Comparing the metabolic profiles of raw and cooked pufferfish (*Takifugu flavidus*) meat by NMR assessment

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Abstract

The difference of metabolite profiles between raw and cooked pufferfish *(Takifugu flavidus)* meat was explored by ^{1}H NMR technique and multivariate statistical methods. The orthogonal projection to latent structure-discriminant analysis (OPLS-DA) results showed an obvious separation between two samples. There were 24 dominating metabolites in the pufferfish muscle extraction, of which 11 metabolites changed significantly ($p < 0.05$), including 9 amino acids (alanine, leucine, methionine, tyrosine, glutamate, glycine, arginine, lysine, taurine), 1 organic acid (lactate) and 1 alkaloid (betaine). Moreover, the Student's *t*-test was performed to identify the different levels of metabolites. Sensory intensity experiments showed that there was a significant difference in the umami taste between raw and cooked pufferfish meat ($p < 0.05$). The content of glutamate, aspartate and 5′-IMP in the cooked meat increased, making the umami taste more intense. This study provided essential information about the metabolites explaining the taste difference between raw and cooked pufferfish meat.

Keywords: *Takifugu flavidus*; Metabolite profile; Umami; NMR; Multivariate data analysis; Sensory evaluation

1 Introduction

Pufferfish (*Takifugu flavidus*) is regarded as one of the most cost-effective fish species in China because of its high economic value and palatable taste. The distribution of *Takifugu flavidus* is mainly in the coastal waters of the East China Sea, yellow sea and Bohai Bay (Ji, Liu, Gong, Zhou, & Wang, 2011). This species is described as one of the most famous food in Asia for the unique delicious taste of the non-toxic muscle part. Pufferfish has special taste properties and the taste-active components were identified as amino acids and nucleotide-related compounds, such as glycine, lysine, alanine and inosine 5′-monophosphate (5′-IMP) which were the predominant compounds in pufferfish meat (Hwang, Chen, Shiau, & Jeng, 2000). Sashimi (raw fish meat) was accepted by consumers thanks to its texture profiles, such as strong hardness and elasticity (Suárez, Abad, Ruiz-Cara, Estrada, & García-Gallego, 2005). However, in China, people are more accustomed to eating cooked meat. Compared with sashimi, heat cooking provides a crispy texture to pufferfish meat and has a specific aroma and flavor, which are favored by Japanese consumers (Yamaguchi et al., 2013).

It is well known that taste is a very important factor to influence food palatability, while *Takifugu flavidus* is famous for its unique umami taste. Umami is considered as one of the five basic sensations, it has special taste characteristics. Besides monosodium glutamate (MSG) (Ikeda, 1908), free amino acids glutamic acid (Glu) and aspartic acid (Asp) all contribute to umami taste. Nucleotide-related compounds, such as inosine monophosphate (IMP) and

guanosine monophosphate (GMP) are also responsible for umami and have a remarkable synergistic effect with glutamic acid (Festring & Hofmann, 2010). Gly, Ala, Val, Met were also recognized to have a synergistic effect on umami in the mixture of MSG-5′-ribonucleotide (Nishimura & Kato, 1988). The perception of umami relies not only on the properties of nonvolatile compounds, but also their availability, which could be regulated by physical and chemical interactions with other food ingredients. Therefore, changes in food composition will affect the taste sensation.

Metabolomics offers potential in the areas of nutritional assessment when combined with multivariate statistical analysis (O'Sullivan, Gibney, & Brennan, 2011). Metabolomics methods was a breakthrough technology in dynamic changes of small molecule metabolites (MW < 1000 Da) in species and quantities (Nicholson, Lindon, & Holmes, 1999). There are many different metabolomics technologies: liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC–MS) and nuclear magnetic resonance (NMR). For example, Han and Xu identified low molecular weight metabolites contained in rice by LC-MS (Han & Xu, 2012) and Yang et al. (2018) assessed the effect of different formulated diets on growth performance of pearl oyster, by GC–MS metabolomics. Compared to these metabolomics analysis, NMR is a non-destructive multi-parameter and dynamic analysis technique, which has both qualitative and quantitative analysis functions (Tugizimana, Piater, & Dubery, 2013). Moreover, NMR detection can be completed in a short time, which is critical for achieving high-throughput sample testing and for maintaining the original properties of the sample during the test period (Xiao, Dai, Liu, Wang, & Tang, 2008). Actually, there have been many reports on the research of NMR combined with multivariate statistical analysis to explore metabolite profile of meat (Wishart, 2008). Lately, ~~Chen et al. (~~Chen, Ye, Chen, Zhan, and Lou (2017) characterized the flavor composition of vinasse pike eel by using NMR spectroscopy. Yoshinori Kodani, Miyakawa, Komatsu, and Tanokura (2017) detected profiles of water-soluble metabolites in Japanese Black cattle based on NMR to evaluate beef flavor quality. Yang et al. (2018) characterized the fact that the umami taste of marinated meat in soy sauce was improved after high pressure treatment by using ^{1}H NMR and multivariate data analysis, and demonstrated that it was economic to improve the taste of marinated meat in soy sauce at 150 MPa. Xiao et al. (2019) used NMR to assess the water-soluble low molecular weight (WLOM) compounds of boiled Wuding chicken, they found out that these main flavor precursors were significantly decreased during cooking progress. These studies revealed that using ^{1}H NMR technology can explain the relationships between metabolite profile and meat flavor quality. With the advantage of metabolomics, it actually offers an excellent opportunity for nutrition scientists to obtain a far more detailed molecular picture of food composition (Wishart, 2008).

