Analysis of key precursor peptides and flavor components of flaxseed derived Maillard reaction products based on iBAQ mass spectrometry and molecular sensory science

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A B S T R A C T

Flaxseed derived Maillard reaction products (MRPs) have typical meaty flavor, but there is no report on comparison of their amino acids and peptides reactivity. The peptides and amino acids of flaxseed protein hydrolysates were separately collected by G-15 gel chromatography. Taste dilution analysis (TDA) showed that peptides-MRPs had high umami, mouthfulness, and continuity enhancement. Further, LC-MS/MS revealed that flaxseed protein hydrolysates consumed 41 peptides after Maillard reaction. Particularly, DLSFIP (Asp-Leu-Ser-Phe-Ile-Pro) and ELPGSP (Glu-Leu-Pro-Gly-Ser-Pro) accounted for 42.22% and 20.41% of total consumption, respectively. Aroma extract dilution analysis (AEDA) indicated that formation of sulfur-containing flavors was dependent on cysteine, while peptides were more reactive than amino acids for nitrogen-containing heterocycles. On the other hand, 11 flavor compounds with flavor dilution (FD) ≥ 64 were identified for flaxseed derived MRPs, such as 2-methylthiophene, 2-methyl-3-furanthiol, furfural, 2-furfurylthiol, 3-thiophenethiol, thieno[3,2-b]thiophene, 2,5-thiophenedicarboxaldehyde, 2-methylthieno[2,3-b]thiophene, 1-(2-methyl-3-furylthio)-ethanethiol, 2-methylthieno[3,2-b]thiophene, and bis(2-methyl-3-furyl)-disulfide. In addition, we further demonstrated the flavors formation mechanism of flaxseed derived MRPs.

Introduction

Flax (Linum usitatissimum L.) is an annual herb belonging to the family Flaxaceae, originating in Central Asia or the Mediterranean region and later spread to China and India. Nowadays, flax is planted in Russia, Canada, United States, and other places. The ancient cultivation of flax was mainly intended for obtaining fiber, but later the main use was extended to the consumption of flaxseed oil (Nemeth et al., 2021). Flaxseed oil is favored by consumers because of its rich α-linolenic acid, which can reduce low-density lipoprotein in the body (Bekhit et al., 2018). At present, flaxseed oil can be directly consumed by humans, and flaxseed cakes after oil extraction are used as animal feed and fertilizer, which causes a waste of useful nutritious resources. Previous studies have reported that the protein content of flaxseed cake is about 32–49%, and different functional properties of flaxseed protein and its active peptides, such as antioxidant, antibacterial, anti-inflammatory, antihypertensive and cholesterol-lowering effects have also been reported (Wang et al., 2017).

Maillard reaction is a non-enzymatic browning reaction, which is essentially a condensation reaction between a carbonyl group and an amino group (Ni et al., 2021). It mainly refers to the occurrence of carbonyl groups between aldehydes, ketones and reducing sugars and amino group (Ni et al., 2021). It mainly refers to the occurrence of carbonyl groups between aldehydes, ketones and reducing sugars and...
free amino groups of amino acids, peptides, and proteins. Maillard re-
action is one of the important factors affecting the color, fragrance, taste, 
and quality stability of food. Therefore, the research on this reaction has 
been carried out in many fields, such as food chemistry, food nutrition, 
flavor and fragrance chemistry to fully understand Maillard reaction 
mechanism to make rational use of the flavor produced by Maillard 
reaction.

Peptides and amino acids are more reactive than proteins in the 
Maillard reaction. Therefore, it is usually necessary to enzymolyze the 
protein when making use of animal or plant protein to prepare Maillard 
reaction essence (Wei, Thakur, Liu, Zhang, & Wei, 2018). However, 
the molecular size, amino acid composition, and high-level structure of 
proteins are diverse and complex, resulting in a variety of complex 
protein enzymatic products. To obtain target peptides with outstanding 
taste and high purity, protein hydrolysates should be separated and 
purified (Chen, Lin, Zhao, & Zhu, 2021). At present, the separation and 
purification of peptides mainly focus on ultrafiltration, macroporous 
resin, gel chromatography, high performance liquid phase, etc. Re-
searchers typically use molecular sensory science and mass spectrometry 
to obtain target peptide components (Xu et al., 2019). However, the 
current analytical method is based on the combination of multiple 
separation techniques and sensory evaluation, and finally, the target 
components after multiple separations are identified by mass spec-
trometry. However, the polypeptide information obtained in this way 
has many drawbacks. First, multiple isolations and purifications result in 
loss of polypeptides and changes in peptides. Secondly, the target 
polypeptides finally obtained are often only individual peptides, and it is 
difficult to represent the entire mixed polypeptide sample. Finally, the 
resulting target polypeptide cannot be quantitated and its content in the 
sample could not be determined, resulting in its undetermined contri-
bution to the overall Maillard reaction products (MRPs).

In recent years, the technology of protein and peptide molecular 
identification based on biological mass spectrometry has made great 
progress. Tandem mass spectrometry is a recently developed method for 
peptide sequence analysis (Simon & Andrews, 2019). It refers to the 
production of a series of fragmentary ions by collision-induced dissocia-
tion or post-source decay processes in biological mass spectrometry, 
and through the analysis of these fragmentary ions, the peptide mole-
cules to be determined are obtained amino acid sequence information. 
This method is especially suitable for peptide mixtures and N-terminal 
blocked polypeptide sequence analysis, with high sensitivity (pmol level 
or even lower), accurate results, easy operation and rapid analysis 
(Simon & Andrews, 2019). Such research tools are often used exten-
sively in proteomics research, while the research on Maillard reaction 
barely appeared. iBAQ (intensity-based absolute quantification) belongs 
to Label-Free proteomics quantitative analysis, which can directly 
reflect the original expression information of the protein or peptide in the 
sample, and its quantitative value has a linear relationship with the 
absolute level of the protein (Schwanhausser et al., 2011).

