2,5-dimethylpyrazine (H3), 4-methyl-pentanoic acid (A8), eucalyptol (C6), *n*-decanoic acid, ethyl ester (D12), 2-nonanone (E1), butanoic acid (A3), heptanoic acid (A4), octadecanoic acid (A9), 1-octen-3-ol (C7), and butyrolactone (D7) on the intake ratio and first choice ratio of CFs showed a positive correlation. Among them, 4-methylvaleric acid (A8) is a compound with a fruit aroma.[34] In the present study, the highest amount of 4-methyl-pentanoic acid (A8) was identified from CF2. Heptanoic acid (A4) and octadecanoic acid (A9) are frequently detected in animal fats and dairy products, which can be attributed to the incorporation of fat and oil during the spraying or preparation processes of cat food attractants.[35] As can be seen from Figure 5B, the flavor compounds influencing the intake and first choice ratios were essentially identical. Based on the previous analysis, it is evident that 3-octen-2-one (E2) and 2-propylpyridine (H4) are the primary flavor-differentiating compounds among the four cat foods, with both compounds being significantly more abundant in CF2 than in the other three cat foods. This is consistent with the results of the CF2 palatability experiments. Combining Tables 1 and 2, and Figure 4B, it was revealed that

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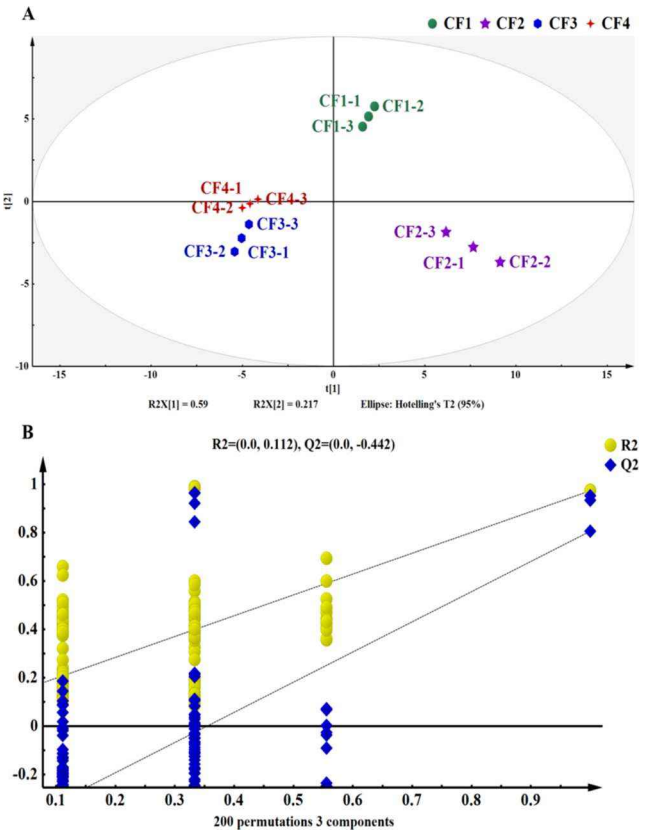

Figure 3. (A) OPLS-DA plots of the volatile compound content of the four CFs. (B) Model replacement test plot.