The changes of metabolite profiles between sashimi and heat cooking pufferfish meat is not very well understood, therefore the aim of this study is to evaluate the difference of metabolic profiling analysis between sashimi and cooked Pufferfish (*Takifugu flavidus*) meat. To understand the taste difference between raw and cooked pufferfish meat, non-volatile components such as amino acids, nucleotide-related taste active components and creatine were studied. Thus, ^{1}H NMR and multivariate statistical methods were used to identify the difference of metabolite profile.

2 Materials and methods

2.1 Sample preparation

Sixteen puffer fishes were purchased from Jiangsu Zhong Hao Import and Export Food Co., Ltd., with an average weight of 65.5 ± 0.2 g in June 2017. *T. flavidus* were butchered by a puffer fish licensed chef from Jiangsu Zhong Hao Import and Export Food Co., Ltd and the fish were ice transported to the laboratory. Eight puffer fish for raw (sashimi) material were separated and wrapped in an aluminum foil bag, stored at −18 °C until analyzed. In order to avoid the loss of weight and metabolite during the heating process, we wrapped each heated sample in a vacuum foil pouch, and then placed this sealed bag in 100 °C water for 25 min. Then stored at −80 °C until analyzed.

2.2 Metabolite extraction

The extraction method which was described by Yang et al. (2018) with minor modification was used: the meat of each fish (400 mg) was homogenized with 600 µL solvent, which contains methanol: water (2:1)~~-~~, for 2 min and discontinuous ultrasonication (2 s ultrasonication and a 2 s break) on ice for 10 min. This process was reproduced twice. Then, the mixture was centrifuged at 12000 rpm for 10 min at 4 °C, and concentrated to remove methanol with the centrifugal concentrator at 1400 rpm for 3 h and the supernatants were lyophilized. The 600 µL recombination solvent necessary for the NMR was composed of 480 µL 99% D_2O and 120 µL sodium 3-(trimethylsilyl)-propionate-2, 2, 3, 3-d4 (TSP) prepared with 99.9% D_2O. The 600 µL recombination of deuterium oxide solvent was added to the freeze-dried powder. Then the mixture was centrifuged at 12,000 rpm at 4 °C for 10 min, the 550 µL supernatant was transferred to the nuclear magnetic tube of 5 mm outer diameter NMR tube for nuclear magnetic analysis.

2.3 NMR detection

The ^{1}H NMR spectra was received by using a Bruker Avance III 600 NMR spectrometer operating at a ^{1}H frequency of 600.13 MHz at 298 K, with a 5-mm CP TCI 1H-13C/15N/D z-gradient probe. The main signal was obtained from small metabolites or fractions with fast motion by a pre-saturated Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence. The acquisition parameters were as follows: the relaxation delay was 2.3 s, the spectrum width was 12019.23 Hz, the pulse width was 8 µs, the number of acquisitions was 1024, the spin-echo delay was 220 µs, the number of loops was 400, the total echo time was 220 ms. The water peak was irradiated during RD.

2.4 Sensory panelists

The sensory evaluation panel was composed of 5 females and 3 males (aged from 22 to 27), who were able to distinguish the basic tastes (bitterness, sweetness, sourness, saltiness and umami) after sensory training. They were recruited from the School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai, China. The sensory panelists were trained to evaluate the taste of standard taste: bitterness was tested with a 0.035% quinine solution, sourness was tested with a 0.08% citric acid solution, saltiness was tested with a 0.35% sodium chloride solution, umami taste was tested with a 0.35% MSG solution, and sweetness was tested with a 1% sucrose solution. Panelists were seating in a sensory panel room at room temperature of 23 ± 2 °C. The panelists were asked to drink one sip of sample and to keep it in mouth for 10 s and spit it out. Therefore, the panelists were asked to wash their mouths with 50–60 ml of pure water between two different samples to avoid aftertaste and sensory fatigue effect.

2.5 Evaluation of taste intensity

Samples from raw pufferfish meat and cooked pufferfish meat were evaluated by the 8 panelists according to the method of Zhuang et al. (2016). The taste profiles used a 10-point intensity scale (0 represents no taste; 10 represents the strongest taste) to analyse the intensity of 1% solution of samples. The reference samples were set up as follows, 0.035% quinine solution for bitter, 0.08% citric acid solution for sour, 1% sucrose solution for sweet, 0.35% sodium chloride solution was for salty, and for umami was 0.35% MSG solution. The taste intensity of reference samples was set as five points. The average rating points of the five tastes of the two samples of pufferfish were scored by the different panelist and recorded.

2.6 Evaluation of taste properties with an electronic tongue

The electronic tongue (Astree, Alpha M.O.S., France) was used in this experiment. Sour taste, salt taste and umami were used as control tastes. The results of the sensory evaluation of taste intensity showed that the taste properties of the two samples had the strongest umami taste, so we used the umami substance MSG as the control sample. The original data were acquired through the E-tongue software (Astree Software V3.0, Alpha M.O.S., France). The system of E-tongue consists of 7 nonspecific chemical sensor arrays, a reference electrode, an electrical signal processor and a pattern recognition system. The concentration of MSG and freeze-dried samples of the raw and cooked pufferfish meat were prepared with ultra-pure water to form 1 mg/ml solution (80 ml), separately. At 26 ± 2 °C, the data acquisition time of each sample was 120 s, one acquisition per second, and the response value of 120 s was selected as the data signal to process. The electronic tongue sensor was cleaned with ultra-pure water once every time after measurement. Each sample was measured 7 times repetitions, and the last 3 times were used as the original data.