At present, the study of flavor substances in food has gone from the 
identification of volatile components to the level of molecular sensory 
science. Molecular sensory science combines instrumental analysis with 
human sensory analysis in the process of food flavor extraction and 
separation to obtain a defined flavor recombiant (Niu, Deng, Xiao, & 
Zhu, 2021). Chromatography-olfactometry (GC-O) combines the sepa-
ration of gas chromatography with the human sense of smell to study the 
odor of the target component. GC-O-based odor analysis methods, such 
as aroma extract dilution analysis (AEDA), can effectively screen and 
identify odor active substances in foods (Peng et al., 2018). In addition, 
taste dilution analysis (TDA) is a method for discovering a target taste 
component for a component having a strong taste activity in a food (Yu, 
Li, Raza, Wang, & Li, 2019).

In this study, we used Gel permeation chromatography (GPC) to 
separate and purify flaxseed protein hydrolysates and flaxseed derived 
MRPs followed by using high-performance liquid chromatography 
(HPLC) combined with the quadrupole orbitrap mass spectrometer. We 
screened differentially peptides between flaxseed protein hydrolysates 
and flaxseed derived MRPs to explore the possible key peptides for 
Maillard reaction. Further, molecular sensory science was applied to 
analyze the sensory properties and flavor differences of MRPs. To further 
reveal the changes in the types, molecular structure, and content of 
volatile flavors of flaxseed derived MRPs, the mechanism of flavor for-
mation was elucidated.

Materials and methods

Materials and chemicals

Flaxseeds were procured from the local market of Xi’an city, Shaanxi, 
China. Defatted flaxseed meal was prepared by removing the oil from 
flaxseed, according to Wei et al. (2018). To identify the generated vol-
atile flavor compounds, L-cysteine (99%), L-xylase (98%), and the 
standard compounds (>95%) were purchased from J&K Chemical Ltd. 
(Beijing, China). The n-alkanes (C5–C30) for retention indices and 1,2-
dichlorobenzene for quantification were purchased from Sigma-
Aldrich (Shanghai, China).

Extraction of high-purity flaxseed protein

Distilled water (DW) with a temperature of 50 °C was added to the 
defatted flaxseed meal at a ratio of material to solution of 1:20, and 
then the pH was adjusted to 9.0. After stirring at 50 °C for 120 min, it 
was filtered, and then the filter residue was repeatedly extracted 3 times 
under the same conditions, and the filtrates were combined. The filtrate 
was allowed to stand for stratification, centrifuged at 5000 r/min for 10 
min, and the supernatant was separated. The supernatant was concen-
trated to 20% of the original volume by rotary evaporation, then the pH 
was adjusted to 5.0, allowed to stand for 180 min, filtered, and the 
precipitate was collected. The precipitate was resuspended in water 
until a concentration of 30–40% (w/v) was reached. High-purity flax-
seed protein powder was prepared by spray drying.

Preparation of high-purity flaxseed protein hydrolysates

According to our previous method, high-purity flaxseed protein hy-
drolysates (FPH) were prepared by spray-drying (Wei et al., 2018). 
Briefly, defatted flaxseed meals were suspended into DW. The samples 
were pre-treated at 85 °C for 30 min. The pH of suspension was accus-
tomed with 1.0 mol/L of sodium hydroxide after cooling to desired 
temperature prior to enzymatic hydrolysis. Enzymatic hydrolysis con-
titions refer to our previous report (Wei et al., 2018). The precipitates 
were separated through centrifugation at 10,000 r/min for 20 min at 
4 °C. Water was added into hydrolysates until the mass concentration 
reached 30–40%.

GPC analysis

The lyophilized sample of 200 mg was taken up in DW (5 mL) and, 
centrifuged at 8000 r/min for 15 min and filtered through 0.22 μm 
microporous membrane. Then the sample was applied on the top of a 
water-cooled 12 mm × 80 cm glass column filled with a slurry of 
Sephadex G-15 (GE Healthcare Bio-Sciences AB. Uppsala, Sweden) 
conditioned with degassed DW. Elution was performed with degassed 
DW at a flow rate of 1 mL/min. The sample was monitored and sepa-
rated at a wavelength of 220 nm using P270 semi-preparative HPLC 
(Aixin, Guangzhou, China).

Preparation of MRPs

For the Maillard reaction, three main ingredients such as 10.0 g of 
high-purity flaxseed protein hydrolysates, 15.0 g of L-xylase, and 5.0 g of 
L-cysteine were mixed with DW for a final concentration of 10% (w/v) at
initial pH of 7.5. The above suspensions were allowed to stand in a thermostatic oil bath at 120 °C with magnetic stirring for 120 min followed by immediate cooling. The obtained MRP powder was screened through 80 mesh size after freeze drying and grinding and then stored at 4 °C prior to the following experiments.

**Molecular weight (MW) distribution**

MW distribution profiles of the prepared samples were determined by Waters e2695 Alliance HPLC system (Moreno-Vilet, Bostyn, Flores-Montano, & Camacho-Ruiz, 2019). The chromatographic conditions were set as follows: TSK gel 2000 SWXL 7.8 i.d. (Tosoh Co., Tokyo, Japan) at 30 °C; acetonitrile/water/trifluoroacetic acid (45/55/0.1, v/v/v) at a flow rate of 0.5 mL/min; injection volume of 10.0 μL.

