Optimization of extraction conditions and determination of purine content in marine fish during boiling (#33036)

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Optimization of extraction conditions and determination of purine content in marine fish during boiling

Tingting Li [1], **Likun Ren** [2], **Dangfeng Wang** [2], **Minjie Song** [2], **Qiuying Li** [2], **Jianrong Li** Corresp. [2]

[1] Department of Life Science, Dalian Minzu University, Dalian, Chian

[2] College of Food Science and Technology, Bohai University, Jinzhou, Chian

Corresponding Author: Jianrong Li
Email address: jwltt@dlnu.edu.cn

Background. Gout is the second most common metabolic disease affecting human health. The disease of gout is closely related to the level of uric acid, which is the end-product of human purine metabolism. Moreover, food is the main way of external ingestion of purine.

Method. A simple and time-saving method was developed to extract purines like adenine, hypoxanthine, guanine, and xanthine from marine fish. The contents of these purines in the edible parts and internal organs of marine fish, as well as *Scophthalmus maximus*, were determined by HPLC to investigate the relationship between the boiling process and purine content.

Result. The total purine content of the edible parts (eyes, dorsal muscles, abdominal muscles, and skin) was the highest in *Scophthalmus maximus*, followed by *sphyraena*, *Sardinella*, *Trichiurus lepturus*, *Scomberomorus niphonius*, *Pleuronectiformes*, *sea catfish*, *Anguillidae*, and *Rajiformes*. Moreover, boiling significantly reduced the purine content in the marine fish because of the transfer of the purines to the cooking liquid during boiling. *Scophthalmus maximus*, *Sphyraena* and *Sardinella* were regard as high-purine marine fish, which we should eat less. We also confirmed that boiling significantly transferred purine bases from fish to cooking liquid. Thus, boiling could reduce the purine content of fish, thereby reducing the risk of hyperuricemia and gout.

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[1] Department of Life Science, Dalian Minzu University, Dalian, Liaoning, China

[2] College of Food Science and Technology, Bohai University, Jinzhou, Liaoning, China

Corresponding Author:

Jianrong Li

19, Keji Rd., New Songshan District, Jinzhou City, Liaoning Province, 121013, China

Email address: lijr6491@163.com

Abstract

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Keywords: marine fish; optimization; purine extraction method; HPLC; purine content; boiling

Introduction

Gout is a type of inflammatory arthritis caused by the deposition of monosodium crystals in tissues. It has become the second-most common metabolic disease affecting human health (Goldberg et al., 2017). According to statistics, about 75 million people in China now suffer from gout or hyperuricemia. These diseases are strongly influenced by uric acid, which is a metabolite of the purine components of RNA and DNA present in foods. Normal serum urate levels in healthy adults are below 0.45 mmol/L for men and below 0.357 mmol/L for women (Zhao et al., 2005). However, when the blood uric acid exceeds normal levels, there is an increased risk for diseases such as gout.

Diet is a key factor in high serum urate levels, and some studies have confirmed that a low-purine diet significantly helps patients with hyperuricemia and gout (Lou, Lin & Benkmann, 2001; Shmerling, 2012). The consumption of food with high purine content can disrupt the balance between uric acid synthesis and metabolism, leading to hyperuricemia or eventual gout (Gowda et al., 2010). Therefore, adherence to a low-purine diet plays an important role in reducing serum uric acid concentration in humans (Suresh & Das, 2012). Compared with other meat products, fish is usually labeled as a healthier food because of its high nutritional value (e.g., high levels of unsaturated fatty acids). However, this nutritional advice may mislead consumers who are not aware of the intrinsic purine content of fish. The quantity and types of purine bases (adenine, guanine, hypoxanthine, and xanthine) in food, however, might be altered

by different cooking methods (Brulé, Sarwar & Savoie, 1989). Previous studies have shown that various processing methods (boiling, steaming, roasting, etc.) can reduce the purine contents in foodstuff; boiling achieved the highest rate of purine removal (Lou et al., 2005). The effect of cooking on purine concentration in beef and chicken has been widely studied, but little information is available on the purine content in the edible parts of marine fish. There are many methods for simultaneous quantification of the purine levels in food. Klampfl, Himmelsbach, Buchberger, and Klein (2002) analyzed the purine and pyrimidine contents in beer by capillary zone electrophoresis. Similarly, an ultra-high-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) method was used to analyze seven purines and pyrimidines in pork products (Clariana et al., 2010). High-performance liquid chromatography (HPLC) has become the most widely used method for purine detection because of its high efficiency, convenience, and accuracy (Rong et al., 2015). Other studies have investigated the purine contents of vegetarian meat analogues, beer, beer-like alcoholic beverages, pork, and beef via HPLC (Jaroslav et al., 2010; Fukuuchi et al, 2013; Rong et al, 2015). Nevertheless, few HPLC methods have been used to quantify the adenine, guanine, hypoxanthine, and xanthine contents in marine fish.

In this paper, a simple and reliable method for purine extraction is reported. Furthermore, the purine contents (adenine, guanine, hypoxanthine, and xanthine) in the edible parts of nine marine fish were measured by HPLC. Finally, the effect of boiling on the purine content in the

edible parts of marine fish was investigated in the context of its viability in decreasing the risk of gout attacks caused by diet.

Material and methods

Chemicals and reagents

Purine standards (adenine, guanine, hypoxanthine, and xanthine) were purchased from Shanghai Aladdin Bio-Chem Technology Co., Ltd (Shanghai, China). The standards were all chromatography-grade with purity >98%. Chromatography-grade acetate, methyl alcohol, tetrabutylammonium hydroxide, and trifluoroacetic acid (TFA) were also obtained from Shanghai Aladdin Bio-Chem Technology Co., Ltd. Analytically pure formic acid (FA) and perchloric acid (PCA) were purchased from Tianjin Fengchuan Chemical Reagent Co., Ltd (Tianjin, China). Water was purified by a Milli-Q system (Millipore, USA).

Sampling and pretreatment

Sample collection

Live marine fish (*Scophthalmus maximus*, *Scomberomorus niphonius, Trichiurus lepturus, Pleuronectiformes, Sea catfish*, *Sardinella*, *Sphyraena*, *Anguillidae*, and *Rajiformes*) were purchased from the local wholesale seafood market in Jinzhou, China, transported to the laboratory where they were killed, and then the dorsal muscles, abdominal muscles, skin, eyes, and viscera were removed for testing. All samples were minced and stored at 0 °C before proceeding.

97 Sample pretreatment

98 The edible parts of the marine fish (dorsal muscles, abdominal muscles, and skin) were
99 boiled in water for 3, 6, 9, 12, and 15 min, and the contents of the purine bases in all samples
100 (edible parts and cooking liquids) were determined. The uncooked samples were used as a
101 control group. Three independent measurements were taken, and the mean and standard
102 deviations were calculated.