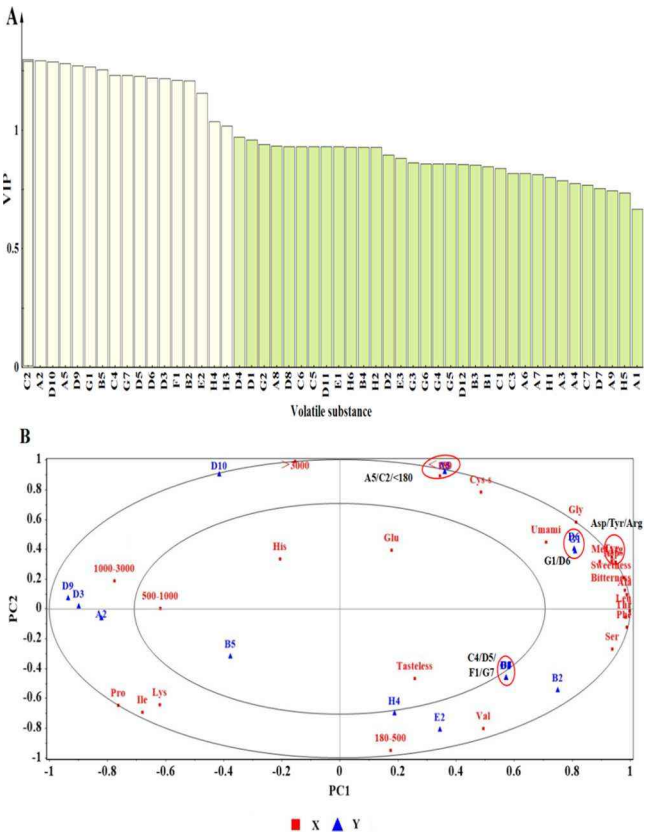

Figure 4. (A) VIP values of each volatile flavor substance in CFs. (B) Correlation loadings plot of the PLSR model between 17 volatile flavor compounds with VIP > 1 (X variable) and free amino acid content and peptide molecular weight distribution of four chicken liver protein hydrolysates (Y variable).

the higher content of peptide molecular mass of 180−500 Da may be favorable for the formation of aroma compounds with strong attraction to cats. An earlier research also reported that the thermal reaction of chicken protein peptides with a relative molecular mass of <500 Da with reducing sugar under suitable conditions was able to generate strong boiled or roasted chicken flavor.[36]

Palatability Validation of Key Volatile Compounds. To verify whether the key aromatic compounds screened by the PLSR model affected the palatability of cat food, a preference test was used for the validation experiment. Three significantly positively correlated compounds, nonanal (B2), 2-propylpyridine (H4), and 3-octen-2-one (E2), two significantly negatively correlated compounds, nonanoic acid, ethyl ester (D9) and 3-methyl-pentanoic acid (A2), and three insignificantly correlated compounds, phenylethyl alcohol, 3-penten-2-ol, and pentanoic acid, 4-oxo-, methyl ester, were selected and added to the base food without the addition of attractants. A control sample was prepared using a basal diet, and the first choice ratio and intake ratio were documented (Table 5). As shown in Table 5, the intake ratio and first choice ratio were significantly higher in the experimental group with the addition of B2, H4, and E2 than those in the control group. The preferred rates for the D9 and A2 treated samples were 40.08 and 47.35%, respectively, which were significantly lower compared to the unflavored substrate control rates of 59.92 and 52.65%. The intake and first choice rates of the three groups with C1, C5, and D11 compound additions were not significantly different from those of the control group. The present validation study demonstrates the effective differentiation capability of OPLS-DA in distinguishing cat food attractants derived from chicken liver protein hydrolysates with varying degrees of hydrolysis based on their respective flavor compounds. Combined with the VIP value above, it can be seen that nonanal (B2), 2-propylpyridine (H4), and 3-octen-2-one (E2) are unique aromatic compounds in CF2. Among them, 2-propylpyridine and 3-octen-2-one are more strongly correlated with 180−500 Da. The peptide content of this fraction was significantly higher in CLPH2 than in CLPH1 and CLPH4, and the intake ratio of CF2 was significantly higher than those of CF1 and CF3. Nonanoic acid, ethyl ester (D9) and 3-methyl-pentanoic acid (A2) were higher in CF1 and strongly correlated with peptides of 1000−3000 Da. The highest content of peptides with 1000−3000 Da in CF1 is associated with its lowest degree of hydrolysis.

3-Methyl-pentanoic acid (A2), α-ethylidene-benzeneacetaldehyde (B5), triacetin (D3), diethyl malonate (D4), and nonanoic acid and ethyl ester (D9) negatively correlated with palatability. 3-Methyl-pentanoic acid (A2) is a carboxylic acid that has been studied and found to be a key flavor substance in tobacco flowers.[37] It is a unique aromatic compound in CF1 and CF3 and may be one of the reasons for the lower intake and first choice ratio of these two cat foods compared to the other three cat foods. α-Ethylidene-benzeneacetaldehyde (B5) is a carbonyl compound containing olefinic (ethylene) and aromatic ring structures and is detected only in CF1 and CF3. The detection of D9 and F2 was absent in CF2, whereas they exhibited the highest level in CF1. According to Figure 4B, the reason for the production of such compounds was may be