**Determination of free amino acid**

For determination of free amino acids generated, samples were prepared as per the previous method described by Checa-Moreno, Marmazo, Miron, and Capitán-Valley (2008). Briefly, 0.02 mol/L HCl was added to 1 g sample and the volume was fixed to 10 mL. The solution was centrifuged for 10 min at 4000 r/min. Then after, 1 mL supernatant was added to 1 mL 7% Sulfosalicylic acid and mixed in dark for 1 h followed by centrifugation for 15 min at 15,000 r/min. Subsequently, supernatant was mixed with 250 μL phenyl isothiocyanate-acetonitrile solution and 250 μL triethylamine-acetonitrile solution for 1 h. Subsequently, n-hexane was added, and the lower solution was taken after stratification, and then analyzed by HPLC. The quantification was performed by C-18 Inertil ODS-SP column and the Waters e2695 Alliance HPLC system (Waters, Milford, MA, USA) as per the previous description (Checa-Moreno et al., 2008). Mobile phase A was 0.1 mol/L sodium acetate solution - acetonitrile (97:3), while mobile phase B was acetonitrile - water (4:1); flow rate was 1 mL/min; column temperature was 40 °C; detection wavelength was 254 nm. The elution procedure was as follows: 0–14 min, A was 100% to 85%; 14–29 min, A was 85% to 66%; 30–37 min, A was 0%; 38–45 min, A was 100%.

**Taste dilution analysis (TDA)**

For sensory evaluation, scores given by well-trained team of 17 personnel (10 females and 7 males) between the age group of 23-48 were applied under suitable laboratory environment. The MRPs of flaxseed protein hydrolysates were analyzed by TDA to evaluate their meaty, umami, mouthfulness, and continuity. MRPs were dissolved in 0.5% mass fraction in the umami soup model (1.0% (w/v) monosodium glutamate and 0.5% (w/v) NaCl) to evaluate the effect of MRPs on meaty, umami, mouthfulness, and continuity. The standard of meaty, umami, mouthfulness, and continuity were in accordance with the method of Ogasawara, Yamada, and Egi (2006). The taste criteria were followed as: meaty was 10.0 g of lean beef which was cooked at 121 °C for 60 min, and tasted at 25 °C. The umami was 1% (w/v) sodium glutamate solution. Mouthfulness and continuity were the taste of adding 1% (w/v) sodium glutamate to beef soup. Specifically, mouthfulness referred to the ability of the taste to fill the mouth, while continuity referred to the ability of the taste to be preserved in the mouth. The members evaluated 10.0 mL of each sample in a triangle test with two umami soup as two blanks. The sensory evaluation panels were evaluated in order of MRPs concentration from low to high. When the difference between the taste of a certain dilution level and the blank umami soup was just recognized, the dilution factor at this time was recorded as the taste dilution (TD).

**LC-MS/MS analysis**

The samples were analyzed by hybrid quadrupole orbitrap mass spectrometer (Q Exactive, Thermo Fisher, USA) using liquid chromatography (Easy-nLC 1000, Thermo Fisher, USA). The two mobile phases were as follow: mobile phase A constituted an aqueous solution of 0.1% formic acid, and mobile phase B was a 0.1% formic acid aqueous solution of acetonitrile (acetonitrile was 84%). After equilibration of the column with 95% of the A solution, the sample was loaded from the autosampler to the Trap column. Samples were analyzed by mass spectrometry with Q-Exactive mass spectrometer after chromatographic separation. The analysis time was 120 min; the detection method was positive ion; the ion scanning range was 300–1800 m/z. The mass spectrometry test raw file was searched using Mascot 2.2 software for the corresponding database.

**Gas chromatography-mass spectrometry (GC-MS)**

The volatiles of each sample were extracted with 75 μm carboxen/poly-dimethylsiloxane SPME-fibre (50 °C, 5 min) and identified through comparison of data obtained from GC–MS (Agilent GC–MS 7890 and DB-WAX 30 m × 0.25 mm × 0.25 μm capillary column, Agilent) and NIST 08 (Gaithersburg, MD, USA). Analyzing Kovats indices (KIs) relative to C7-C39 n-alkanes on the capillary column. Two micro liters of 1,2-dichlorobenzene (50 μg, in 1 mL of methanol) was added to each sample as an internal control. All compounds were analyzed with the available standard compounds for identification. Chromatographic conditions were used as per the methods given by Cai, Zhu, Ma, Thakur, and Wei (2020). The column flow rate was set as 1.0 mL/min, using helium as a carrier gas. The column temperature program was maintained at 40 °C for 2 min, 40–80 °C at 3 °C/min, 80–120 °C at 4 °C/min, and 120–230 °C at 10 °C/min for DB-WAX column. The GC was equipped with a mass spectrometric detector which was set at a scanning range of 35 to 450 m/2.

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CV = \frac{Sv}{Si} \cdot Ci
\]

where CV and Ci represent the concentration of volatile compound and internal standard, respectively; Si and Si corresponded to the peak area of volatile compound and internal standard.

**Aroma extract dilution analysis (AEDA)**

Volatile flavors were analyzed by AEDA (Fan et al., 2019). The volatile compounds were sampled with SPME-fibre (75 μm, carboxen/polydimethylsiloxane). Three trained evaluators performed the gas chromatography–olfactometry (GC-O) (HP-5 capillary column, 30 m × 0.25 mm × 0.25 μm, Agilent) analyses using a DATU 2000 high-resolution olfactometry system (DATU Inc., Durham, NC). The dilutions were achieved through a step-wise increase of the injector split ratio from 2:1, 4:1, … to 128:1 obtaining flavor dilution (FD). Nitrogen was used as carrier gas at the rate of 1.0 mL/min. The used capillary column, programmed oven temperatures and desorption of the fiber were similar to those in the aforementioned GC–MS analysis.

**Statistical analysis**

Data analysis was obtained by according one-way ANOVA, which was followed by Duncan’s multiple-range tests through SPSS Statistics 20.0 (SPSS, Inc., Chicago, IL, USA). Experiments were repeated three times and less than 0.05 was defined as significant (P ≤ 0.05). Data were showed as mean ± standard deviation.