2.7 Data analysis

The software package TOPSPIN (V. 3.2; Bruker BioSpin, Ettlingen, Germany) was used to process all NMR spectra. The obtained 1D-CPMG ^{1}H NMR spectra for the samples were processed with a 1.0-Hz line-broadening factor before Fourier Transformation, and then manually corrected for phase and baseline distortions and referenced to the chemical shift of TSP (CH_3 δ 0). All spectra were bucketed and automatically integrated using an automation routine in AMIX 3.9.14. Each ^{1}H NMR spectrum was segmented into regions of 0.004 ppm. The integrals of these buckets covered the region δ 8.99–0.70 and were normalized to the total sum of the spectral integrals according to the previous methods (Song, Liu, Li, & Gu, 2016; Violetta Aru, Khakimov, Toldam-Andersen, & Engelsen, 2018; Zanardi et al., 2015). The resulting normalized integral data were saved in an Excel format (Microsoft, 2016) and then submitted to MVSA using the software package SIMCA (Version 14.0; Umetrics, Umea, Sweden). The chemical shifts were assigned to individual metabolites based on the Human metabolites database (HMDB) and literature (Chen, Ye, Chen, & Yan, 2016; Dai, Xiao, Liu, Hao, & Tang, 2010; Jung et al., 2010; Yang et al., 2018). Principal component analysis (PCA) was performed with the SIMCA-Soft Independent Modeling of Class Analogy, CAMO to analyze the difference in metabolite profiles between the pufferfish samples. To minimize the influence of the baseline noise regions of the NMR spectra or weak signals whose variation was dominated by baseline noise, UV-scaled data were used in the multivariate statistical analysis (MVSA).

An orthogonal projection to latent structures discriminant analysis (OPLS-DA) was used to analyze the data to maximize the difference between raw pufferfish meat and cooked pufferfish meat. R^2X and R^2Y, as the fractions of the sum of squares for the selected component, represented the variance of X and Y. Q^2 was the predictive ability parameter for the model. In our study, R^2X, R^2Y, and Q^2 were used to evaluate the model quality. All of OPLS-DA models were validated by ANOVA of the cross-validated residuals (CV-ANOVA) approach with $p < 0.05$ as significant level. The scores and loadings plots combined with the variable importance in the projection (VIP) were used to interpret various OPLS-DA models. Metabolites of which $p < 0.05$ and VIP > 1 were identified as the significantly disturbed metabolites.

The semi-quantitative analysis of the metabolites in pufferfish meat was carried out by taking the NMR peak integral of the internal standard (TSP) as a reference, selecting the integrated value of the NMR signal of the metabolite (the minimum overlap value), and comparing the relative contents of the metabolites between the two groups of samples.

For sensory data, means were calculated and statistically tested using analysis of variance to determine if a statistical difference existed at $p < 0.05$. The statistical analysis for Student's *t*-test was performed with the software SPSS 17.0.

3 Results and discussion

3.1 ^{1}H NMR data of aqueous extract of *Takifugu flavidus*

^{1}H NMR spectra were obtained from the D_2O extracts of *Takifugu flavidus* samples. Representative one-dimensional NMR spectra of the muscle aqueous *Takifugu flavidus* extraction from the raw pufferfish meat and the cooked pufferfish meat were shown in Fig. 1. All the metabolites identified using a list of two-dimensional NMR experiments by both ^{1}H and ^{13}C data (Table 1). Based on the 2D NMR analysis, the metabonome of *Takifugu flavidus* extracts is dominated by 24 metabolites, including 13 amino acids (valine, isoleucine, methionine, glutamate, arginine, lysine, aspartate, leucine, glycine, tyrosine, tryptophan, alanine and taurine), 5 organic acids (succinate, lactate, acetate, creatine and fumarate), 4 nucleic acids (inosine, 5′-IMP, uracil and hypoxanthine), and 2 alkaloids (betaine and choline). Significant differences among the features of ^{1}H NMR spectra of *Takifugu flavidus* samples were detected (VIP > 1, $p < 0.05$). To investigate the change of metabolites between raw and cooked pufferfish meat, multivariate statistical analyses were conducted (You can precise where we can find these analyses §3.2).

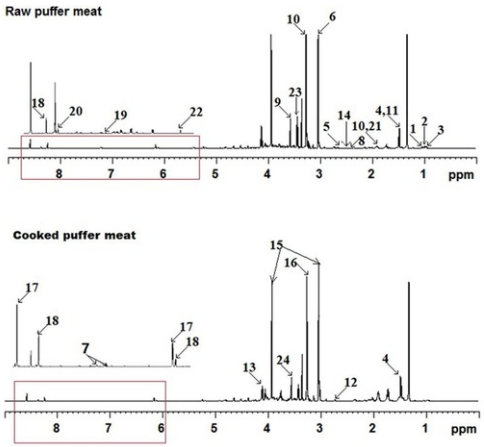

Fig. 1 Representative 600 MHz ^{1}H NMR spectra of meat in *Takifugu flavidus* extracts from sashimi and cooked puffer meat. Resonance assignments are given in Table 1. Key signals: 1 valine, 2 isoleucine, 3 leucine, 4 alanine, 5 methionine, 6 tyrosine, 7 tryptophan, 8 glutamate glutamine, 9 glycine, 10 arginine, 11 lysine, 12 aspartate, 13 lactateα-glucose, 14 succinate, 15 creatine, 16 betaine, 17 5′-IMP, 18 inosine, 19 uracil formate, 20 hypoxanthine, 21 acetate, 22 fumarate, 23 choline, 24 taurine.

Table 1 ^{1}H NMR and ^{13}C NMR data for metabolites in *Takifugu flavidus* extracts.