103 Establishment of purine extraction method

104 The purine bases in the samples were extracted according to the method of Piñeiro-Sotelo,
105 López-Hernández, and Simal-Lozano (2002), with some modifications. First, 200 mg of sample
106 was added to a centrifuge tube (50 mL) with 10 mL of acid, and heated at 90 °C in a water bath
107 for 15 min. Next, the acid hydrolysate was placed in a rotary evaporator at 75 °C to remove the
108 volatiles, and then redissolved in 10 mL of the HPLC mobile phase (water-methanol-glacial
109 acetic acid-20% tetrabutylammonium hydroxide (v/v = 879/100/15/6)). Finally, the sample was
110 centrifuged at 8000 *g* for 10 min at 4 °C and filtered through a 0.22-μm filter before analysis by
111 HPLC.

112 Optimization of the PCA method

113 The optimum conditions for the purine bases extraction by the PCA method were
114 determined by the single factor method and orthogonal test method. The hydrolysis was
115 performed following the procedure reported by Piñeiro-Sotelo, López-Hernández, and Simal-

Lozano (2002), with some modifications. A single factor method was employed in this study. Four factors were investigated which include PCA concentrations (5, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100%), the temperature of water bath (30, 40, 50, 60, 70, 80, and 90 °C), hydrolysis time (5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 min) and liquied-solid ratio (10:1, 20:1, 30:1, 40:1, 50:1, 60:1, 70:1, 80:1, 90:1), three levels were selected for each factor. When one factor was studies, others were fixed in the optimum value determined in this paper. Then an $L_9(3^4)$ orthogonal test was then used to determine the optimal conditions of the method as determined by the extraction rate.

Optimization of the mixed-acid method

The mixed-acid method was optimized in a similar manner as the PCA method. Single - factor experiments were performed to examine the influence of temperature in water bath (30, 40, 50, 60, 70, 80, 90, and 100 °C), Liquid-solid (10:1, 20:1, 30:1, 40:1, 50:1, 60:1, 70:1, 80:1, 90:1), the concentration of TFA and FA (10, 20, 30, 40, 50, 60, 70, 80, 90, and 100%) and hydrolysis time (5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 min) in extraction efficiency. The effect of each factor was explored by changing the factor while keeping other factors constant. To analyze the data of single factor to carrying out orthogonal test. An orthogonal analysis was then performed using an $L_{16}(4^5)$ test design. The optimum condition was determined based on the extraction rate.

HPLC conditions

A Shimadzu LC-2030 HPLC system (Shimadzu Corporation, Kyoto, Japan) consisting of

136 an LC-20AD pump unit, an SPD-20AV UV detector, and a CTO-20AC column heater, was used
137 to identify the purine bases extracted. An Agilent Eclipse XDB-C18 column (4.6 mm × 250.0
138 mm × 5.0 μm, Agilent Technologies, Germany) was used as the analytical column and
139 maintained at 28 °C during operation. The mobile phase was water-methanol-glacial acetic acid-
140 20% tetrabutylammonium hydroxide (v/v = 879/100/15/6), and the 10 μL sample was eluted at a
141 flow rate of 0.8 mL/min. At the end of each procedure, the analytical column was washed with
142 the mobile phase for 30 min and equilibrated before the next run. The detector measured
143 absorbance at 254 nm and data was analyzed using Shimadzu analysis software (Shimadzu
144 Corporation, Kyoto, Japan).

145 Method evaluation

146 The purine base standards (adenine, guanine, hypoxanthine, and xanthine) were dissolved in
147 ultrapure water at concentrations of 0.1, 0.5, 1, 5, 10, 50, 100, 200, and 300 mg/L and detected
148 by HPLC according to the method described above. Purine bases in the samples were identified
149 by comparing the peak retention times with those of the standard solutions. Quantification of the
150 purine bases was based on the regression analysis of peak area against concentration. The
151 linearity, range, squared correlation coefficient values (R^2), and limits of detection (LODs) were
152 determined (Li, 2015). The LOD was determined using standard solutions, calculated as 3 times
153 the baseline noise signal. The precision of the method and the repeatability of the purine
154 extraction process were evaluated by testing the mixed purine base standard solution and the
155 extraction sample from *Scophthalmus maximus* (dorsal muscles) six times, and the relative

156 standard deviation (RSD%) was calculated. To determine the percent recovery of the method,
157 known quantities of individual purine base standards (0.5, 1, and 2 times the amounts found in
158 the samples) were added to the samples, and the percent recovery was calculated according to the
159 following formula (Peng et al., 2008):

160 $$\%\ \text{Recovery} = \frac{\text{test sample - baseline sample}}{\text{content added}} \times 100$$

161 Statistical analysis

162 All measurements were performed in triplicate and the standard deviation was determined
163 using SPSS 20 for Windows (SPSS Inc., Chicago, IL, USA). Graphs were drawn with the
164 OriginPro 8.5 software package (OriginLab, Northampton, MA, USA). Statistical significance
165 was assessed via analysis of variance (ANOVA). Differences with $P < 0.05$ were considered
166 statistically significant.

167 **Results and discussion**

168 HPLC method

169 The HPLC method described in Section 2.4 were used to simultaneously quantify the
170 adenine, guanine, hypoxanthine, and xanthine contents in marine fish. As shown in Fig. 1, the
171 four purine bases were baseline separated within 10 min.

172 Establishment of the purine extraction method

173 Optimization of the PCA method

174 The results showed that acid concentration, hydrolysis temperature, hydrolysis time, and the
175 liquid-solid ratio had important influences on the extraction rates of the purines. As shown in Fig.
176 2, the extraction rate of the purines decreased gradually with increasing PCA concentration,
177 eventually beginning to increase once the PCA concentration reached 60%. The extraction rate
178 also reached a maximum at a hydrolysis temperature of 80 °C, beyond which point it decreased
179 slightly. This may be due to the destruction of some purine bases at high temperatures (Jamil,
180 Halim & Sarbon, 2016). In addition, the extraction with the PCA method was the best with a
181 hydrolysis time of 45 min and a liquid-solid ratio of 60:1. Finally, nine tests were carried out at
182 acid concentrations (A) of 70 (A_1), 80 (A_2), and 90% (A_3), temperatures (B) of 70 (B_1), 80 (B_2),
183 and 90 °C (B_3), hydrolysis times (C) of 40 (C_1), 45 (C_2), and 55 min (C_3), and liquid-solid ratios
184 (D) of 50:1 (D_1), 60:1 (D_2), and 90:1 (D_3). The results of the orthogonal test and the *Kij*, *kij*, and
185 R values presented in Table 1 indicated that the maximum combined extraction yield of the
186 purines was 82.68%. The extraction yields of adenine, guanine, hypoxanthine, and xanthine
187 obtained from these nine runs were 4.61–10.31, 8.54–14.42, 48.95–54.11, and 2.06–4.16%,
188 respectively. According to the R value, acid concentration was the dominant factor influencing
189 purine extraction in the PCA method. Therefore, the selected PCA extraction method was as
190 follows: acid concentration 80%; temperature 80 °C; hydrolysis time 55 min; solid-to-liquid ratio
191 50:1.