**Results and discussion**

**Isolation of flaxseed protein hydrolysates**

Molecular exclusion chromatography separates the sample from the largest to the smallest according to MW. The high MW components were
Eluted at first, and the low MW components were later eluted. The Sephadex G-15 dextran gel used in this experiment was suitable for separating and purifying peptides having a MW of 100–1500 Da. As shown in Fig. 1, the flaxseed protein hydrolysates were separated into three components by molecular exclusion chromatography, namely F1, F2 and F3, respectively. The ratios of F1, F2 and F3 in the chromatogram was 11.40%, 84.30% and 4.30%, respectively. The separation and purification operation were repeated to collect a large number of samples. The components were vacuum freeze dried and stored at 4 °C for subsequent experiments.

**Change in MW distribution and free amino acid composition**

Enzymatic hydrolysis and Maillard reaction have an important influence on the flavor of flaxseed derived MRPs, including both odor and taste. Previous studies have shown that by controlling the enzymatic hydrolysis conditions, the 30–50 kDa flaxseed protein is hydrolyzed into short peptides and free amino acids with a MW of less than 3000 Da (Wei et al., 2018). This amino mixture containing short peptides and free amino acids were thermally reacted after the addition of xylose and cysteine to prepare a meaty seasoning. However, many studies have shown that different short peptides and free amino acids have different Maillard reactivity and effect on flavor (Hou et al., 2017). As shown in Table 1, short peptides and free amino acids of MW less than 1000 Da were the main components of FPH. Since a component of less than 128 Da was regarded as a free amino acid, the FPH can be regarded as a mixture of short peptides and amino acids having a MW of less than 1000 Da, wherein the content of the short peptide was higher. We compared the components (F1, F2 and F3) separated by G-15 gel chromatography with FPH. The results showed that the F1 has more components with a MW greater than 1000 Da (10.43%) compared to the F2 (3.28%) and the F3 (0.00%), while the F3 was mainly composed of free amino acids (63.27%) and contained partial short peptides of less than 500 Da (34.23%). The F2 was the main component obtained by molecular exclusion chromatography, accounting for 84.30% of the total peak area, and its MW distribution was close to that of FPH (Table 1). As an important component in the flaxseed protein hydrolysates, free amino acids are one of the flavor precursors of Maillard reaction and have a significant effect on the sensory properties of MRPs (Cao et al., 2017). We divided free amino acids into total free amino acids, umami amino acids (Glu and Asp) , bitter amino acids (Tyr, Ile, Leu, Val, Phe, and Lys) and sulfur-containing amino acids (Cys and Met) (Liu, Liu, He, Song, & Chen, 2015). As shown in Table 1, it was found from the analysis of free amino acids that the separation of the molecular exclusion chromatography resulted in a large loss of free amino acids. Therefore, the F2 component can be regarded as a component mainly composed of short peptides having a MW of less than 1000 Da in the flaxseed protein hydrolysates (68.27%), while the F3 component was regarded as a component mainly composed of free amino acids in the flaxseed protein hydrolysates (63.27%). In addition, the F1 was more complex, and it contained more peptides with a MW greater than 1000 Da, but the content of the peptide itself was low (10.43%). We prepared enough F1, F2 and F3 to further analyze the flavor formation mechanism of the “FPH - xylose - cysteine” reaction system by molecular sensory science in the following experiment.

**Comparative sensory characteristics by TDA**

TDA is a test method for analyzing the taste or taste enhancement of samples (Istiqamah, Nuryani Lioe, & Adawiyah, 2019). It should be noted that the umami soup (blank control) used in this experiment has a umami, mouthfulness, and continuity, while it has no meaty. Therefore, TDA evaluation of meaty is based on the strength of the meaty itself, and the evaluation of umami, mouthfulness, and continuity is aimed at the enhancement of the taste of MRPs. As shown in Fig. 2 A, we performed a TDA test on the taste standard solution, such as meaty, umami, mouthfulness, and continuity and then determined the TD factor of the taste standard solution. Through the comparison of Pr-MRPs and Re-MRPs, we found that we reorganized F1, F2, and F3 according to 11.40%, 84.30%, and 4.30% to carry out Maillard reaction. Re-MRPs is basically the same as Pr-MRPs in sensory evaluation. We prepared the MRPs (F1-MRPs, F2-MRPs, F3-MRPs, and Pr-MRPs) from F1, F2, F3 and flaxseed protein hydrolysates, respectively. TDA test results for F1-MRPs, F2-MRPs, F3-MRPs, and Pr-MRPs were shown in Fig. 2 B. The results showed that compared with Pr-MRPs, F1-MRPs were significantly lower in meaty, umami enhancement, mouthfulness enhancement, and continuity enhancement (P < 0.05); F2-MRPs showed no statistical difference in meaty, and mouthfulness enhancement (P > 0.05), but significantly higher in umami enhancement, mouthfulness enhancement and continuity enhancement (P < 0.05). Therefore, it can be concluded that the formation of meaty mainly depends on the Maillard reaction between cysteine and xylose in order to produce a sulfur-containing volatile component, which tends to

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**Table 1** Change in molecular weight distribution (%) and free amino acid (mg/g) as function of gel permeation chromatography.