Key signal	Metabolites	Moieties	δ^{1}H(ppm) and multiplicity	δ^{13}C(ppm)
1	Valine	γCH_3, βCH, αCH	0.99(d), 2.27(m), 3.62	19.0, 29.3, 62.4, 174.0
2	Isoleucine	δCH_3, β,CH_3, βCH_2, γCH_2, αCH	0.96(t), 1.22(d), 1.48(m), 2.02(m), 4.04(d)	14.7, 17.0, 27.8, 35.5, 64.0, 172.5
3	Leucine	δCH_3, βCH_2,αCH	0.94(t),1.73(m),3.72(d)	26.0, 28.2, 43.1, 54.3
4	Alanine	αCH,βCH_3	1.48(d),3.82	18.0, 52.3, 176.1
5	Methionine	S-CH_3, γCH_2, βCH_2, αCH	2.14(s),2.65, 2.35, 3.60	15.0, 31, 32.5, 57.2
6	Tyrosine	βCH_2, αCH, Ring C_1,C_4 Ring$C_{3,5}$H,Ring$C_{2,6}$H	3.15,3.05,,3.90(d)6.90(d),7.20(d)	36.7, 57.6, 116, 133, 177.8
7	Tryptophan	RingC_3,C_4,C_9,RingC_5H, RingC_6H,RingC_7H, RingC_8H	7.73(d),7.19(d),7.28(t),7.53(d)	122, 124.5, 115.8, 113.2
8	Glutamate	αCH,βCH_2,γCH_2	3.80(t), 2.06(m), 2.36(m)	187.8, 57.2, 29.9, 32.2, 184.5
9	Glycine	αCH_2	3.56(s)	43.4, 175.5
10	Arginine	βCH_2, γCH_2, δCH_2, αCH	1.92(m), 1.66(m), 3.26(t), 3.76(t)	32.7, 27.5, 38.1, 51.5
11	Lysine	αCH,βCH2,γCH2, δCH2, εCH2	3.76(t), 1.91(m), 1.48(m),1.72(m), 3.03	53.3, 32.7, 28.2, 32.5, 178.2

12	Aspartate	αCH,βCH, β,CH	3.89(dd), 2.68(d), 2.83(dd)	56.9, 37.3, 177.5
13	Lactate	αCH,βCH_3	4.11(q), 1.33(d)	77.2, 24.5, 179.8
14	Succinate	αCH_2	2.41(s)	35.0, 176.7
15	Creatine	αCH_2,βCH_3	3.93(s),3.03(s)	172.8, 57.0, 38.4, 157.8
16	Betaine	αCH_2,βCH_3	3.26(s),3.93(s)	53.5, 75.0, 175.2
17	5′-IMP	$C_4C_6C_9H$	8.56(s),8.23(s),6.15(d)	140.7, 150.8, 98.4
18	Inosine	C_4,H, C_2, H,C_1,H,C_5,C_2,C_3,C_2H,C_5H	3.89(d), 4.43(t), 4.79(t), 6.10(d), 8.23(s), 8.35(s)	65.7, 90.1, 75.5, 99.8, 150.8, 140.7
19	Uracil	C_3H, C_4H	7.53(d),5.80(d)	143.4, 103.4
20	Hypoxanthine	C_2H, C_4H	8.21(s),8.23(s)	148.5, 150.6
21	Acetate	αCH_3	1.91(s)	26.7, 184.1
22	Fumarate	CH = CH	6.52(s)	131.9, 169.5
23	Choline	$N(CH_3)^+$, βCH_2, αCH_2	3.20(s), 3.43(t)	57.0, 68.8
24	Taurine	CH_2NH_2, CH_2SO_3H	3.27(s), 3.43(t)	51.6, 37.8

3.2 Analysis of metabolites in *Takifugu flavidus*

Multivariate statistical analyses were carried out to expound the effect of different processing methods on the metabolite profile of *Takifugu flavidus*. In order to highlight the differences between the raw pufferfish meat and the cooked pufferfish meat, the OPLS-DA of the spectral data was performed for further explanation (Fig. 2). The values for R^2X represented the interpretation rate of the model and Q^2 indicated the prediction rate of the model, the ratio of R^2X to Q^2 close to 1 indicated a more reliable model. The s-line corresponding color-coded correlation coefficient loadings plots indicated the contribution of the metabolites, the color-coded loadings plots with the absolute number of correlation coefficients, where the hot color mark denoted that the contribution to the category difference was more obvious than the cool color mark. The uptrend metabolites mean that their contents in the heat cooking samples were higher than those in the raw samples, while the downtrend mean that their contents in the cooked samples were lower than those in the raw samples.

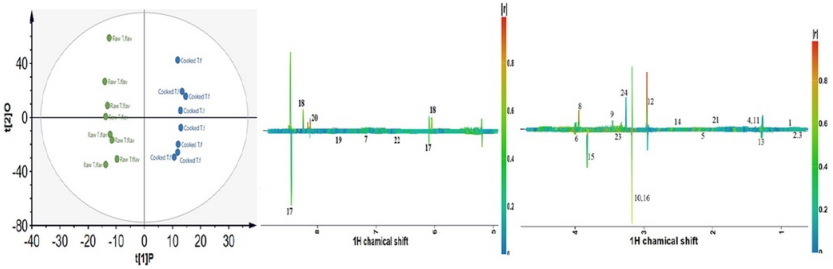

Fig. 2 OPLS-DA scores plots and S-line corresponding color-coded loadings plots in *Takifugu flavidus* extracts.

According to our data, the OPLS-DA model (Fig. 2) revealed R^2X, Q^2 and CV-ANOVA of p values were 0.696, 0.802 and 2.35×10^{-2}, respectively, which all described the discrimination of the samples and the predictability of the model. OPLS-DA score plot provided a comparison and showed obvious intergroup metabonomic differences between the raw and heat cooked pufferfish samples. As can be seen from the S-line plot, there were 11 metabolites in the heat cooked samples higher than those in the raw group, identified as Val, Ala, Glu, Gly, Lys, Asp, Succinate, Inosine, Hypoxanthine, Acetate and Taurine, while the remaining components in the cooked pufferfish meat were lower than those in raw puffer meat.