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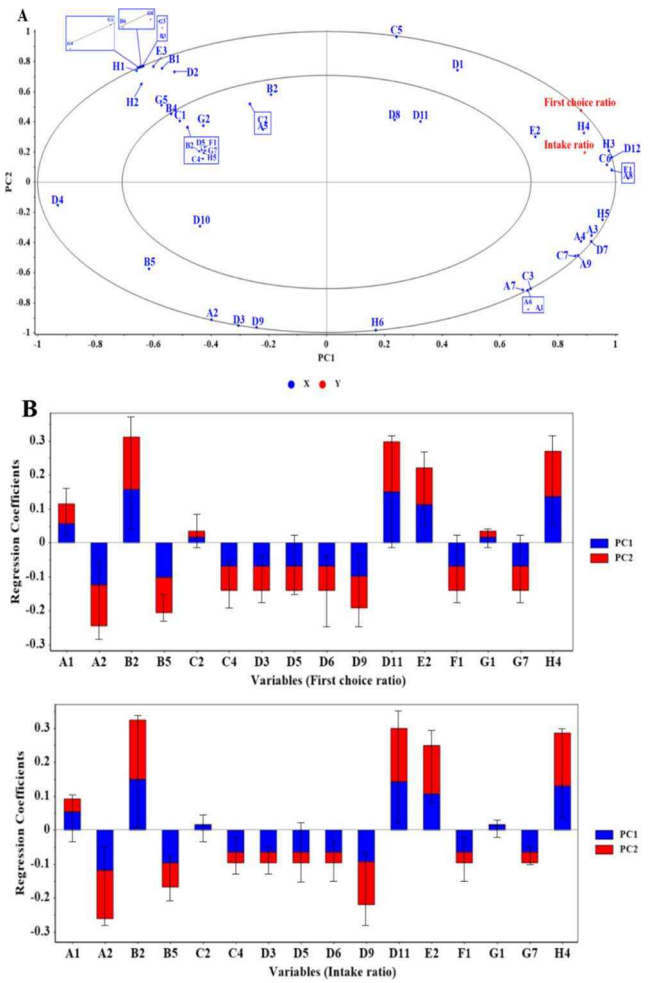

Figure 5. (A) Correlation loading plot of the PLSR model between volatile flavor substances (*X* variable) and experimental results in preference testing (*Y* variable). (B) RCs of aromatic compounds in CFs in the PLSR model.

related to the peptides with the molecular mass of 1000−3000 Da, and the highest molecular mass content of peptides with 1000−3000 Da was found in CLPH1, which was related to the low hydrolysis degree of CLPH1.

In this study, four CF attractants were prepared using four chicken liver protein hydrolysates with different degrees of hydrolysis by the Maillard reaction. Fifty-one volatile flavor compounds were identified in the four CFs. OPLS-DA combined with VIP value results showed 17 key differential compounds among the four CFs attractants. Palatability test results showed that CF2 had the highest intake ratio and CF4 had the highest first choice ratio. The amino acid content and peptide molecular mass distribution of four chicken liver protein hydrolysates and 17 key differential compounds were analyzed by PLSR. It was found that propanoic acid, 2-methyl-, dodecyl ester (D6) and styrene (G1) had the strongest correlation with umami amino acids. Peptides with molecular mass <180 are associated with nonanoic acid (A5) and cedrol (C2). Peptides with molecular mass >3000 correlated with hexanoic acid, ethyl ester (D10). Validation experiments confirmed that nonanal (B2), 2-propylpyridine (H4), and 3-octen-2-one (E2) had a significant effect on enhancing the palatability of cat food. These compounds correlate well with 180−500 Da in chicken liver protein hydrolysates. Nonanoic acid, ethyl ester and 3-methyl-pentanoic acid have significant effects on reducing the palatability of cat food. These compounds are related to the 1000−3000 Da peptide content of chicken liver protein hydrolysates.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.4c02871.