| Molecular weight distribution (%) | F1        | F2        | F3        | FPH       |
|----------------------------------|-----------|-----------|-----------|-----------|
| <128 Da                          | 19.16 ±   | 28.45 ±   | 63.27 ±   | 32.15 ±   |
| 128-500 Da                       | 0.22a     | 0.79b     | 1.08c     | 1.27d     |
| 500-1000 Da                      | 25.59 ±   | 34.61 ±   | 34.23 ±   | 37.65 ±   |
| 1000-3000 Da                     | 44.82 ±   | 33.66 ±   | 2.50 ±    | 25.08 ±   |
| >3000 Da                         | 0.72 ±    | –         | 0.11 ±    | 0.03      |

| Free amino acid (mg/g)           |           |           |           |           |
|----------------------------------|-----------|-----------|-----------|-----------|
| Free amino acids                 | 6.15 ±    | 16.44 ±   | 35.06 ±   | 165.44 ±  |
| Umami amino acids                | 0.20a     | 0.26b     | 0.95c     | 0.36d     |
| Bitter amino acids               | 0.58 ±    | 1.46 ±    | 2.55 ±    | 13.76 ±   |
| Sulfur-containing amino acids    | 1.83 ±    | 5.09 ±    | 8.38 ±    | 42.00 ±   |
|                                  | 0.10a     | 0.14b     | 0.14c     | 0.80d     |
|                                  | 0.19 ±    | 0.52 ±    | 1.09 ±    | 5.44 ±    |
|                                  | 0.01a     | 0.04b     | 0.05c     | 0.14d     |

Means within different letters are significantly (P < 0.05) different in the same line. “-”, not detected.

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**Fig. 1.** Chromatograms representing the fraction of high-purity flaxseed protein hydrolysates using Sephadex G-15 chromatography.

**Table 1** Change in molecular weight distribution (%) and free amino acid (mg/g) as function of gel permeation chromatography.
MRPs were analyzed. As shown in Table 2, mass spectrometry peptide mixtures. The 41 peptides with the largest difference in iBAQ
The identified peptide sequence belonged to the corresponding proteome, and its value was consistent with the iBAQ value of the proteome, approximately equal to the absolute concentration of the protein in the sample. For this, we can focus our analysis on the contribution of peptides with a MW of less than 1000 Da to the flavor in the Maillard reaction. Lancker, Adams, and De Kimpe (2012) reported that the MRPs prepared from dipeptides with different structures, and then the structure and content of pyrazine in the MRPs were studied. Authors found that the content of alkylpyrazines produced by the dipeptide reaction system was higher than the corresponding pure amino acid system, which proved that the peptide can directly participate in the Maillard reaction without degradation, so the peptide and amino acid have different Maillard reaction mechanisms (Lancker et al., 2012). Similarly, Wang, Yang, and Song (2012) found that glutathione breaks down during the Maillard reaction and forms Cys-Gly, which is directly involved in the Maillard reaction. It was worth noting that TDA is a large number of studies have reported the sulfur-containing volatile components, such as thiophenes, mercaptans, etc., which are closely related to meat flavor (Zhao, Wang, Xie, Xiao, Cheng et al., 2019; Zhao, Wang, Xie, Xiao, Du et al., 2019). The MRPs of flaxseed protein hydrolysates and xylose has an effect on the improvement of umami, mouthfulness, and continuity. It was further found that, as flavor precursor, short peptides with a MW of less than 1000 Da (accounting for 68.27% of the F2 component) has a greater effect on the improvement of umami, mouthfulness, and continuity, while the effect of free amino acids (accounting for 63.27% of the F3 component) were less. On the other hand, peptides containing more MW greater than 1000 Da (accounting for 10.43% of the F1 component) have no significant effect on these flavors. Therefore, the flavor of the MRPs should be emphasized that the analysis of the polypeptide sequences was obtained based on the iBAQ algorithm, and its value was approximately equal to the absolute concentration of the protein in the sample. The identified peptide sequence belonged to the corresponding proteome, and its value was consistent with the iBAQ value of the proteome, thereby realizing the quantification of the main peptide sequence in the peptide mixtures. The 41 peptides with the largest difference in iBAQ values between flaxseed protein hydrolysates and flaxseed derived MRPs were analyzed. As shown in Table 2, mass spectrometry information and iBAQ values of the top 10 peptides in the 41 peptides were listed. From the molecular ion peak m/z, it can be seen that the main kinds of peptide consumed by the reaction system were those with MW less than 1000 Da, which is consistent with the results of our MW distribution test. Quantitative results showed that the top 10 peptides of iBAQ value accounted for 88.86% of the iBAQ value of 41 peptides. Thus, the peptides listed in Table 2 can reflect the overall situation of flaxseed protein hydrolysates consumed by Maillard reaction. This means that these 10 peptides can be considered as key precursors of flaxseed derived MRPs. Supplementary Fig. 1 represented the mass spectrum of the top 10 peptides; Supplementary Fig. 2 presented the order of the iBAQ values of the 41 peptides from high to low. In particular, DLSFIP (Asp-Leu-Ser-Phe-Ile-Pro) and ELPGSP (Glu-Leu-Pro-Gly-Ser-Pro) peptides accounted for 42.22% and 20.41% of the total peptide content, respectively. Peptides are widely found in the thermal reactions of various food materials. As a flavor precursor, peptides have different reactivity with amino acids in the Maillard reaction, and their involvement in the Maillard reaction is much more complicated than free amino acids (Zou, Kang, Yang, Song, & Liu, 2019). Lancker et al. reported that the N-terminal amino acid of the peptide chain has a more important role in the Maillard reaction to produce pyrazine-like flavors than the C-terminal amino acid (Lancker et al., 2012). Schlichther-Cerny et al. identified several N-terminal Glu peptides after hydrolysis of wheat gluten protein, such as pGlu-Pro-Ser, pGlu-Pro, pGlu-Pro-Glu, and pGlu-Pro-Gln (Schlichther-Cerny & Amadò, 2002). Stark et al. reported that a series of 2,5-diketopiperazines produced during roasting cocoa beans were associated with flavor precursor peptide Phe-Glu (Stark & Hofmann, 2005). The results of this experiment indicated that the N-termini of the peptides involved in the Maillard reaction in the flaxseed protease were mainly D-X (Asp-X), E-X (Glu-X), G-X (Gly-X), Q-X (Gln-X), A-X (Ala-X), and V-X (Val-X). This means that these types of peptides are easily involved in the Maillard reaction. In addition, it is worth noting that the two most consumed peptides in the Maillard reaction, QTVGQAP (Gln-Thr-Val-Gln-Gly-Ala-Pro) and ELPGSP (Glu-Leu-Pro-Gly-Ser-Pro), have N-terminal amino acids that are umami-related amino acids. Some studies have reported that these amino acids are associated with the full mouthfeel and continuity of MRPs (Song et al., 2016). However, it should be emphasized that the analysis of the polypeptide sequences used in this study does not have the ability to analyze peptides with a MW of less than 350. Therefore, the 41 peptides identified in this experiment had 5 or more amino acids. Therefore, the flavor of the MRPs of each component was further quantitatively analyzed by AEDA. Selective and comparative key flavor components