192 Optimization of the mixed-acid method

193 The effects of hydrolysis temperature, the liquid-solid ratio, FA concentration, TFA
194 concentration, and hydrolysis time on the extraction rates of the four purine bases are shown in
195 Fig. 3. An increase in hydrolysis temperature improved the extraction efficiency gradually, until
196 a maximum extraction rate was reached at about 90 °C. The extraction rate of the purines
197 fluctuated with increasing TFA concentration. The highest extraction rate was obtained when the
198 sample was hydrolyzed with 50% FA for 10 min in a liquid-solid ratio of 30:1. Finally,
199 hydrolysis temperatures (A) of 50 (A_1), 80 (A_2), 90 (A_3), and 100 °C (A_4), liquid-solid ratios (B)
200 of 30:1 (B_1), 40:1 (B_2), 50:1 (B_3), and 70:1 (B_4), FA concentrations (C) of 50 (C_1), 70 (C_2), 80
201 (C_3), and 100% (C_4), TFA concentrations (D) of 40 (D_1), 60 (D_2), 70 (D_3), and 90 (D_4), and
202 hydrolysis times (E) of 5 (E_1), 10 (E_2), 15 (E_3), and 20 min (E_4) were evaluated according to the
203 results of the single-factor experiment.

204 A further orthogonal analysis was performed using an L_{16} (4^5) test design. The *Kij*, *kij*, and
205 R values of the mixed-acid extraction were calculated and are listed in Table 2. The results of the
206 orthogonal test indicated that the factor combination of Test 9 achieved the highest purine
207 extraction rate. Furthermore, according to the R values, the influence of each parameter on the
208 extraction yield of the purine bases followed the order A > E > D > B > C. Therefore, the
209 parameters of Test 9 ($A_3B_1C_3D_4E_2$) were selected for the mixed acid extraction method. The
210 method used was as follows: mixture acid 90% TFA/80% FA (v/v, 1:1); temperature 90 °C;
211 hydrolysis time 10 min; solid-to-liquid ratio 30:1.

212 Determination of the optimal method

213 In order to determine the best extraction method, the extraction yields of the two methods
214 were compared; the results are shown in Table 3. The extractions of the four purine bases by the
215 mixed-acid method were higher than those by the PCA method. The extraction yield of xanthine
216 by the mixed-acid extraction was nearly two times that achieved by the PCA extraction. These
217 differences were likely due to protective effects of FA on purine bases. Moreover, TFA could
218 promote complete dissolution of purine bases. In addition, the mixed-acid treatment was
219 timesaving and could effectively avoid the production of toxic chlorine. Therefore, the mixed-
220 acid method was chosen for the extraction of purine bases from different marine fish.

221 Method Validation

222 The linearity, range, and LOD of the method were subsequently calculated; results are
223 shown in Table 4. Excellent linearity was observed for the quantification of the four purine bases.
224 The LODs ranged from 0.0118 to 0.0774 mg/L, which were considered excellent. Furthermore,
225 the R^2 values were all above 0.9999, indicating a good linearity.

226 Standards were used to evaluate the precision of the method. For the repeatability and
227 recovery tests, *Scophthalmus maximus* (dorsal muscles) samples were prepared by the method
228 described previously. As shown in Table 5, the the deviation in results were less than 0.69%,
229 which indicated that the HPLC method was highly reproducible. The mean repeatability of the
230 method for quantification of adenine, guanine, hypoxanthine, and xanthine were 1.32%, 0.87%,
231 0.83%, and 1.77%, respectively. By this method, average recoveries ranged from 94.90% to

104.51%. These results suggest that the mixed-acid method avoided damaging the purine bases, and confirm the validity of the method.

Purine contents in edible parts of marine fish

The contents of the four purines in different edible parts of marine fish were determined by the method described above; results are shown in Table 6. Among the marine fish evaluated, the total purine content (dorsal muscles + abdominal muscles + skin + entrails + eyes) was highest in *Sphyraena*. In contrast, *Rajiformes* exhibited the lowest total purine content. Considering only the edible parts of the fish (muscles, skin, and eyes) *Scophthalmus maximus*, *Sphyraena*, and *Sardinella* had the highest purine contents. *Rajiformes* and *Anguillidae* had the lowest purine contents and would thus be more suitable for a low-purine diet. To control the concentration of serum uric acid and avoid hyperuricemia and gout, it is best to reduce the consumption of high-purine fish such as *Scophthalmus maximus* and *Sphyraena*.

The purine contents in fish viscera were significantly higher than those in muscles. The total purine content in viscera ranged from 1085.61 mg/kg to 2719.72 mg/kg, but the muscle samples contained only 551.33–1188.12 mg/kg (dorsal muscles) and 376.94–1302.82 mg/kg (abdominal muscles) total purines, with *Sphyraena* and *Scophthalmus maximus* exhibiting higher total purine contents than the muscles samples of other fish. This may be due to the higher visceral metabolic rate in the intestine of these species, which could promote the formation of purine bases. Furthermore, all muscle samples (dorsal muscles and abdominal muscles) were found to contain higher amounts of hypoxanthine than the other three purine bases. This observation is consistent

252 with the report by Qu et al. (2016), who found that hypoxanthine content was the dominant
253 purine in the muscles of *Lateolabrax japonicus*, followed by adenine, guanine, and xanthine.

254 The skins of the marine fish contained large amounts of guanine and hypoxanthine, which
255 accounting for 74.22 to 95.48% of the total purine content. The total purine content of skin
256 varied significantly between the different species of marine fish. *Anguillidae* skin exhibited total
257 purine content of 486.56 mg/kg, whereas the purine content of *Sphyraena* skin was ten times
258 higher. This might be related to the different habitats and growth patterns of the marine fish.
259 Theoretically, the four purine bases can be transformed to uric acid in an equal manner, but
260 hypoxanthine and adenine exhibit the greatest hyperuricemic effect (Clifford et al., 1976). The
261 main difference between the purine contents in viscera compared with that of the eyes was the
262 dominant purine. As seen in Table 6, viscera were rich in adenine, guanine, and hypoxanthine,
263 whereas eyes contained the highest level of guanine. Furthermore, it is worth noting the purine
264 content in fish eyes, which ranged from 1010.64 mg/kg to 3990.19 mg/kg. In some regions,
265 especially in China, consumers commonly eat fish eyes. Considering the high purine content of
266 fish eyes and the pain and potential joint damage caused by hyperuricemia and gout, the
267 consumption of fish eyes should be avoided, even though they are rich in nutrients such as
268 collagen and unsaturated fatty acids.