Palatability test results from the individual cats (Table S1) (PDF)

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Table 5. Validation Experiments of Key Volatile Compounds on the Palatability of Attractants

correlation[a]	number	aroma compounds	groups[b]	intake ratio (%)[c]	first choice ratio (%)
significantly positive correlation	B2	nonanal	control	32.54[b]	36.38[b]
			treatment	67.46[a]	63.62[a]
	H4	2-propylpyridine	control	40.43[b]	41.13[b]
			treatment	59.57[a]	58.87[a]
	E2	3-octen-2-one	control	34.62[b]	28.78[b]
			treatment	65.38[a]	71.22[a]
nonsignificant correlation	C1	phenylethyl alcohol	control	50.43[a]	48.91[a]
			treatment	49.57[a]	51.09[a]
	C5	3-penten-2-ol	control	47.32[a]	46.27[a]
			treatment	52.68[a]	53.73[a]
	D11	pentanoic acid, 4-oxo-, methyl ester	control	49.77[a]	52.22[a]
			treatment	50.23[a]	47.78[a]
significantly negative correlation	D9	nonanoic acid, ethyl ester	control	63.3[a]	59.92[a]
			treatment	36.7[b]	40.08[b]
	A2	3-methyl-pentanoic acid	control	56.17[a]	52.65[a]
			treatment	43.83[b]	47.35[b]

[a]The correlations shown here represent the correlation with palatability. [b]Groups: control, without addition of the aroma compound. [c]Different letters (a,b) represent significant differences ($p \leq 0.05$).

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[§]Y.W. and L.X. contributed equally to this work.

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Notes

The authors declare no competing financial interest.

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Supporting Information

Generation of olfactory compounds in cat food attractants: Chicken liver-derived protein hydrolysates and their contribution to enhancing palatability

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Table S1 Palatability test results from the individual cats

No[a]	Intake ratio（%）[b]								First choice ratio（%）[c]							
	CF0	CF1	CF0	CF2	CF0	CF3	CF0	CF4	CF0	CF1	CF0	CF2	CF0	CF3	CF0	CF4
1	54.0%[d]	76.0%	26.0%	67.0%	47.0%	86.0%	22.0%	67.0%		√		√		√		√
2	19.0%	67.0%	86.0%	32.0%	22.0%	63.0%	52.0%	28.0%		√	√			√	√	
3	67.0%	15.0%	68.0%	53.0%	71.0%	14.0%	65.0%	26.0%	√		√		√		√	
4	50.0%	74.0%	23.0%	62.0%	11.0%	94.0%	29.0%	60.0%		√		√		√		√
5	68.0%	49.0%	44.0%	93.0%	21.0%	89.0%	32.0%	94.0%	√			√		√		√
6	39.0%	69.0%	49.0%	30.0%	59.0%	84.0%	16.0%	57.0%		√	√			√		√
7	79.0%	37.0%	14.0%	69.0%	63.0%	22.0%	0.0%	89.0%	√			√	√			√
8	21.0%	57.0%	12.0%	96.0%	2.0%	67.0%	29.0%	59.0%		√		√		√		√
9	66.0%	20.0%	91.0%	65.0%	83.0%	4.0%	33.0%	91.0%	√		√		√			√
10	0.0%	87.0%	42.0%	95.0%	24.0%	61.0%	40.0%	83.0%		√		√		√		√
11	44.0%	83.0%	76.0%	24.0%	40.0%	89.0%	59.0%	28.0%		√	√			√	√	
12	19.0%	59.0%	77.0%	20.0%	48.0%	17.0%	72.0%	12.0%		√	√		√		√	

13	60.0%	17.0%	33.0%	75.0%	54.0%	21.0%	28.0%	67.0%	√			√	√			√
14	18.0%	91.0%	8.0%	71.0%	7.0%	93.0%	26.0%	55.0%		√		√		√		√
15	81.0%	12.0%	31.0%	95.0%	83.0%	2.0%	36.0%	92.0%	√			√	√			√
16	65.0%	29.0%	77.0%	0.0%	96.0%	18.0%	50.0%	17.0%	√		√		√		√	
17	2.0%	97.0%	22.0%	49.0%	0.0%	77.0%	9.0%	73.0%		√		√		√		√
18	20.0%	54.0%	78.0%	27.0%	15.0%	52.0%	30.0%	64.0%		√	√			√		√
19	73.0%	4.0%	54.0%	93.0%	68.0%	2.0%	97.0%	34.0%	√			√	√		√	
20	26.0%	58.0%	38.0%	94.0%	58.0%	10.0%	38.0%	76.0%		√		√	√			√

a. 1-10 represents the first day results of the double-bowl test in 10 cats, and 11-20 represents the second day results of the double-bowl test in 10 cats.

b. Indicates the intake ratio in the two-bowl test.

c. "√" indicates the first choice ratio of the two-bowl method test, here are the results of the first set of experiments.

d. The average of 3 test intake ratio data for individual cat.