The volatile components were extracted by SPME and further

Fig. 2. Taste dilution (TD) factor of taste criteria (A) and Maillard reaction products (MRPs) in umami soup (B). The values followed by different letters were significantly different (P < 0.05).
Table 2 (continued)

| Polypeptide sequence | Molecular ion peak m/z ([M + H]⁺) | Primary fragment ion peak m/z | iBAQ value (×10⁶) | Proportion (%)¹ |
|----------------------|----------------------------------|------------------------------|-----------------|---------------|
| AVNDGL               | 588.30                           | b2 (171.08); b3 (285.16); b4 (400.18); b5 (229.13) and 457.25; y2 (189.12); y3 (304.15); y4 (517.27); [M–H]₂O (570.29) | 11.01 | 1.39 |
|                      |                                  |                              |                 |               |

¹ Proportion (%) is the ratio of iBAQ values of each of the 41 polypeptides sequences identified by LC-MS/MS.

analyzed by GC–MS. Furthermore, GC-O was used to analyze the odor characteristics, and then the compounds were identified based on mass spectrometry, KI, odor characteristics, and standard substances to ensure the accuracy of the identification results, in order to facilitate targeted screening of flavor components in a large number of volatile components. As shown in Table 3, 40 flavor-active compounds were identified. From the analysis of Pr-MRPs, the sulfur-containing flavor compounds were the most abundant flavor components (25 species, 1075 ng/g), followed by oxygenates (4 species, 673 ng/g) and nitrogen-containing heterocycles (8 species, 247 ng/g). High content (50 ng/g) in all flavors was 2-methylthiophene, furfural, 2-furfurylthiol, thieno[3,2-b]thiophene, bis(2-methyl-3-furyl)-disulfide, and bis(2-furfuryl) disulfide.

Sulfur-containing compounds are typical meat flavor substances. In the reaction of cysteine and xylene, the Strecker degradation of cysteine produces H₂S and NH₃ (Yu, Tan, & Wang, 2012). Further, 2-furfurylthiol is formed by reacting furfural with H₂S (Liu, Wang, Hui, Fang, & Zhang, 2021); thiophenes are formed by reacting 1,4-dicarbonyl compound with H₂S (Mottram & Elmore, 2010); and thiazoles are formed by reacting aldehydes, dicarbonyl compounds, H₂S and NH₃ (Mottram & Elmore, 2010; Mottram, 1998). As shown in Table 3, we identified 7 pyrazines and 1 pyridine, all of which contained less than 128. For F1-MRPs, F2-MRPs, and F3-MRPs, no obvious change in sulfur-containing flavor components was observed. This is because the main source of the sulfur-containing flavor component is the Maillard reaction of cysteine and xylene, while the flavexseed protein hydrolysates has less effect on the sulfur-containing flavor components.

Nitrogen-containing heterocycles, such as pyrazine, pyrrole, etc., are characteristic products of a type of Maillard reaction, usually having a barbecue taste and a nutty taste, which can cause people to feel the taste of barbecue (Guerra & Yaylayan, 2010). As shown in Table 3, we identified 7 pyrazines and 1 pyridine, all of which contained less than 50 ng/g. Pyrazines are the most important nitrogen-containing heterocycles, usually produced by condensation of aminoketones formed by Strecker degradation (Wen, Ontaíñez, Ferreira, & Lopez, 2018). However, for nitrogen-containing heterocycles, F2-MRPs were significantly more than F3-MRPs in species and content. Therefore, for the Maillard reaction to produce pyrazine compounds, the reactivity of peptides with xylene was higher than that of amino acids and xylene in flaxseed protein hydrolysates. Previous studies have shown that when amino acids participate in the Maillard reaction in the form of lysyl dipeptides, the amount of pyrazines produced is significantly greater than the corresponding free amino acids (Lancker, Adams, & De Kimpe, 2010). From Table 3, it is shown that the content of sulfur-containing flavor substances was close to that of Re-MRPs. On the other hand, sulfur-containing flavor components generally had a high FD factor (≥8). It is worth noting that we identified seven FD ≥ 128 and three FD = 64 sulfur-containing flavors as key meat-flavor compounds. In addition, the FD factor of the flavor component depends not only on the content but also on the flavor threshold. Such as 2-methyl-3-furanthiol, 1-(2-methyl-3-furylthio)ethanethiol, and 3-thiophenethiol, although the odor content were less than 50 ng/g, the FD factor were greater than 128. For F1-MRPs, F2-MRPs, and F3-MRPs, no obvious change in sulfur-containing flavor components was observed. This is because the main source of the sulfur-containing flavor component is the Maillard reaction of cysteine and xylene, while the flaxseed protein hydrolysates has less effect on the sulfur-containing flavor components.
Change in key flavor components of Maillard reaction products in content and FD values as function of gel permeation chromatography.