269 Effect of boiling on purine content

270 Changes in the purine content of muscle during boiling

271 Previous studies have demonstrated that processes such as boiling, steaming, and

272 microwaving can significantly reduce the purine content in foods. Among these processes,
273 boiling is one of the most effective approaches to decreasing purine content. However, most
274 studies on the effect of boiling on purine levels have mainly focused on meats such as chicken
275 and beef, while less attention had been paid to the effect in aquatic organisms (Brulé, Sarwar
276 & Savoie 1989; Young, 1983). The changes in purine content during the boiling of marine fish
277 measured during this study are shown in Fig. 4.

278 Hypoxanthine was the major purine base measured in the muscle samples (Fig. 4). In the
279 dorsal and abdominal muscles of *Scophthalmus maximus*, the amounts of adenine, guanine, and
280 hypoxanthine decreased after boiling in water, but no changes were observed in the amount of
281 xanthine present (Fig. 4). Among the purines measured, hypoxanthine was found to undergo the
282 greatest reduction, from 867.90 mg/kg to 258.24 mg/kg (70.24%) in dorsal muscles and from
283 933.94 mg/kg to 442.98 mg/kg (52.57%) in abdominal muscles. Several studies have determined
284 that hypoxanthine and free hypoxanthine-related compounds have good solubility and can be
285 released easily from foods during cooking (Brulé, Sarwar & Savoie 1989; Young, 2015). Our
286 findings are also comparable to those of Lou, Lin, and Benkmann (2001), who found that free
287 purine bases in grass shrimp could be easily released and transferred to the cooking liquid during
288 the cooking process. Furthermore, the contents of adenine and guanine decreased slightly after
289 boiling; the amounts of adenine and guanine released into the cooking water were 35.89% and
290 15.92%, respectively, in dorsal muscles, and 32.21% and 29.18%, respectively, in abdominal
291 muscles. The xanthine contents in muscles were relatively low to begin with, and exhibited

292 nearly no change after boiling.

293 As for the effect of boiling time on changes in purine content, the contents of the four
294 purine bases decreased more extensively with prolonged treatment time, and most purines in the
295 dorsal muscles were reduced within 12–15 min. In comparison, the total purine and
296 hypoxanthine contents in abdomen muscles decreased significantly within 0–3 minutes,
297 declining by 64.63% and 49.13%, respectively (Fig. 4).

298 Changes in purine content of skin during boiling

299 Changes in the purine content of the skins were similar to those of muscles. Boiling reduced
300 the amounts of purine base presents. As shown in Fig. 5, boiling led to a slight decrease in total
301 purine content, from 1596.68 to 1117.41 mg/kg in *Scophthalmus maximus* skin. Hypoxanthine
302 decreased rapidly (by 41.47%) in the first 3 min, after which time the rate of removal slowed
303 down. Guanine was the major purine base in the skin samples; however, adenine and guanine
304 decreased only slightly after boiling, declining by 35.71 and 23.20%, respectively. Boiling had
305 the greatest effect on the removal of hypoxanthine among the four purines, with the content
306 decreasing from 324.04 mg/kg to 82.51 mg/kg.

307 Change in purine content of the cooking liquid during boiling

308 In order to explore the transfer of purine between the fish parts and the cooking water
309 during boiling, the changes in purine content of the cooking water were also determined. As
310 shown in Fig. 6, with increased boiling time, the contents of the four purines in the cooking

311 liquid increased gradually over 15 min. The total purine content of the water increased
312 remarkably within the first 3 min. The cooking liquid contained abundant levels of hypoxanthine,
313 which was measured at concentrations approximately three times higher than those of the other
314 three purines combined. The hypoxanthine content significantly increased within the first 9 min
315 and remained stable thereafter. There was no significant change in xanthine content over 15 min
316 of boiling. The adenine and guanine contents in the cooking liquid increased sharply from 3 to
317 15 min, by 161.99 mg/kg and 180.10 mg/kg, to final contents of 200.26 mg/kg and 230.94 mg/kg,
318 respectively. These results confirm that the purines contained in the edible parts of the marine
319 fish were transferred to the cooking liquid. This result agrees with the findings of Young (2015),
320 who reported that the level of hypoxanthine in tissues decreased during cooking as it was
321 removed by the cooking liquids.

322 **Conclusions**

323 The results indicated that marine fish contained high purine levels, with *Scophthalmus*
324 *maximus*, Sphyraenus, and Sardine containing higher purine levels than the other fish tested.
325 Moreover, the dominant purine and content of each purine varied significantly between the
326 different parts of the marine fish, where purine content in the viscera and eyes of the fish were
327 higher than that in muscles. The muscle samples (dorsal and abdominal muscles) were found to
328 contain higher amounts of hypoxanthine, whereas the major purine base in the eyes was guanine,
329 the skin of the marine fish contained large amounts of guanine and hypoxanthine, and the
330 viscera were rich in adenine, guanine, and hypoxanthine. We confirmed that boiling significantly

reduced the purine contents in the fish, as the purines were transferred to the cooking liquid. Thus, boiling fish before eating could reduce the purine content, thereby reducing the incidence of hyperuricemia and gout.

Acknowledgment

We thank all people who contributed directly or indirectly to this work, We are grateful to National & Local Joint Engineering Research Center of Storage, Processing and Safety Control Technology for Fresh Agricultural and Aquatic Products-Bohai University for infrastructure and for partially financing this study.

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Figure 1(on next page)

HPLC chromatogram of standards' solution

a: Adenine b: Guanine c: Hypoxanthine d: Xanthine

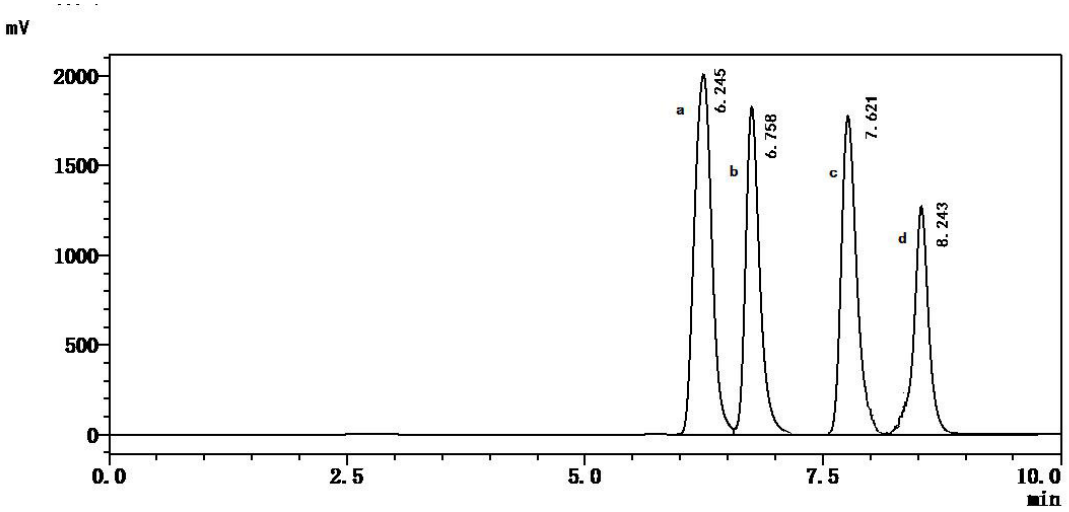

mV
2000
1500
1000
500
0
0.0
2.5
5.0
7.5
10.0
min
a
6.245
b
6.758
c
7.621
d
8.243

Figure 2(on next page)