The relative concentrations of metabolites were also determined, and these relative concentrations were calculated with reference to the internal standard TSP (Fig. 3). Compared with the metabolites in the raw puffer meat, there was a higher amount of 11 metabolites in the heated puffer meat, and the remaining 13 metabolites were reduced. Due to the overlapping of characteristic peaks of metabolites, only semi-quantitative studies were performed. Through the integral value of the internal standard TSP, calculate the content of different metabolites (the least overlap of NMR signals), and compare the changes of metabolite content between raw and cooked puffer meat. The

difference level of metabolites between raw and cooked puffer meat were presented in Table 2. The contents of 11 metabolites were found to change significantly ($p < 0.05$), including alanine, glutamate, glycine, lysine and taurine increased ($p < 0.05$), while leucine, methionine, tyrosine, lactate, arginine and betaine decreased ($p < 0.05$), other 13 metabolites also changed, but there was no significant difference (Table 2). Among these amino acids, alanine, glutamate, glycine greatly contributed to the taste of puffer meat.

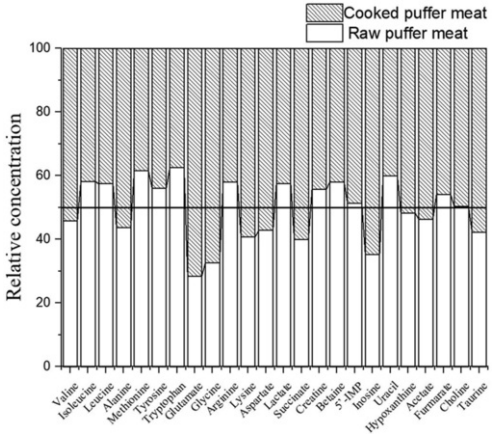

Fig. 3 Relative concentration of metabolites between raw and cooked pufferfish meat.

Table 2 Comparison of metabolite content and p-value for Student's *t*-test in raw puffer meat and cooked puffer meat.

Key signal	Metabolites	Mean ± SD		*p-value	VIP value
		Raw pufferfish meat	Cooked pufferfish meat		
1	Valine	0.11 ± 0.01[a]	0.13 ± 0.03[a]	0.23	1.1
2	Isoleucine	0.32 ± 0.07[a]	0.23 ± 0.01[a]	0.11	2.5
3	Leucine	0.23 ± 0.02[a]	0.17 ± 0.01[b]	0.012	2.9
4	Alanine	2.41 ± 0.01[b]	3.10 ± 0.26[a]	0.016	1.4
5	Methionine	0.08 ± 1.15[a]	0.05 ± 0.01[b]	0.033	1.3
6	Tyrosine	10.05 ± 0.40[a]	7.87 ± 0.89[b]	0.018	1.1
7	Tryptophan	0.05 ± 0.02[a]	0.03 ± 0.001[a]	0.16	1.5
8	Glutamate	0.29 ± 0.01[b]	0.73 ± 0.01[a]	0.000	2.2
9	Glycine	0.8 ± 0.1[b]	1.65 ± 0.09[a]	0.001	1.4
10	Arginine	11.39 ± 0.24[a]	8.21 ± 0.77[b]	0.002	2.6
11	Lysine	2.01 ± 0.27[b]	2.90 ± 0.48[a]	0.048	1.7
12	Aspartate	0.03 ± 0.001[a]	0.04 ± 0.01[a]	0.18	2.2
13	Lactate	17.04 ± 1.97[a]	12.60 ± 1.24[b]	0.03	1.4
14	Succinate	0.02 ± 0.001[a]	0.03 ± 0.01[a]	0.15	1.9
15	Creatine	9.57 ± 0.59[a]	7.62 ± 1.32[a]	0.08	1.6

16	Betaine	11.39 ± 0.24[a]	8.22 ± 0.77[b]	0.002	1.6
17	5′-IMP	0.39 ± 0.03[a]	0.37 ± 0.05[a]	0.29	1.9
18	Inosine	0.06 ± 0.01[a]	0.11 ± 0.04[a]	0.11	2.5
19	Uracil	0.01 ± 0.001[a]	0.0067 ± 0.0057[a]	0.42	1.1
20	Hypoxanthine	0.27 ± 0.06[a]	0.29 ± 0.01[a]	0.64	1.2
21	Acetate	0.58 ± 0.17[a]	0.67 ± 0.17[a]	0.44	0.4
22	Fumarate	0.02 ± 0.001[a]	0.02 ± 0.006[a]	0.42	1.3
23	Choline	3.33 ± 0.34[a]	3.28 ± 0.82[a]	0.93	1.8
24	Taurine	1.27 ± 0.15[b]	1.74 ± 0.03 [a]	0.002	3.3

The content of each metabolite were obtained from 8 parallel samples and selected the area of the least overlapping peak in the NMR spectrum.

The letter (a) represents a significant difference $p > 0.05$, there was no significant difference in the metabolite content of raw and cooked meat; (b) represents a significant difference $p < 0.05$ and there was a significant difference in the metabolite content of raw and cooked meat.

*p-value were results of Student's *t*-test by SPSS 17.0 variable importance in projection (VIP).