| No. | Components | χ1s | Amounts (ng/g) | FD values | Odors | Identification methods |
|-----|------------|-----|---------------|-----------|-------|-----------------------|
|     |            | F1-MRPs | F2-MRPs | F3-MRPs | Pr-MRPs | Re-MRPs | F1-MRPs | F2-MRPs | F3-MRPs | Pr-MRPs | Re-MRPs |               |
| 1   | 2-methylthiophene | 785 | 131 | 156 | 200 | 150 | 162 | 64 | ≥128 | 64 | ≥128 | 128 | onion, KI/MS/O/S |
| 2   | 2-methylthiazole  | 801 | 33  | 38  | 27  | 34  | 31  | 8  | 8    | 8    | 8    | 8    | grass, KI/MS/O/S |
| 3   | 2-methyl-3-furanyl | 855 | 20  | 41  | 50  | 34  | 39  | 32 | ≥128 | 64 | 128 | 128 | meaty, KI/MS/O/S |
| 4   | 2-ethylthiazole   | 872 | 427 | 534 | 483 | 607 | 596 | ≥128 | 128 | ≥128 | 128 | 128 | roasted, KI/MS/O/S |
| 5   | Furural         | 886 | 23  | 22  | 31  | 17  | 29  | 4  | 4    | 4    | 8    | 8    | garlic, KI/MS/O/S |
| 6   | 3-methyl-1,2-dithiane | 896 | 125 | 137 | 85  | 75  | 121 | 64 | ≥128 | 64 | 128 | 128 | meaty, KI/MS/O/S |
| 7   | 2-furfurythiol    | 953 | 19  | 31  | 24  | 24  | 28  | 4  | 16   | 4    | 8    | 8    | roasted, KI/MS/O/S |
| 8   | 2-ethyl-3-(methylthio)furan | 969 | -   | 14  | 14  | 25  | 17  | 4  | 4    | 4    | 16   | 4    | roasted, KI/MS/O/S |
| 9   | 1-octen-3-ol      | 1060 | 23  | 30  | 20  | 26  | 29  | 2  | 8    | 2    | 8    | 8    | caramel, KI/MS/O/S |
| 10  | 3-thiophenecarboxaldehyde | 1080 | -   | 23  | 12  | 34  | 37  | 16 | 16   | 16   | 16   | 16   | onion, KI/MS/O/S |
| 11  | 1-(methylthio)-1-hexanethiol | 1108 | -   | 20  | 6   | 15  | 10  | 8  | 8    | 8    | 8    | 8    | roasted, KI/MS/O/S |
| 12  | Pyrazine         | 1117 | 75  | 115 | 87  | 84  | 107 | 16 | ≥128 | 128 | ≥128 | 128 | roasted, KI/MS/O/S |
| 13  | 5-ethyl-2,5-dimethylpyrazine | 1217 | 22  | 17  | 25  | 38  | 49  | 16 | 32   | 16   | 64   | 64   | roasted, KI/MS/O/S |
| 14  | 3-methyl-2-thieno[2,3-c]pyridine | 1223 | -   | 46  | -   | -   | -   | 8  | 8    | 8    | 8    | 8    | roasted, KI/MS/O/S |
| 15  | 1,2,3-trihexadiene | 1226 | -   | 17  | 15  | 27  | 21  | 2  | 4    | 4    | 4    | 4    | roasted, KI/MS/O/S |
| 16  | Thioene[2,3-b]thiophene | 1231 | -   | 46  | -   | -   | -   | 8  | 8    | 8    | 8    | 8    | roasted, KI/MS/O/S |
| 17  | 2,5-thiophenedicarboxaldehyde | 1232 | -   | 46  | -   | -   | -   | 8  | 8    | 8    | 8    | 8    | roasted, KI/MS/O/S |
| 18  | 2-oxo-1-methyl-3-isopropylisoxazole | 1241 | -   | 22  | 18  | 29  | 23  | 8  | 8    | 8    | 8    | 8    | roasted, KI/MS/O/S |
| 19  | 2-pentylpyridine | 1248 | 21  | 25  | 46  | 37  | 8  | 8    | 16   | 8    | 32   | 32   | roasted, KI/MS/O/S |
| 20  | 3-acetyl-2,5-dimethylthiophene | 1259 | -   | 20  | -   | 37  | 52  | -  | 2    | 8    | 8    | 8    | pungent, KI/MS/O/S |
| 21  | 2,3-dimethylpyrazine | 1263 | -   | 28  | 6   | 15  | 10  | 8  | 8    | 4    | 4    | 4    | green, KI/MS/O/S |
| 22  | 2-tetrahydrothiofuran | 1274 | 35  | 25  | 24  | 37  | 55  | 64 | 16   | 8    | 32   | 32   | roasted, KI/MS/O/S |
| 23  | 2-methylthieno[2,3-b]thiophene | 1285 | 41  | 33  | 44  | 30  | 39  | 64 | 32   | 64   | 64   | 64   | burnt, KI/MS/O/S |
| 24  | 1-(2-methyl-3-furyl)-thiophene | 1322 | 44  | 37  | 31  | 48  | 64 | ≥128 | 64   | ≥128 | ≥128 | ≥128 | garlic, KI/MS/O/S |
| 25  | 2-methylthieno[3,2-b]thiophene | 1337 | 45  | 56  | 30  | 44  | 61  | 64 | 64   | 64   | 64   | 64   | roasted, KI/MS/O/S |
| 26  | 2-methyl-3-(2-methyltetrahydro-2-thienyl)thiophene | 1403 | -   | 25  | -   | -   | -   | 8  | 8    | 8    | 8    | 8    | roasted, KI/MS/O/S |
| 27  | Bis(2-methyl-3-furyl)difuran | 1534 | 30  | 92  | 70  | 79  | 87  | 64 | ≥128 | ≥128 | ≥128 | ≥128 | roasted, KI/MS/O/S |
| 28  | 2-methyl-3-[(tetrahydro-2-thienyl)methyl]thio]furans | 1573 | 21  | 23  | 33  | 35  | 40  | 8  | 16   | 16   | 16   | 16   | onion, KI/MS/O/S |
| 29  | Bis(2-furanyl)difurans | 1639 | 53  | 47  | 42  | 66  | 57  | 4  | 8    | 4    | 8    | 16   | roasted, KI/MS/O/S |
| 30  | 2,3-dihydro-5-methyl-4-(2-methyl-3-furyl)dithio]furans | 1688 | 25  | 44  | 26  | 42  | 53  | 8  | 32   | 8    | 32   | 32   | onion, KI/MS/O/S |