Influence of different conditions on the extraction yield using the PCA method

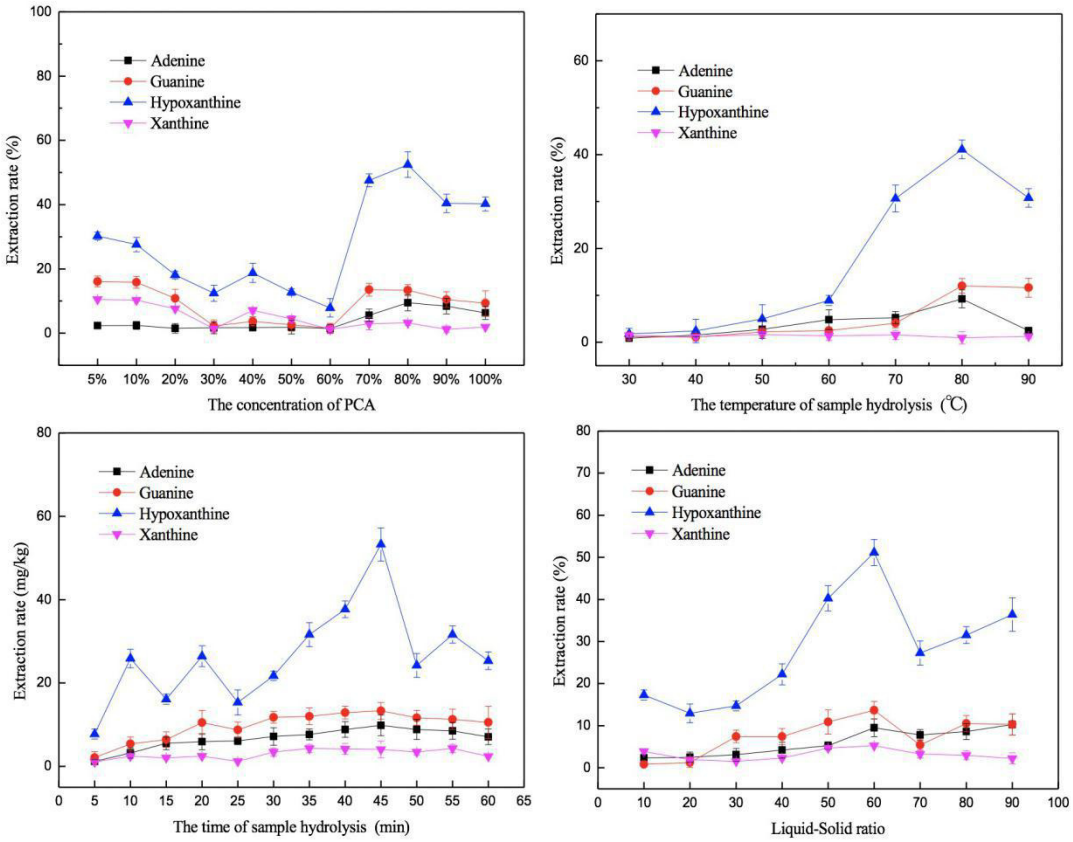

Adenine
Guanine
Hypoxanthine
Xanthine
Extraction rate (%)
The concentration of PCA
5% 10% 20% 30% 40% 50% 60% 70% 80% 90% 100%
Extraction rate (%)
The temperature of sample hydrolysis (℃)
Extraction rate (mg/kg)
The time of sample hydrolysis (min)
Extraction rate (%)
Liquid-Solid ratio

Figure 3(on next page)

Influence of different conditions on the extraction yield using the mixed-acid method