3.3 Assessment of taste-active metabolites in *Takifugu flavidus*

It is well known that amino acids and nucleotide related components are taste active compounds and they play an important role in the taste characteristics of food. The hydrophilic amino acids (Asp, Glu, Lys and Arg) are responsible for the salty, sour and umami taste in pork meat. While the hydrophobic amino acids (Ala, Val, Leu, Pro and Phe) are responsible for the bitter and sweet taste (Keska & Stadnik, 2017). Generally, the tastes of the individual amino acids are complex and need to be described by more than one taste characteristic. Thus, alanine is involved in producing sweet and umami taste, arginine imparts a bitter taste and slight sweetness. Serine gives a sweet taste accompanied by sourness and umami taste. Glutamate has a sour taste combination with umami taste (Jiro Kirimura, Kimizuka, Ninomiya, & Katsuya, 1969). In our data, Table 2 showed that glutamate, alanine and glycine had an increasing trend in cooked meat ($p < 0.05$). Glutamate was especially abundant in foods, such as beef, pork, fish and crab (Ninomiya, 2002), which can improve the palatability of food. T1R functional expression and mouse knockout studies indicated that glutamate taste perception was transduced by the heterodimeric T1R1/T1R3 (Li et al., 2002) and mGluR4 receptor (Reilly, 2000). Cooked pufferfish meat also had a stronger sweet taste ($p < 0.05$) than raw pufferfish meat except for umami, due to the content increase of sweet amino acids glycine and alanine ($p < 0.05$). Glycine and alanine were recognized as sweet amino acids, they had a synergistic effect with monosodium glutamate (Tanaka, Okuhara, & Yokotsuka, 1969), which provides a positive effect on the taste of food. The synergistic effect soothes the sour flavors, and significantly improve the flavor of food.

The taste of umami was not the function of a single substance, but the combined synergistic effect of several substances. 5′-IMP was one of the nucleotide related components, which can synergize with glutamic acid to potentiate the umami taste of food. Synergies between MSG and IMP could be explained by T1R1/T1R3 signal recognition mechanism (Li et al., 2002). T1R1/T1R3 responds to glutamate, and this response can be enhanced by IMP. When glutamate molecule and IMP bind to the region of Venus flytrap domain (VFT) in the T1R1, glutamate combines with VFT near the hinge region, which induces the closing of the VFT structure. While IMP promotes the closure of the VFT region after glutamic acid acts on VFT, which binds closer to the opening of VFT, thus achieving the function of umami enhancement (Kurihara, 2015). The contents of inosine and hypoxanthine increased while that of IMP decreased with higher temperature in water systems (Table 2). The taste contribution of hypoxanthine was studied in heat-stable sarcoplasmic fraction, with the addition of hypoxanthine, and it was shown that it can enhance the taste of meat (Ichimura, Nakamura, Yoshida, & Hattori, 2017).

Importantly, taurine was also abundant in pufferfish meat (Table 2). Compared with raw pufferfish meat, taurine increased significantly in cooked pufferfish meat ($p < 0.05$). Although taurine was recognized as tasteless, it was involved in numerous physiological and biological functions (Shiau, Hsu, Cheng, & Huang, 2018). Particularly, taurine decreases concentration of cholesterol and triglyceride in blood vessels (Chung, Kim, Nam, & Kang, 2010). It also affects brain development and improves immune function. It has been reported that the accumulation of taurine in mammalian systems was capable of forming bile salts, regulating osmotic pressure and maintaining calcium homeostasis (Pi, Shiau, Chang, & Sung, 2017).

3.4 Sensory evaluation

Sensory evaluation was performed to explore the overall taste of pufferfish after different processing methods. Results of pufferfish meat (Fig. 4A) showed that the cooked pufferfish meat had the higher umami taste (almost 94% as umami as the 0.35% MSG solution) and the umami taste of cooked meat was significantly higher different from that of the raw pufferfish meat ($p < 0.05$). The saltiness, sourness and bitterness were not affected by heat cooking. In addition, the sweetness (36% of the sweetness of the 0.1% sucrose solution) of cooked pufferfish meat is also significantly higher than raw pufferfish meat through different processing methods ($p < 0.05$). The contribution of different processing methods to the taste of umami was further verified by electronic tongue. The results (Fig. 4B) showed that the umami intensity of cooked pufferfish was higher than sashimi (6.1 and 3.7 respectively), and the umami taste intensity of intelligent senses was consistent with the results of artificial senses. At the same concentration, the umami taste of MSG was obviously stronger than that of pufferfish meat, but the umami taste of cooked meat (6.1) was closer to MSG (8.2). The increased intensity of umami taste was closely related to the changes of taste compounds, particularly amino acids related compounds and nucleotide-related taste component.

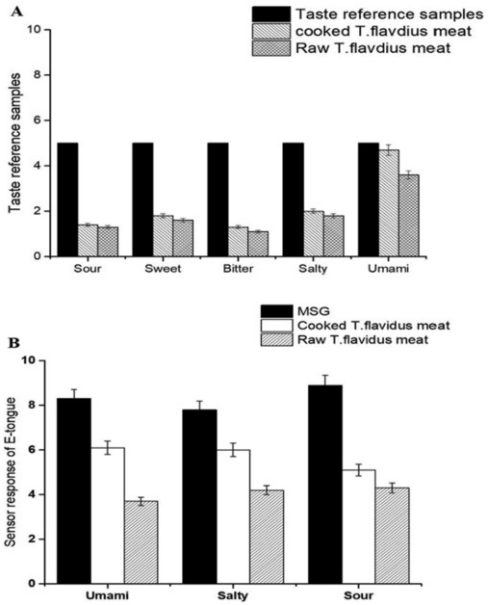

Fig. 4 Sensory evaluation of raw pufferfish meat and cooked pufferfish meat (A. taste intensity of two samples compared with taste reference samples B. Sensory evaluation of electronic tongue for raw and cooked pufferfish meat).