3Odor detected by the panelists in GC-O analysis using the DB-Wax column.

4K1, identified by Kovats indices (K1); MS, identified by search of mass spectra in the NIST 08 database and manual interpretation; O, identified by odor characteristics; and S, identified by comparison of the abovementioned analytical parameters with the authentic chemicals injected.

1K1s, Kovats indices determined using the n-alkanes C7–C40 on DB-Wax column (30 m × 0.25 mm × 0.25 μm) in the GC–MS and GC-O analysis.

2F1-MRPs, F2-MRPs, F3-MRPs, Pr-MRPs, and Re-MRPs indicated that MRPs are prepared by reacting F1, F2, F3, FPH, and recombinant component with xylose and cysteine, respectively. Means within different letters are significantly (P < 0.05) different in the same line. “–”, not detected.
showed that the FD factor (generally 4–16) of the nitrogen-containing heterocycles were generally lower than the sulfur-containing flavors. However, the barbecue and nutty flavors of pyrazines are also important ingredients in meat flavors in many reports (Alim et al., 2018; Fadel, Lotfy, Asker, Mahmoud, & Al-Okbi, 2018).

Oxygen-containing flavors, including oxygen-containing heterocycles, alcohols, and ketones, most have a high flavor threshold, so the flavor activity is not obvious in MRPs. In our previous study, the oxygen-containing flavors were found to be the most volatile components in the Maillard reaction system of the flaxseed protein hydrolysates - xylose - cysteine (Wei et al., 2019), while only the flavor active ingredients were listed in Table 3. Furfural is the highest content oxygen-containing heterocycle and has an FD factor greater than 128, and its flavor profile is caramel. Furfural is formed by dehydration of 3-deoxyllose or xylose in the Maillard reaction, and is an intermediate component formed by many flavor components such as 2-furfurylthiol (Ricci, Piccolella, Pepi, Garzoli, & Giacomello, 2013). 1-Octene-3-ol, another flavor component with a high FD factor (FD = 16) usually has a mushroom flavor and is used as a flavor in meats such as lamb and chicken (Fan et al., 2019).

After analyzing flavor compounds, a proposed flow diagram involving flaxseed derived MRPs mechanisms was presented in Fig. 3. Zhao et al. (2019) reported the formation of 3-thiophenethiol and 2-methyl-3-furanthiol in the Maillard reaction by [13C5]-xylose isotope labeling (Zhao, Wang, Xie, Xiao, Cheng et al., 2019). Mottram (1998) reviewed the flavor and formation pathways of 2-furfurylthiol, 2-methyl-3-furanthiol, bis(2-methyl-3-furyl)-disulfide, and bis(2-furfuryl) disulfide produced by Maillard reaction in cooked meat. Zhao et al. (2019a) analyzed the flavor and formation of 2,5-thiophenedicarboxaldehyde, 2-methylthieno[2,3-b]thiophene, thieno[3,2-b]thiophene, and 2-methylthieno[3,2-b]thiophene by [13C5]-xylose isotope labeling in the cysteine-xylose-glycine Maillard reaction system.

Conclusion

Flaxseed protein hydrolysates were separated into F1, F2, and F3 by GPC, which accounted for 11.40%, 84.30%, and 4.30%, respectively. F2 was defined as the peptide component and F3 was defined as the free amino acid component. After preparation of F1-MRPs, F2-MRPs and F3-MRPs, F2-MRPs had high umami, mouthfulness, and continuity enhancement. Further, the LC-MS/MS combined with the iBAQ algorithm revealed that flaxseed protein hydrolysates consumed the most 41
peptides after Maillard reaction. The top 10 peptides in the 41 peptides accounted for 88.86% of the total consumption. In addition, DLSFP (Asp-Leu-Ser-Phe-Ile-Pro) and ELPGSP (Glu-Leu-Pro-Gly-Ser-Pro) accounted for 42.22% and 20.41% of the total consumption, respectively. AEDA results indicate that sulfur-containing flavors were not significantly different in peptide and amino acid systems; for nitrogen-containing heterocycles, peptide systems were significantly higher than amino acid systems. This is because the formation of sulfur-containing flavors was dependent on cysteine, while for nitrogen-containing heterocycles, peptides were more reactive than amino acids. On the other hand, 11 kinds of flavor compounds with FD ≥ 64 were identified for flaxseed derived MRPs, such as 2-methylthiophene, 2-methyl-3-furathion, furfural, 2-furfurylthiol, 3-thiophenethiol, thieno[3,2-b]thiophene, 2-thiophenicarboxyldehyde, 2-methylthieno[3,2-b]thiophene, 1-(2-methyl-3-furlyhio)ethanethiol, 2-methylthiophene[3,2-b]thiophene, and 6(2-methyl-3-furyl)disulfide. Moreover, this study revealed the flavors formation mechanism of flaxseed derived MRPs.

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Declaration of Competing Interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.100224.

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