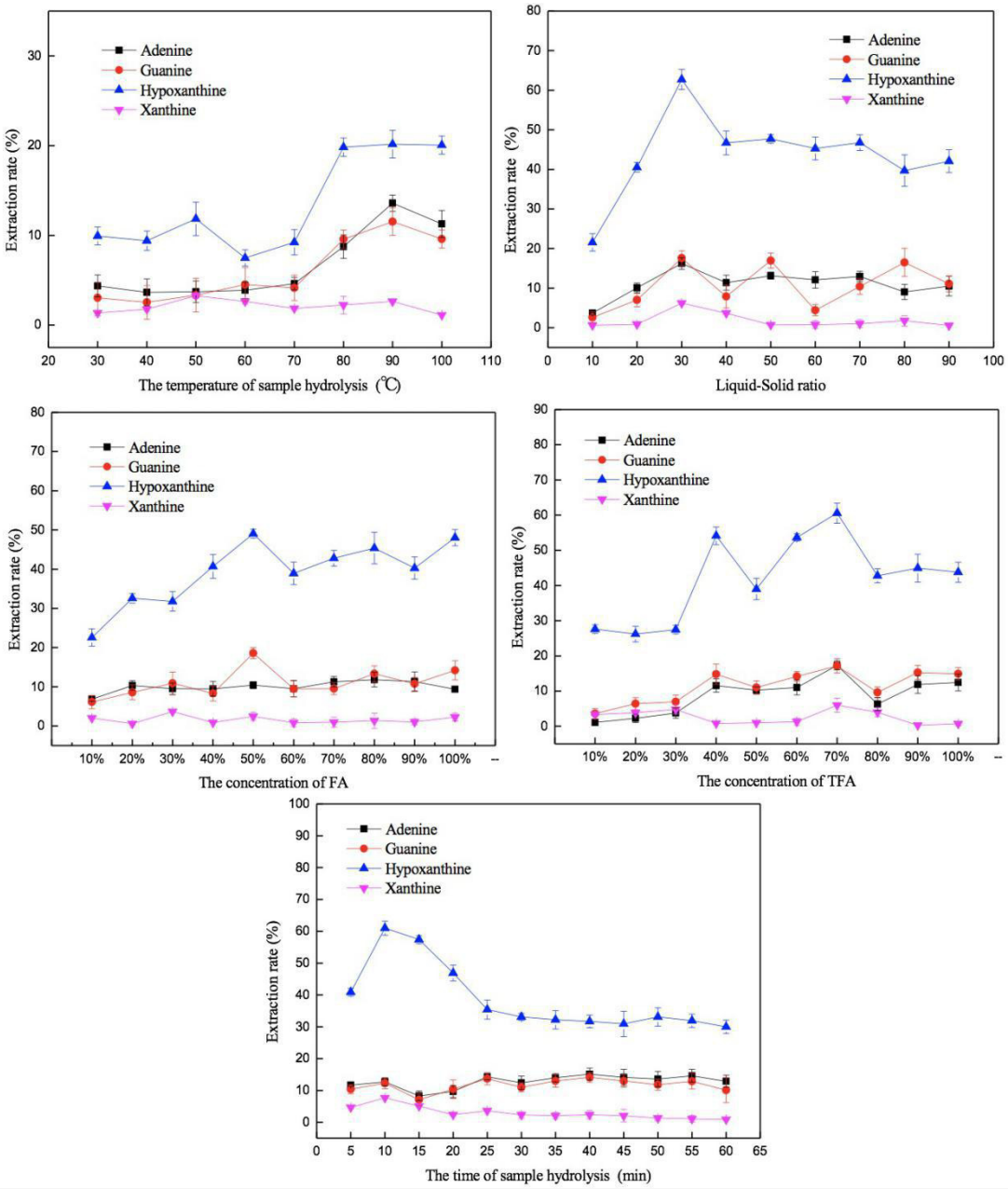
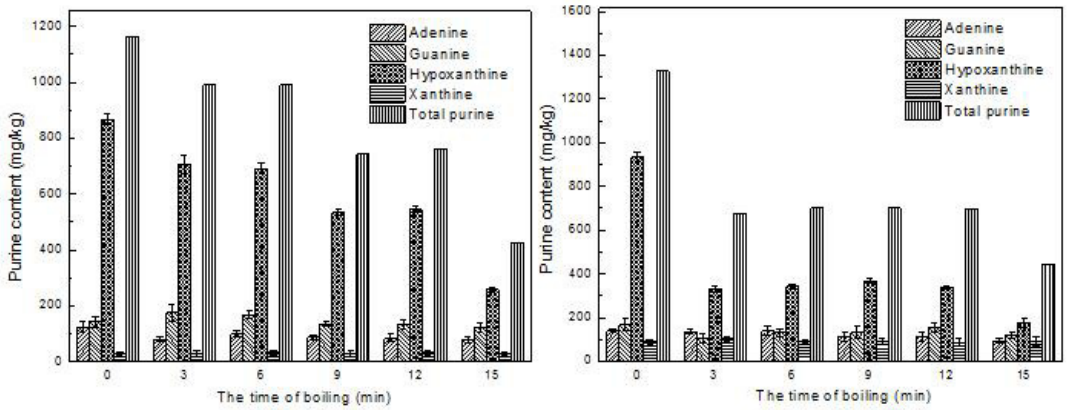

Adenine
Guanine
Hypoxanthine
Xanthine
Extraction rate (%)
The temperature of sample hydrolysis (℃)
Liquid-Solid ratio
The concentration of FA
The concentration of TFA
The time of sample hydrolysis (min)

Figure 4(on next page)

Effects of boiling on the purine contents in muscles

a: dorsal muscles b: abdominal muscles


Adenine
Guanine
Hypoxanthine
Xanthine
Total purine
Purine content (mg/kg)
The time of boiling (min)
0
3
6
9
12
15
200
400
600
800
1000
1200
1400
1600

a b

Figure 5(on next page)

Effect of boiling on the purine contents in skin

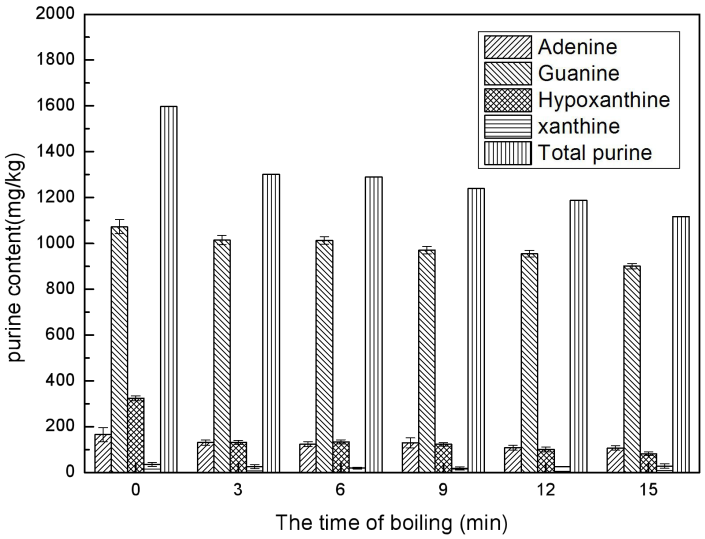

Adenine
Guanine
Hypoxanthine
xanthine
Total purine
purine content(mg/kg)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0
3
6
9
12
15
The time of boiling (min)

Figure 6(on next page)

Effect of boiling on the purine contents in boiling liquid

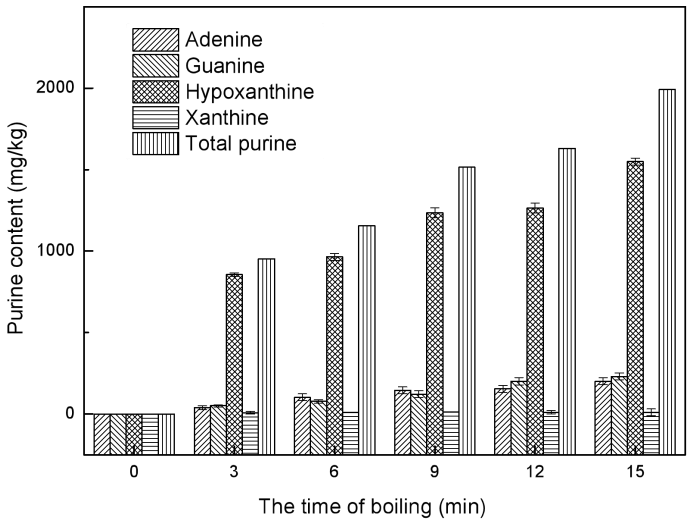

Adenine
Guanine
Hypoxanthine
Xanthine
Total purine
Purine content (mg/kg)
2000
1000
0
0
3
6
9
12
15
The time of boiling (min)

Table 1(on next page)