In combination with the results of NMR, the content of metabolites such as Glu in the cooked pufferfish meat was significantly higher than the raw samples ($p < 0.05$), which could contribute to the stronger umami taste. The degradation of 5′-IMP, produced higher inosine and hypoxanthine, which contributed to the taste of cooked meat, and caused the overall flavor changes. Sweet amino acids, such as glycine and alanine, can act synergistically with umami amino acids to contribute to the umami flavor.

4 Conclusion

After heat cooking treatment of pufferfish meat, metabolites changed. Among them, the content of glutamate, aspartate, alanine and glycine in the cooked pufferfish meat significantly increased ($p < 0.05$), making the umami taste more intense than the raw pufferfish meat. In addition to these amino acid, taste-active substances, like nucleotide related components (inosine, 5′-IMP and hypoxanthine) played a role in enhancing the taste of meat. To the best of the authors' knowledge, this is the first study comparing the metabolic profiles of raw and cooked Pufferfish (*Takifugu flavidus*) meat by NMR assessment, including primary and secondary metabolites throughout different processing methods, even if the NMR data were incomplete. The sensory evaluation combined with ^{1}H NMR and multivariate statistical method all demonstrated that the effects of metabolite changes on food flavor could be analyzed more intuitively. As sensitivity of NMR increases, NMR metabolomics will make a significant contribution to the characterization of food analysis.

Acknowledgments

This work was funded by The National Key R&D Program of China (2016YFD0400803) and National Natural Science Foundation of China (Grant Nos. 31622042, 31371790, 31271900). Additionally, we also thank the interdisciplinary training "Network to address key questions in plant development for Food Security" (Net4FS) for their financial support covering Dr. Ayed's travel costs.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2019.03.128.

References

Chen D., Ye Y., Chen J. and Yan X., Evolution of metabolomics profile of crab paste during fermentation, *Food Chemistry* **192**, 2016, 886–892.

Chen D., Ye Y., Chen J., Zhan P. and Lou Y., Molecular nutritional characteristics of vinasse pike eel (*Muraenesox cinereus*) during pickling, *Food Chemistry* **224**, 2017, 359–364.

Chung S.I., Kim S.Y., Nam Y.J. and Kang M.Y., Development of surimi gel from king oyster mushroom and cuttlefish meat paste, *Food Science and Biotechnology* **19** (1), 2010, 51–56.

Dai H., Xiao C.N., Liu H.B., Hao F.H. and Tang H.R., Combined NMR and LC-DAD-MS analysis reveals comprehensive metabonomic variations for three phenotypic cultivars of salvia miltiorrhiza bunge, *Journal of Proteome Research* **9** (3), 2010, 1565–1578.

Festring D. and Hofmann T., Discovery of N-2-(1-carboxyethyl)guanosine 5 '-monophosphate as an umami-enhancing maillard-modified nucleotide in yeast extracts, *Journal of Agricultural and Food Chemistry* **58** (19), 2010, 10614–10622.

Han F. and Xu Y., Identification of low molecular weight peptides in chinese rice wine (Huang Jiu) by UPLC-ESI-MS/MS, *Journal of the Institute of Brewing* **117** (2), 2012, 238–250.

Hwang D., Chen T., Shiau C. and Jeng S., Seasonal variations of free amino acids and nucleotide-related compounds in the muscle of cultured Taiwanese puffer *Takifugu rubripes*, *Fisheries Science* **66** (6), 2000, 1123–1129.

Ichimura S., Nakamura Y., Yoshida Y. and Hattori A., Hypoxanthine enhances the cured meat taste, *Animal Science Journal* **88** (2), 2017, 379–385.

Ikeda K., On a new seasoning, *Journal of Tokyo Chemical Society* **30**, 1908, 820–836.

Ji Y., Liu Y., Gong Q., Zhou L. and Wang Z., Toxicity of cultured puffer fish and seasonal variations in China, *Aquaculture Research* **42** (8), 2011, 1186–1195.

Jiro Kirimura A.S., Kimizuka Akimitsu, Ninomiya Tsunehiko and Katsuya Noboru, The contribution of peptides and amino acids to the taste of foodstuffs, *Journal of Agricultural and Food Chemistry* **17** (4), 1969, 689–695.

Jung Y., Lee J., Kwon J., Lee K.S., Ryu D.H. and Hwang G.S., Discrimination of the geographical origin of beef by H-1 NMR-based metabolomics, *Journal of Agricultural and Food Chemistry* **58** (19), 2010, 10458–10466.

Keska P. and Stadnik J., Taste-active peptides and amino acids of pork meat as components of dry-cured meat products: An in-silico study, *Journal of Sensory Studies* **32** (6), 2017.

Kodani Yoshinori, Miyakawa Takuya, Komatsu Tomohiko and Tanokura Masaru, NMR-based metabolomics for simultaneously evaluating multiple determinants of primary beef quality in Japanese Black cattle, *Scientific Reports* **7** (1), 2017, https://doi.org/10.1038/s41598-017-01272-8.

Kurihara K., Umami the fifth basic taste: History of studies on receptor mechanisms and role as a food flavor, *Biomed Research International* **2015**, 2015, 189402.

Li X., Staszewski Lena, Xu H., Durick Kyle, Zoller Mark and Adler E., Human receptors for sweet and umami taste, *PNAS* **99** (7), 2002, 4692–4696.

Nicholson J.K., Lindon J.C. and Holmes E., 'Metabonomics': Understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data, *Xenobiotica* **29** (11), 1999, 1181–1189.

Ninomiya K., Umami: A universal taste, *Food Reviews International* **18** (1), 2002, 23–38.