Orthogonal test results

1 Table 1. Orthogonal test results

Test No.	Parameters				Yield[a] (%)				
	A	B	C	D	Total[b]	Adenine	Guanine	Hypoxanthine	Xanthine
1	A_1	B_1	C_1	D_1	71.58^{cd}	4.61^{c}	9.65^{bc}	54.11^{a}	3.21^{ab}
2	A_1	B_2	C_2	D_2	69.87^{d}	6.68^{b}	8.54^{c}	50.54^{bc}	4.11^{a}
3	A_1	B_3	C_3	D_3	68.43^{d}	6.74^{b}	8.88^{c}	48.95^{c}	3.86^{ab}
4	A_2	B_1	C_2	D_3	73.00^{cd}	8.63^{ab}	9.96^{bc}	51.65^{b}	2.76^{ab}
5	A_2	B_2	C_3	D_1	82.68^{a}	10.31^{a}	14.42^{a}	53.79^{ab}	4.16^{a}
6	A_2	B_3	C_1	D_2	74.04^{b,c}	9.31^{a,b}	10.43^{b,c}	50.54^{b,c}	3.76^{a,b}
7	A_3	B_1	C_3	D_2	75.76^{b}	8.52^{ab}	11.27^{b}	53.31^{ab}	2.66^{ab}
8	A_3	B_2	C_1	D_3	73.17^{c}	9.43^{ab}	9.54^{bc}	50.44^{bc}	3.76^{ab}
9	A_3	B_3	C_2	D_1	69.53^{d}	7.85^{b}	10.64^{bc}	48.98^{c}	2.06^{b}
K_1	209.88^{c}	220.34	218.79	223.79					
K_2	229.72	225.72	212.40	219.67					
K_3	218.46	212.00	226.87	214.6					
k_1	69.96^{d}	73.45	72.93	74.60					
k_2	76.57	75.24	70.80	73.22					
k_3	72.82	70.67	75.62	71.53					
R	6.61^{e}	4.57	4.82	3.07					

2 [a]Extraction yield (%) = (purine content in sample (mg)/sample mass (kg)) × 100

3 [b]Total extraction yield (%) = Adenine extraction yield + Guanine extraction yield + Hypoxanthine extraction yield + Xanthine

4 extraction yield

5 [c] $K^A_i = \Sigma$ the amount of target compounds at Ai.

6 [d] $k^A_i = K^A_i/3$.

7 [e] $R^A_i = \max\{k^A_i\} - \min\{k^A_i\}$.

8 Values within a column marked with different letters are significantly different ($p < 0.05$).

Table 2(on next page)

Results of the orthogonal test

1 Table 2. Results of the orthogonal test

Test No.	Parameters					Yield[a] (%)				
	A	B	C	D	E	Total[b]	Adenine	Guanine	Hypoxanthine	Xanthine
1	A_1	B_1	C_1	D_1	E_1	16.21^{g}	3.02^{e}	4.00^{e}	6.85^{e}	2.34^{cd}
2	A_1	B_2	C_2	D_2	E_2	15.19^{g}	2.07^{e}	2.75^{e}	7.14^{e}	3.23^{bc}
3	A_1	B_3	C_3	D_3	E_3	25.98^{fg}	5.47^{de}	6.39^{de}	11.16^{de}	2.96^{c}
4	A_1	B_4	C_4	D_4	E_4	36.25^{ef}	6.94^{de}	7.39^{d}	15.66^{de}	6.26^{ab}
5	A_2	B_1	C_2	D_3	E_4	78.14^{c}	13.73^{bc}	15.75^{bc}	47.44^{bc}	1.22^{cd}
6	A_2	B_2	C_1	D_4	E_3	95.85^{b}	15.27^{ab}	14.91^{c}	64.98^{a}	0.69^{d}
7	A_2	B_3	C_4	D_1	E_2	49.91^{de}	7.24^{d}	7.47^{d}	34.24^{c}	0.96^{d}
8	A_2	B_4	C_3	D_2	E_1	30.61^{f}	4.82^{de}	4.77^{de}	18.23^{d}	2.79^{cd}
9	A_3	B_1	C_3	D_4	E_2	108.50^{a}	17.59^{a}	18.84^{b}	64.39^{a}	7.68^{a}
10	A_3	B_2	C_4	D_3	E_1	58.08^{d}	11.07^{c}	13.39^{cd}	28.12^{c,d}	5.50^{b}
11	A_3	B_3	C_1	D_2	E_4	79.22^{c}	12.95^{bc}	16.31^{bc}	43.14^{bc}	6.82^{ab}
12	A_3	B_4	C_2	D_1	E_3	96.55^{b}	14.41^{b}	15.24^{bc}	61.60^{ab}	5.30^{b}
13	A_4	B_1	C_4	D_2	E_3	97.53^{b}	15.09^{ab}	24.15^{a}	51.63^{b}	6.66^{ab}
14	A_4	B_2	C_3	D_1	E_4	100.94^{ab}	17.23^{ab}	23.42^{ab}	58.25^{a,b}	2.04^{c,d}
15	A_4	B_3	C_2	D_4	E_1	72.54^{c}	12.03^{bc}	16.20^{bc}	37.62^{c}	6.69^{ab}
16	A_4	B_4	C_1	D_3	E_2	40.61^{e}	14.82^{ab}	4.77^{de}	18.23^{d}	2.79^{cd}
K_1	93.63^{c}	300.38	231.89	263.61	177.44					
K_2	254.51	270.06	262.42	222.55	214.21					
K_3	342.35	227.65	266.03	202.81	315.91					
K_4	311.62	204.02	313.14	313.14	294.55					
k_1	23.41^{d}	75.10	57.97	65.90	44.36					
k_2	63.63	67.52	65.61	55.64	53.55					

k_3	85.59	56.91	66.51	50.70	78.98
k_4	77.91	51.01	78.29	78.29	73.64
R	62.18[e]	24.09	20.31	27.58	34.62

[a]Extraction yield (%) = (purine content in sample (mg)/sample mass (kg)) × 100

[b]Total extraction yield (%) = Adenine extraction yield + Guanine extraction yield + Hypoxanthine extraction yield + Xanthine extraction yield

[c] $K^A_i = \Sigma$ the amount of target compounds at Ai.

[d] $k^A_i = K_i^A/4$.

[e] $R^A_i = \max\{k_i^A\} - \min\{k_i^A\}$.

Values within a column marked with different letters are significantly different ($p < 0.05$).