Nishimura T. and Kato H., Taste of free amino acids and peptides, *Food Reviews International* **4** (2), 1988, 175–194.

O'Sullivan A., Gibney M.J. and Brennan L., Dietary intake patterns are reflected in metabolomic profiles: Potential role in dietary assessment studies, *American Journal of Clinical Nutrition* **93** (2), 2011, 314–321.

Pi T.H., Shiau C.Y., Chang C.J. and Sung W.C., Studies on the development and quality of fish chiffon cake and its storage stability, *Journal of Aquatic Food Product Technology* **26** (8), 2017, 969–978.

Reilly C.E., L-glutamate receptor identified as taste receptor of umami, *Journal of Neurology* **247** (5), 2000, 402–403.

Shiau C.Y., Hsu M.H., Cheng A.C. and Huang Y.R., Comparative chemical, taste, and functional components in different tissues of giant grouper (*Epinephelus lanceolatus*), *Journal of Aquatic Food Product Technology* **27** (3), 2018, 358–368.

Song J., Liu C., Li D. and Gu Z., Postharvest changes in physicochemical characteristics and free amino acids content of immature vegetable soya bean (Glycine max L.) grains, *International Journal of Food Science and Technology* **51** (2), 2016, 461–469.

Suárez M.D., Abad M., Ruiz-Cara T., Estrada J.D. and García-Gallego M., Changes in muscle collagen content during post mortem storage of farmed sea bream (*Sparus aurata*): Influence on textural properties, *Aquaculture International* **13** (4), 2005, 315–325.

Tanaka T.S.N., Okuhara A. and Yokotsuka T., Studies on the taste of α-amino acids. II. Ternary synergism of palatable taste of α-amino acids (1), *Nippon Nogeikagaku Kaishi (in Japanese)* **43**, 1969, 171–176.

Tugizimana F., Piater L. and Dubery I., Plant metabolomics: A new frontier in phytochemical analysis, *South African Journal of Science* 2013, 1–11.

Violetta Aru K.M.S., Khakimov Bekzod, Toldam-Andersen Torben Bo and Engelsen Soren Balling, Cool-climate red wineschemical composition and comparison of two protocols for 1H-NMR analysis, *Molecules* **23** (160), 2018, 1–18.

Wishart D.S., Metabolomics: Applications to food science and nutrition research, *Trends in Food Science & Technology* **19** (9), 2008, 482–493.

Xiao C., Dai H., Liu H., Wang Y. and Tang H., Revealing the metabonomic variation of rosemary extracts using 1H NMR spectroscopy and multivariate data analysis, *Journal of Agricultural and Food Chemistry* **56** (21), 2008, 10142–10153.

Xiao Z., Luo Y., Wang G., Ge C., Zhou G., Zhang W. and Liao G., 1H-NMR-based water-soluble low molecular weight compound characterization and fatty acid composition of boiled Wuding chicken during processing, *Journa of the Science of Food and Agriculture* **99** (1), 2019, 429–435.

Yamaguchi H., Nakaya M., Kaneko G., Yoneda C., Mochizuki T., Fukami K., ... Watabe S., Comparison in taste and extractive components of boiled dorsal muscle and broth from half-smooth golden puffer *Lagocephalus spadiceus* caught in Japan with those of the same fish imported, *Fisheries Science* **79** (2), 2013, 327–334.

Yang C., Hao R., Du X., Wang Q., Deng Y., Sun R. and Zheng Z., GC–TOF/MS-based metabolomics studies on the effect of protein sources in formulated diet for pearl oyster Pinctada fucata martensii, *Aquaculture* **486**, 2018, 139–147.

Yang Y., Ye Y., Pan D., Sun Y., Wang Y. and Cao J., Metabonomics profiling of marinated meat in soy sauce during processing, *Journal of the Science of Food and Agriculture* **98** (4), 2018, 1325–1331.

Yang Y., Ye Y., Wang Y., Sun Y., Pan D. and Cao J., Effect of high pressure treatment on metabolite profile of marinated meat in soy sauce, *Food Chemistry* **240**, 2018, 662–669.

Zanardi E., Caligiani A., Palla L., Mariani M., Ghidini S., Di Ciccio P.A., ... Ianieri A., Metabolic profiling by 1H-NMR of ground beef irradiated at different irradiation doses, *Meat Science* **103**, 2015, 83–89.

Zhuang M., Lin L., Zhao M., Dong Y., Sun-Waterhouse D., Chen H., ... Su G., Sequence, taste and umami-enhancing effect of the peptides separated from soy sauce, *Food Chemistry* **206**, 2016, 174–181.

Appendix A. Supplementary data

The following are the Supplementary data to this article:

Multimedia Component 1

Supplementary data 1

Multimedia Component 2

Supplementary data 2

Multimedia Component 3

Supplementary data 3

Highlights

- The effects of heat processing on the metabolites identified by NMR such as amino acid-related taste components and nucleotide-related components. It was also decided to highlight that the NMR analyses were in good agreement with the results of the sensory part.
- This is the first research to compare the metabolic profiles of Raw and cooked Pufferfish (*Takifugu flavidus*) meat by NMR assessment.
- This article combines chemical detection methods with flavor sensation to explain the effects of different processing methods on puffer fish flavor from the perspective of metabolites.
- Sensory analysis combines the taste intensity of artificial senses and the sensitive sensor response of intelligent senses to comprehensively explain the effects of metabolite changes on the taste of pufferfish.

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1. Metabolic profiles of pufferfish meat were firstly illuminated by NMR.
2. The taste metabolites were in good agreement with sensory evaluation.
3. The process effects on pufferfish taste were partly explained by their metabolites.
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