Table 3(on next page)

Extraction yields of four purine bases by different methods

Table 3. Extraction yields of four purine bases by different methods (mean (%) ± S.D, n = 3)

	Adenine	Guanine	Hypoxanthine	Xanthine
Perchloric acid extraction	10.31±1.40	14.42±0.90	53.79±2.60	4.11±1.70
Mixed-acid extraction	17.59±0.90	18.84±4.50	64.39±3.40	7.68±0.20

Table 4(on next page)

Linear relation results

1 Table 4. Linear relation results (n=3).

Purine	Regression equation[a]	R^2	Linear range (mg/L)	LOD（mg/L）
Adenine	y = 126196x - 16483	1.0000	0.1-300	0.0774
Guanine	y = 90966x - 8366.6	1.0000	0.1-300	0.0178
Hypoxanthine	y = 104998x - 8604.2	1.0000	0.1-300	0.0118
Xanthine	y = 67689x - 1249.5	0.9999	0.1-300	0.0555

2 [a]Variables: y, peak area (mV); x, concentration of each analyte (mg/L)

3

Table 5(on next page)

Precision, repeatability, and recovery results

1 Table 5. Precision, repeatability, and recovery results (n=6)

Purine	Precision (%)[a]	Repeatability (%)[b]	Recovery mean (%)[c]
Adenine	0.13	1.32	95.31
Guanine	0.69	0.87	94.90
Hypoxanthine	0.02	0.83	104.51
Xanthine	0.06	1.77	95.48

2 [a]Expressed as RSD by mixed purine base standards solution repeated six times.

3 [b]Expressed as RSD by *Scophthalmus maximus* (dorsal muscles) repeated six times.

4 [c]Average of recoveries at three spiked levels.

5

6

Table 6(on next page)

Purine content in different parts of marine fish

1 Table 6. Purine content in different parts of marine fish (mean ± S.D.)

Sample	Adenine	Guanine	Hypoxanthine	Xanthine	Total
Scophthalmus maximus (dorsal muscles)	127.50±1.32	143.51±4.52	820.71±20.57	96.39±5.33	1188.12
Scophthalmus maximus (abdominal muscles)	135.33±2.74	155.33±2.72	920.61±2.63	91.55±1.85	1302.82
Scophthalmus maximus (skin)	247.04±4.57	1190.03±24.06	206.01±5.55	42.65±0.92	1685.73
Scophthalmus maximus (viscera)	378.05±3.16	467.58±13.23	256.01±16.76	114.72±8.87	1216.36
Scophthalmus maximus (eyes)	6.55±0.00	3820.77±28.33	117.39±0.53	_[b]	3944.71
Scomberomorus niphonius (dorsal muscles)	121.17±0.34	135.86±7.76	704.17±23.14	22.32±3.70	983.53
Scomberomorus niphonius (abdominal muscles)	148.01±2.42	177.65±15.17	651.59±7.80	17.66±0.16	994.91
Scomberomorus niphonius (skin)	194.95±7.09	588.67±26.14	314.62±4.93	23.29±1.34	1121.53
Scomberomorus niphonius (viscera)	326.38±0.14	822.00±9.37	1015.87±1.48	498.97±0.44	2663.22
Scomberomorus niphonius (eyes)	140.63±2.68	1636.54±69.73	255.58±2.82	50.29±0.80	2083.04
Pleuronectiformes (dorsal muscles)	110.85±4.85	109.43±3.42	503.69±18.61	70.14±2.12	794.11
Pleuronectiformes (abdominal muscles)	157.77±2.22	398.17±1.10	645.52±8.28	7.39±0.41	1208.85
Pleuronectiformes (skin)	83.70±4.77	234.05±19.29	385.25±9.18	2.31±0.13	705.31

Pleuronectiformes (viscera)	673.41±8.00	980.73±9.34	205.48±0.56	223.56±5.30	2083.18
Pleuronectiformes (eyes)	157.62±0.56	1390.48±5.31	454.60±10.66	3.69±0.57	2006.39
Sea catfish (dorsal muscles)	107.96±0.46	110.48±0.13	525.52±0.71	7.63±0.01	751.59
Sea catfish (abdominal muscles)	84.53±0.78	101.70±0.26	325.97±1.09	6.99±0.01	519.20
Sea catfish (skin)	244.51±3.73	463.46±13.39	380.49±0.07	48.56±0.27	1137.02
Sea catfish (viscera)	496.76±0.47	482.56±0.09	305.94±0.04	1.89±0.01	1287.15
Sea catfish (eyes)	56.56±0.01	807.51±0.19	114.82±0.05	31.73±0.04	1010.64
Sardinella (dorsal muscles)	88.30±0.05	62.15±0.08	486.70±0.80	37.47±0.01	674.62
Sardinella (abdominal muscles)	136.73±0.15	106.22±0.06	835.42±0.78	52.04±0.15	1130.41
Sardinella (skin)	118.90±0.12	1100.16±0.35	918.66±2.01	121.20±0.05	2258.91
Sardinella (viscera)	429.03±4.72	607.65±2.39	549.02±2.34	257.57±0.46	1843.26
Sardinella (eyes)	69.94±0.35	1156.23±0.52	161.82±0.03	56.40±0.05	1444.39
Sphyraena (dorsal muscles)	110.57±0.24	84.06±0.84	715.71±0.49	8.85±0.33	919.19
Sphyraena (abdominal muscles)	101.74±0.02	314.09±0.70	656.65±3.02	185.27±0.10	1257.75
Sphyraena (skin)	130.45±0.01	3651.71±0.95	567.29±0.23	69.19±0.26	4418.64
Sphyraena (viscera)	722.00±2.58	952.23±12.49	901.25±0.33	144.24±0.41	2719.72

Sphyraena (eyes)	51.70±3.08	1131.60±0.46	51.47±1.33	20.80±0.00	1255.57
Anguillidae (dorsal muscles)	280.04±0.01	94.15±0.50	302.94±0.52	8.95±0.05	686.07
Anguillidae (abdominal muscles)	186.96±0.10	112.08±0.00	227.65±0.13	_	527.61
Anguillidae (skin)	33.50±0.12	173.94±0.65	219.41±3.01	59.71±0.20	486.56
Anguillidae (viscera)	761.48±0.70	915.74±1.99	275.33±0.30	29.97±0.07	1982.53
Anguillidae (eyes)	68.76±0.02	1252.41±3.76	65.93±0.08	19.08±0.15	1406.19
Rajiformes (dorsal muscles)	89.50±0.06	88.93±0.16	372.90±0.10	_	551.33
Rajiformes (abdominal muscles)	74.51±0.14	52.91±0.32	249.52±0.13	_	376.94
Rajiformes (skin)	130.46±0.03	514.27±0.67	451.36±0.14	13.89±0.03	1109.97
Rajiformes (viscera)	277.50±1.52	363.22±0.37	349.68±1.73	95.21±0.15	1085.61
Rajiformes (eyes)	54.51±0.06	704.83±0.48	250.32±0.81	16.10±0.16	1025.75
Trichiurus lepturus (muscles)	134.50±3.85	170.59±0.77	878.57±1.88	14.31±3.84	1197.97
Trichiurus lepturus (viscera)	83.21±0.31	736.40±2.41	491.41±1.70	166.93±5.78	1477.93
Trichiurus lepturus (eyes)	609.51±8.72	2579.80±60.13	418.67±12.05	382.21±2.44	3990.19

[a]Total purine = Adenine + Guanine + Hypoxanthine + Xanthine

[b]not detected

5

6